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# Comparative Genomics and Site-Directed Mutagenesis Support the Existence of Only One Input Channel for Protons in the C-Family ($cbb_3$ Oxidase) of Heme−Copper Oxygen Reductases†

James Hemp,‡,§ Huazhi Han,‡,|| Jung Hyeob Roh,⊥ Samuel Kaplan,⊥ Todd J. Martinez,‡,§ and Robert B. Gennis*,‡,§,||

*Center for Biophysics and Computational Biology and Departments of Chemistry and Biochemistry, University of Illinois, 600 South Mathews Street, Urbana, Illinois 61801, and Department of Microbiology and Molecular Genetics, University of Texas Health Science Center, Houston, Texas 77030*

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ABSTRACT: Oxygen reductase members of the heme−copper superfamily are terminal respiratory oxidases in mitochondria and many aerobic bacteria and archaea, coupling the reduction of molecular oxygen to water to the translocation of protons across the plasma membrane. The protons required for catalysis and pumping in the oxygen reductases are derived from the cytoplasmic side of the membrane, transferred via proton-conducting channels comprised of hydrogen bond chains containing internal water molecules along with polar amino acid side chains. Recent analyses identified eight oxygen reductase families in the superfamily: the A-, B-, C-, D-, E-, F-, G-, and H-families of oxygen reductases. Two proton input channels, the K-channel and the D-channel, are well established in the A-family of oxygen reductases (exemplified by the mitochondrial cytochrome *c* oxidases and by the respiratory oxidases from *Rhodobacter sphaeroides* and *Paracoccus denitrificans*). Each of these channels can be identified by the pattern of conserved polar amino acid residues within the protein. The C-family ($cbb_3$ oxidases) is the second most abundant oxygen reductase family after the A-family, making up more than 20% of the sequences of the heme−copper superfamily. In this work, sequence analyses and structural modeling have been used to identify likely proton channels in the C-family. The pattern of conserved polar residues supports the presence of only one proton input channel, which is spatially analogous to the K-channel in the A-family. There is no pattern of conserved residues that could form a D-channel analogue or an alternative proton channel. The functional importance of the residues proposed to be part of the K-channel was tested by site-directed mutagenesis using the $cbb_3$ oxidases from *R. sphaeroides* and *Vibrio cholerae*. Several of the residues proposed to be part of the putative K-channel had significantly reduced catalytic activity upon mutation: T219V, Y227F/Y228F, N293D, and Y321F. The data strongly suggest that in the C-family only one channel functions for the delivery of both catalytic and pumped protons. In addition, it is also proposed that a pair of acidic residues, which are totally conserved among the C-family, may be part of a proton-conducting exit channel for pumped protons. The residues homologous to these acidic amino acids are highly conserved in the cNOR family of nitric oxide reductases and have previously been implicated as part of a proton-conducting channel delivering protons from the periplasmic side of the membrane to the enzyme active site in the cNOR family. It is possible that the C-family contains a homologous proton-conducting channel that delivers pumped protons in the opposite direction, from the active site to the periplasm.

The heme−copper superfamily is structurally and catalytically diverse, with members that perform either oxygen reductase or nitric oxide reductase reactions. The oxygen reductases are terminal oxidases in the respiratory chains of mitochondria and aerobic bacteria and archaea. These enzymes catalyze the reduction of $O_2$ to $H_2O$ utilizing a bimetallic active site that contains a high-spin heme and a copper ion. The oxygen reductases couple the chemical reaction to an electrogenic proton pump in which one proton is pumped across the membrane per electron transferred to the active site (*1*−*3*). The proton electrochemical gradient (protonmotive force) produced can be coupled to chemical synthesis, membrane translocation processes, and bacterial locomotion. The net reaction is

$$O_2 + 8H^+_{IN} + 4e^- \leftrightharpoons 2H_2O + 4H^+_{OUT}$$

where the subscripts IN and OUT refer to the cytoplasm and periplasm, respectively, for the bacterial and archaeal enzymes.

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* Address correspondence to this author at the Department of Biochemistry, University of Illinois. E-mail: r-gennis@uiuc.edu. Fax: 217-244-3186. Tel: 217-333-9075.

‡ Center for Biophysics and Computational Biology, University of Illinois.

§ Department of Chemistry, University of Illinois.

|| Department of Biochemistry, University of Illinois.

⊥ University of Texas Health Science Center.

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The nitric oxide reductases catalyze the reduction of NO[1] to $N_2O$ at an active site consisting of a high-spin heme and an iron ion. The nitric oxide reductases are not electrogenic and do not pump protons or generate a protonmotive force (*4*). The protons used for chemistry in the nitric oxide reductases come from the periplasm, the opposite side of the membrane than in the oxygen reductases (*5*).

$$2NO + 2H^+_{OUT} + 2e^- \leftrightarrows N_2O + H_2O$$

The oxygen reductases are multisubunit integral membrane complexes and have been previously classified into three families based on structural analyses: the A-, B-, and C-families of oxygen reductases (*6*). More recent work (Hemp and Gennis, manuscript in preparation) has identified five additional oxygen reductase families: the D-, E-, F-, G-, and H-families. It appears that enzymes from each of the eight oxygen reductase families couple the reduction of oxygen to proton pumping and the generation of a transmembrane voltage (*7*−*13*), whereas nitric oxide reductases from either of the known families (cNOR and qNOR families) do not pump protons and are not electrogenic (*5*, *14*−*16*).

Subunit I is the core of the multisubunit complex and defines the heme−copper superfamily. It is the only subunit common to all of the oxygen reductase families and contains the ligands for three redox-active metals: a low-spin heme (two histidine ligands) and the bimetallic catalytic site composed of a high-spin heme (one histidine ligand) and a nearby copper ion, $Cu_B$ (three histidine ligands). The low-spin heme accepts electrons from the electron donor specific for the particular complex and transfers them to the catalytic site for oxygen reduction. X-ray crystallographic structures have been reported for members of both the A-type (*17*−*21*) and B-type (*22*) oxygen reductase families. These structures show that the core of subunit I is formed by 12 transmembrane helices arranged in a pseudo-3-fold rotational symmetry with the symmetry axis perpendicular to the membrane. The 3-fold symmetry produces three pores (A, B, and C) which span the length of the protein (Figure 1). The low-spin heme is located in pore C, whereas the binuclear center is in pore B. In members of the A- and B-families X-ray crystallography (*17*, *18*, *21*) and mass spectrometry (*23*, *24*) have identified a novel cross-inked histidine−tyrosine cofactor in the active site. The cofactor is formed between a conserved active site tyrosine and one of the histidine ligands to $Cu_B$. Recently, a similar cross-linked cofactor has been found in the C-family of oxygen reductases (*23*, *25*). However, in this family the tyrosine residue involved in the cross-link is located within a different transmembrane helix in comparison to the A- and B-families (*26*).

Mutagenesis studies of members of the A-family have identified two conserved proton input channels necessary for function: the D-channel and the K-channel (*27*−*31*). The K-channel (located in pore B, Figure 1) leads from a glutamic acid residue on the cytoplasmic side of the membrane, near the interface of subunits I and II, to the conserved His-Tyr cofactor in the active site (*32*, *33*). The K-channel is

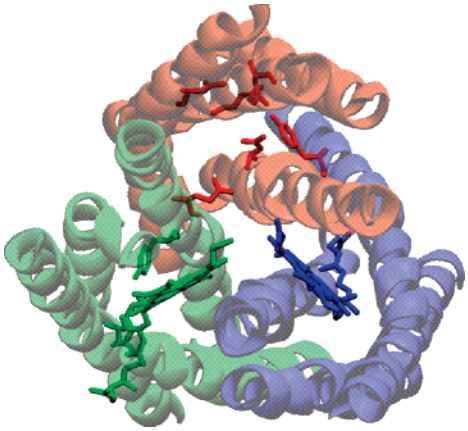

FIGURE 1: Pore structure of cytochrome *c* oxidase. Cytochrome *c* oxidase from *R. sphaeroides* viewed perpendicular to the membrane from the periplasmic side showing the structure of the three pores. Each pore is formed by four transmembrane helices (pore A, red; pore B, green; pore C, blue). The D-channel (red residues) is located within pore A and the K-channel (green residues) within pore B.

responsible for the delivery of at least one, and likely two, of the protons used in catalysis to form $H_2O$ (*34*). The D-channel (located in pore A, Figure 1) leads from an aspartate on the cytoplasmic surface of subunit I to a proton-accepting residue (a glutamate in some A-type subfamilies or a tyrosine in others) near the active site. This channel has been implicated in the transfer of both catalytic protons and protons which are pumped across the membrane (*34*). A third proton-conducting channel (H-channel) has been proposed on the basis of the structure of the bovine cytochrome *c* oxidase (*17*, *35*−*37*); however, strong supporting evidence for a functional role is lacking, and the putative pathway is not found in the microbial A-type oxygen reductases (*38*, *39*).

Both the D- and K-channels contain highly conserved polar and ionizable residues that guide the formation of hydrogen-bonded water chains, assuring kinetically competent proton transfer (*21*, *40*, *41*). The polar and ionizable amino acid side chains also can electrostatically stabilize the transferred proton along the water chain within the channel (*42*, *43*). Without the electrostatic stabilization provided by the protein, protons could be excluded from water-containing pores (*40*).

The residues which form the proton channels in the A-family are not conserved in the C-family of oxygen reductases. Since the C-type oxygen reductases have been shown to pump protons (*9*, *44*−*47*), it is certain that there must be proton-conducting channels in this family that play analogous roles to the D- and/or K-channels. In this work, sequence analyses and structural modeling are used to identify residues which might form putative proton channels in the C-family of oxygen reductases. The functional importance of the identified residues was then examined by site-directed mutagenesis of the $cbb_3$-type respiratory oxidases from *Vibrio cholerae* and *Rhodobacter sphaeroides*. The data support the proposal that the C-type oxygen reductases have a proton-conducting channel that is spatially analogous to the K-channel in the A-type oxygen reductases. However, there are no conserved hydrophilic residues in the C-type oxidases that could form either a channel analogous to the D-channel or an alternative channel from the cytoplasmic surface. Hence, in the C-type oxygen reductases, it is suggested that only one channel is used for the input of

[1] Abbreviations: HC, heme−copper; NO, nitric oxide; NOR, nitric oxide reductase; cNOR, cytochrome *c* nitric oxide reductase; qNOR, quinol nitric oxide reductase.

both catalytic and pumped protons. In addition, a possible proton exit pathway for pumped protons in the C-type oxygen reductases is proposed on the basis of homology to a putative proton input channel in the cNOR nitric oxide reductase family (*14*).

## MATERIALS AND METHODS

*Sequence Analysis*. DNA sequences from over 850 prokaryote genomes and 9 metagenomic projects were analyzed for the presence of C-type oxygen reductase genes using BLAST (*48*). Supporting Information Table S1 lists the genome and metagenomic sequences analyzed along with their accession information. Sequence alignments were performed using MUSCLE 3.52 (*49*) and manually adjusted with Jalview (*50*). Transmembrane regions of the C-type oxygen reductase family were predicted from multiple sequence alignments using TMAP (*51*).

*Homology Modeling*. Structural models of subunit I for various members of the C-type oxygen reductase family were generated using homology modeling techniques as described in Hemp et al. (*26*). Swiss-Model (*52*) was used to generate homology models based on crystal structures of members of the heme−copper superfamily. The high-spin heme, low-spin heme, and copper ion were incorporated into the modeled protein structures, and water molecules were predicted using DOWSER (*53*). Minimization of the models was performed with NAMD2 (*54*) using a quenched MD simulated annealing procedure. A modified version of the CHARMM27 Proteins and Lipids release force field (*55*) that included modified parameters for heme *b* and $Cu_B$ (*26*) was used in the calculations.

*Mutagenesis of the C-Type Oxygen Reductase from V. cholerae*. The oligonucleotides used for mutagenesis were synthesized by IDT. Site-directed mutagenesis was performed using Stratagene QuikChange kits as previously reported (*26*). Sequence verification of the mutagenesis reactions was performed at the Biotechnology Center at the University of Illinois, Urbana−Champaign.

*Mutagenesis of the C-Type Oxygen Reductase from R. sphaeroides*. The expression vector pUI2803NHIS (*56*) was used for site-directed mutagenesis. pUI2803NHIS encodes subunit I with a C-terminal polyhistidine tag. Point mutations of subunit I (*fixN*) were made by recombinant PCR and then subcloned into pUI2803NHIS. Sequence verification of the mutagenesis products was performed at the Molecular Genetics Core Facility, Department of Microbiology and Molecular Genetics, The University of Texas Health Science Center at Houston.

*Purification of Recombinant Proteins*. The mutant proteins with polyhistidine tags were overexpressed and purified as previously described (*26*). Briefly, *V. cholerae* cells were grown at 37 °C in LB media (USB Corp.) with 100 μg/L ampicillin (Fisher Biotech) and 100 μg/L streptomycin (Sigma). Protein expression was induced with 0.2% L-(+)-arabinose (Sigma). *R. sphaeroides* was grown semiaerobically at 30 °C in Sistron media with 2 mg/L tetracycline.

The cells were lysed and centrifuged at 40000 rpm to collect the membranes. The membranes were solubilized with 0.5% dodecyl β-D-maltoside (Anatrace). Nonsolubilized membranes were removed by centrifuging at 40000 rpm for 30 min. The protein was then purified using a nickel affinity column (Qiagen, CA) and eluted using a stepped gradient of imidazole.

*Heme Analysis of Proteins*. Heme staining was used to identify subunits II and III, CcoO and CcoP, containing covalently attached heme *c* (*57*). GeneMate Express PAGE gels from ISC BioExpress were used to separate the purified protein complexes. The gels were then incubated in 3 parts 6.3 mM 3,3′,5,5′-tetramethylbenzidine (TMBZ from Sigma) and 7 parts 0.25 M sodium acetate, pH 5.0, for 1 h. The gels were then stained for heme *c* by adding $H_2O_2$ to 30 mM.

*Spectroscopic Analysis*. Spectra were acquired using an Agilent Technologies 8453 UV−visible spectrophotometer running ChemStation software. Twenty-five microliters of the protein sample at 130 mM was mixed with 100 μL of 50 mM sodium phosphate buffer and 0.05% DM at pH 6.5. The enzymes were oxidized with 2 μL of 1 mM $Fe(CN)_6$ and reduced with dithionite, both obtained from Sigma. Spectra were measured from 375 to 850 nm and analyzed using Matlab.

*Oxidase Activity Measurements*. A YSI model 53 oxygen monitor was used to polarographically measure steady-state oxidase activity. The buffer used for oxidase activity measurements was 50 mM sodium phosphate and 50 mM NaCl at pH 6.5. Ten microliters of 1 M ascorbate, pH 7.4, and 18 μL of 0.1 M TMPD were mixed with 1.8 mL of buffer in the sample chamber at 25 °C. The reaction was initiated by adding 10 μL of 1 μM enzyme, and oxygen consumption was monitored.

## RESULTS

Genomic and sequence analyses, structural modeling, and mutagenesis were performed to investigate the properties of proton channels in the C-type oxygen reductase family. Evolutionarily conserved residues were mapped onto structural models of the C-type family in order to identify potential proton channels. Mutagenesis studies were then performed to verify the roles of the predicted residues in channel formation.

*Sequence and Structural Analysis of the C-Family of Oxygen Reductases*. DNA sequences from over 850 prokaryotic genomes, nine metagenomic projects, and individually submitted sequences to Genbank were analyzed for members of the C-type oxygen reductase family. BLAST analysis identified 380 (275 genomic/90 metagenomic/15 individual submissions) putative members of the C-type family (Supporting Information Table S2). The 275 genomic members represent 23.5% of the total number of oxygen reductases and 20.7% of the total number of heme−copper superfamily members identified. The C-type family has a broad phylogenetic distribution with members from sequenced genomes being unevenly distributed among 14 of the 23 officially named bacterial phyla currently recognized by the NCBI Taxonomy Database. [It should be noted that there are likely to be over 100 bacterial phyla, many of which are not represented in the NCBI Taxonomy Database (*58*).] The majority (89%) were found within the proteobacteria (Supporting Information Table S2); however, the skewed phylogenetic distribution toward proteobacteria is clearly due to the disproportionate genomic sequencing efforts on members of this phylum. To date no members of the C-type

oxygen reductase family have been found in archaea or eukaryotes. A complete genomic and phylogenetic analysis along with subfamily classification of the C-type oxygen reductase family will be presented elsewhere.

Sequence alignments were used to identify conserved amino acid residues which may play important functional roles in members of the C-type oxygen reductase family (Supporting Information Figure S1). Table 1 lists the completely conserved (>99%) and partially conserved (>95%) residues within the family. The conserved residues are classified into four categories: (1) residues conserved in all members of the superfamily; (2) residues conserved within all of the oxygen reductase families but not the NO reductases; (3) residues conserved between the C-type oxygen reductases and at least one of the other families within the superfamily (either oxygen or nitric oxide reductases); and (4) residues conserved only within the C-type oxygen reductase family.

(1) There are only five residues completely conserved in all members of the superfamily. These five residues are the histidines that ligate the low-spin heme (two histidines), high-spin heme (one histidine), and active site metal ion (two histidines), which is copper, $Cu_B$, in the oxygen reductases or an iron, $Fe_B$, in the nitric oxide reductases. The active site metal ion is usually ligated by three histidines; however, a new heme−copper family has been found in which one of the histidines is replaced by an aspartate (to be described elsewhere).

(2) There are no residues uniquely conserved in all of the oxygen reductases that are not also conserved within the nitric oxide reductases. This implies that for functions that are unique to the oxygen reductases, such as proton pumping, different amino acids may play the same structural/functional role in different oxygen reductase families.

(3) There are numerous residues conserved between the C-type family of oxygen reductases and at least one other family of either oxygen reductases or nitric oxide reductases within the superfamily (Table 1). Four residues are particularly interesting: E126, E129, Y227, and T297 (*V. cholerae* numbering). Residues homologous to E126 and E129 are also conserved in the cNOR family of NO reductases, where they have recently been shown (*59*) to be components of a proton input pathway from the periplasmic surface to the active site (*5*, *14*, *15*). Y227 is homologous to a conserved tyrosine in the B-type oxygen reductase family that has been proposed to be part of the putative K-channel, and T297 is homologous to a conserved S/T located within the K-channel (*6*) in the A-type oxygen reductases.

(4) There are ten residues with >99% conservation unique to the C family (Table 1). One of these residues is a glycine (G199). Three of the residues (W166, F248, and W249) form a conserved hydrophobic cluster and are likely to be important for structural reasons. The other six conserved residues unique to the C-family are polar amino acids that are located within pore B (Figure 1) and are candidates for being part of a proton-conducting K-channel analogue: T219, Y228, S240, Y255, N293, and Y321. Y255 is the conserved cross-linked active site tyrosine, previously identified (*26*).

To identify amino acids which could form proton channels analogous to those in the A- and B-type oxygen reductase families, we mapped conserved residues onto structural models of members of the C-type oxygen reductase family.

Table 1: Conserved Residues in the C-Family of Heme−Copper Oxygen Reductases[a]

| residue | structural/ functional role | superfamily conservation |
|---|---|---|
| | >99% Conservation | |
| **H64** | high-spin heme ligand | conserved in all HC's |
| **E126** | proton exit | conserved in NOR's and C-family |
| **E129** | proton exit | conserved in NOR's and C-family |
| W166 | hydrophobic cluster | unique to C-family |
| G199 | structural | unique to C-family |
| **H211** | $Cu_B$ ligand | conserved in most HC's |
| **T219** | K-channel analogue | unique to C-family |
| **Y227** | K-channel analogue | conserved in B- and C-families |
| **Y228** | potential hydrogen bond to R156 | unique to C-family |
| P231 | structural | conserved in B- and C-families |
| **S240** | K-channel analogue entrance | unique to C-family |
| F248 | hydrophobic cluster | unique to C-family |
| W249 | hydrophobic cluster | unique to C-family |
| **Y255** | K-channel analogue/ active site cofactor | unique to C-family |
| G259 | structural | conserved in NOR's and C-family |
| **H261** | $Cu_B$ ligand | conserved in all HC's |
| **H262** | $Cu_B$ ligand | conserved in all HC's |
| P286 | structural | conserved in most HC's |
| **N293** | K-channel analogue | unique to C-family |
| **T297** | K-channel analogue entrance | conserved in A- and C-families |
| **Y321** | K-channel analogue | unique to C-family |
| **H349** | low-spin heme ligand | conserved in all HC's |
| **H351** | high-spin heme ligand | conserved in all HC's |
| G407 | structural | conserved in most HC's |
| R441 | salt bridge with D344 | conserved in NOR's and C-family |
| G445 | structural | conserved in NOR's and C-family |
| | 95% Conservation | |
| R59 | helix cap | unique to C-family |
| R61 | low-spin heme propionate hydrogen bond | conserved in NOR's and C-family |
| R87 | helix cap | unique to C-family |
| R156 | potential hydrogen bond to Y228 | unique to C-family |
| W207 | active site | conserved in all HC's |
| N212 | unknown | unique to C-family |
| **S244** | K-channel analogue entrance | unique to C-family |
| S280 | hydrogen bond H261 | conserved in most HC's |
| G328 | structural, allows for high-spin heme | conserved in all HC's |
| **H341** | cation binding site/ high-spin heme propionate | conserved in most HC's |
| T343 | cation binding site | conserved in most HC's |
| Y367 | unknown | partially conserved in some HC's |
| P371 | structural | partially conserved in some HC's |
| F389 | structural | partially conserved in A-family |
| W390 | structural | partially conserved in some HC's |
| G395 | structural | unique to C-family |
| F427 | unknown | unique to C-family |
| G451 | structural | unique to C-family |

[a] The conserved residues for the C-family were determined by analysis of all 275 genomic sequences (*V. cholerae* numbering). The residues in bold are from published site-directed mutagenesis studies. A likely structural and/or functional role was assigned to the conserved residues on the basis of structural analysis and mutagenesis studies. The conservation of the conserved residues in the C-family relative to other members of the heme−copper superfamily is given.

We identified nine conserved residues which could form a proton channel analogous to the K-channel: T219, Y227, Y228, S240, S244, Y255, N293, T297, and Y321. A model of the *V. cholerae* C-type oxygen reductase highlighting these

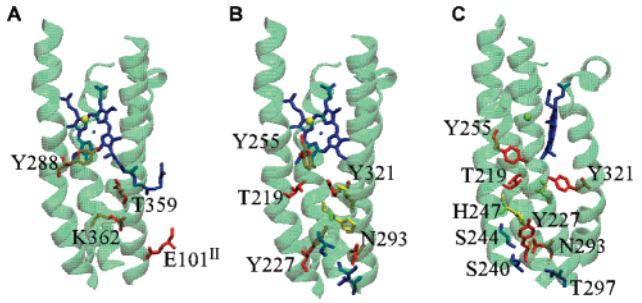


FIGURE 2: K-channel analogue in the C-family of heme−copper oxygen reductases. (A) A-family K-channel. Residues forming the K-channel in the A-type cytochrome *c* oxidase from *R. sphaeroides* are shown in red (Y288, T359, K362, and E101$^{II}$). The $Cu_B$ ion is in yellow, and the helices forming pore B are in green (see Figure 1). (B) C-family K-channel analogue. K-channel analogue in the C-type $cbb_3$ oxidase from *V. cholerae*. Amino acids in red are conserved residues identified by mutagenesis as playing a role in channel formation (Y255, Y321, T219, Y227, and N293). (C) C-family K-channel analogue rotated by 90°. Amino acids in blue are conserved residues which individually had no effect on oxidase activity; however, they may collectively form an entrance to the channel (S240, S244, and T297). Amino acids in yellow are residues which are not conserved in the C-family (H247 and S287); however, they may play a role in channel formation in the individual sequences which contain them.

residues is shown in Figure 2. Sequence alignments of the whole family show that there are no conserved or partially conserved residues that could form a channel analogous to the D-channel (Supporting Information Figure S1). Since the heme−copper superfamily is ancient, it is possible that residues forming a second proton channel might only be conserved within individual subfamilies of the C-type oxygen reductases and that different subfamilies may have channels composed of different conserved residues. Sequence, structural, and phylogenetic analyses have identified at least 13 subfamilies of C-family oxygen reductases. Sequence analyses of these subfamilies revealed no conserved residues even within subfamilies which could form a channel analogous to the D-channel.

*Site-Directed Mutagenesis of Conserved Residues Proposed To Form a Proton Input Channel*. Sequence analysis and structural modeling (described above) identified the following nine conserved polar residues as likely forming a proton-conducting channel analogous to the K-channel in the A-family of oxygen reductases: T219, Y227, Y228, S240, S244, Y255, N293, T297, and Y321. Previously, Y255 was demonstrated to be essential for catalytic function, and this residue was shown to be part of the cross-linked cofactor in the enzyme active site (*23*, *25*). The remaining eight conserved residues in subunit I (CcoN) of the *V. cholerae* C-type oxygen reductase were each replaced by site-directed mutagenesis to assess their functional importance: T219V, Y227F/Y228F (double mutant), N293D, Y321F, S240A, S244A, and T297V. All of the mutants were expressed and assembled correctly, as determined by UV−vis spectroscopy and heme analysis (Figure 3). Oxygen reductase activity assays were performed to assess the effect of each mutation on catalysis (Table 2). Four of the mutants [T219V, Y227F/Y228F (double mutant), N293D, and Y321F] had significantly reduced catalytic activity or were completely inactive, whereas three of the mutations (S240A, S244A, and T297V) had no effect on oxidase activity. The locations of some of the residues are shown in Figure 2.

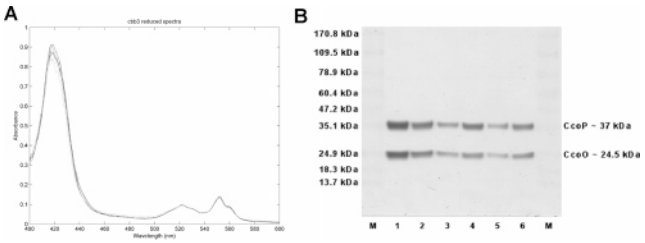


FIGURE 3: (A) Reduced spectra of mutant proteins. (B) Heme-stained PAGE gel of selected mutants of *V. cholerae* C-type oxygen reductase. Heme analyses of all other mutants were identical. Lane assignments: M, marker; 1, wild type; 2, T219V; 3, S240A; 4, N293D; 5, T297V; 6, Y321F.

Table 2: Relative Oxygen Reductase Activity of Mutant C-Type Oxygen Reductases[a]

| *V. cholerae* | | *R. sphaeroides* | | |
|---|---|---|---|---|
| mutant | activity (%) | mutant | activity (%) | location |
| WT | 100 | WT | 100 | |
| E126Q | <1 | E180G | <1 | exit channel |
| E129Q | 47 | E183G | 11 | exit channel |
| T219V | 48 | T275G | <1 | K-channel |
| Y227F/Y228F | 57 | | | K-channel |
| S240A | 100 | | | |
| S244A | 100 | | | |
| | | H303G | 4 | K-channel |
| Y255F | <1 | Y311G | <1 | K-channel |
| T297V | 110 | | | |
| N293D | 26 | N349G | <1 | K-channel |
| Y321F | <1 | Y377G | <1 | K-channel |
| S244A N293D | 25 | | | |

[a] The residues in each row are structurally equivalent. The numbering is for the respective sequences.

Mutations were also made at four sites in the C-type oxidase from *R. sphaeroides*: T275G, Y311G, N349G, and Y377G. Y311 is the active site tyrosine, equivalent to *V. cholerae* Y255, and as expected, the Y311G mutant is virtually inactive (Table 2). This has been shown previously (*47*). The T275G, N349G, and Y377G mutants also have low catalytic activity, qualitatively consistent with the results from the mutations in the equivalent sites (T219V, N293D, and Y321F) in the *V. cholerae* enzyme. The influence of these three mutations in the *R. sphaeroides* oxidase is much more dramatic than the mutations in the equivalent positions in the *V. cholerae* oxidase (Table 2). This may be due to the fact that glycine was placed in each position in the *R. sphaeroides* oxidase, which may cause structural perturbations. Heme analysis of the mutants from the *R. sphaeroides* oxidase is consistent with structural perturbations resulting from the glycine substitutions. Whereas the wild-type *R. sphaeroides* oxidase has a measured heme *c*:heme *b* ratio of 3:2, this ratio is altered in the mutants: T275G (2:1), Y311G (3:1), N349G (4:1), and Y377G (4:1). This is not observed with the mutants of the *V. cholerae* oxidase (T219V, N293D, Y311F, and Y321F).

Previously, H303 was proposed to be part of the K channel in the $cbb_3$-type oxidases (*47*). However, the larger data set used in the current work shows that H303 is not conserved either within the family of C-type oxygen reductases or within any of the subfamilies. The H303G mutant in the current work (Table 2) has very low catalytic activity (4%), and the heme *c*:heme *b* ratio is the same as the wild type, suggesting no major problem of assembly. However, it has

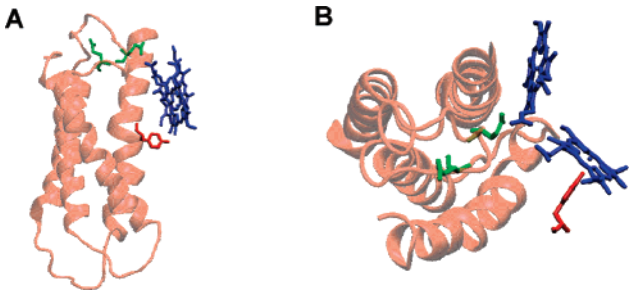


FIGURE 4: Potential exit pathway. The residues identified as forming a possible exit pathway analogous to that in the cNOR family are shown in green (E126 and E129). These residues are located in the loop between helices III and IV at the top of pore A. Modeling of this region is very difficult due to the lack of homology with known structures. This figure shows one possibility of the location of these important residues. A crystal structure of a member of the C-family will be necessary to determine their actual location.

recently been shown (*56*) that the H303A mutant in the same *R. sphaeroides* oxidase is fully active, indicating that the histidine at this location is not critical for activity.

*Site-Directed Mutagenesis of Conserved Residues Proposed To Form a Proton Exit Channel*. Two acidic residues, E126 and E129 (*V. cholerae*), are totally conserved in the C-family oxygen reductases and in the cNOR family of nitric oxide reductases (Supporting Information Figure S2). Mutations were made of both glutamates in the *V. cholerae* (E126Q and E129Q) and in the *R. sphaeroides* (E180G and E183G) C-type oxygen reductases to determine their functional importance. The E126Q (*V. cholerae*) and E180G (*R. sphaeroides*) mutants are virtually inactive. The *V. cholerae* E129Q mutant has about 47% of the wild-type oxidase activity, whereas the equivalent *R. sphaeroides* E183G mutant is virtually inactive (Table 2). The mutants in the *V. cholerae* oxidase have no effect on the heme content or UV−vis spectrum of the enzyme. However, the glycine substitutions in the *R. sphaeroides* oxidase may have caused structural effects, altering the heme ratio for E180G (2:1) and E183G (4:1).

The loss of function obtained with the *V. cholerae* oxidase mutants (Table 2) is very similar to that previously published on the effects of the equivalent mutations (E122Q and E125Q) in the NO reductase activity of a member of the cNOR family (*59*). In the cNORs, the conserved glutamates are proposed to be part of a proton input pathway (*14*, *59*), so it is reasonable to suggest that in the C-type oxygen reductases the two glutamates may form an exit pathway for pumped protons delivered to the periplasm. The location of the two glutamates in a model of the *V. cholerae* $cbb_3$ oxidase is shown in Figure 4. The loop region between helix III and helix IV in the C-type oxygen reductases is difficult to model due to the lack of homology of this region with known crystal structures. This should be kept in mind when proposing structural/functional features of this loop.

## DISCUSSION

Members of the heme−copper superfamily are divided into two classes: the oxygen reductases and the nitric oxide reductases. The active sites for enzymes from both of these classes are buried within the proteins; therefore, the protons required for their chemistries must be provided via proton-conducting channels from the aqueous phase. It has been demonstrated experimentally that the required protons are taken from the inside (bacterial cytoplasm or mitochondrial matrix) for the oxygen reductases and from the outside (bacterial periplasm) for the nitric oxide reductases. In this work sequence analyses, structural modeling, and site-directed mutagenesis were used to identify proton-conducting channels in the C-family of heme−copper oxygen reductases ($cbb_3$-type respiratory oxidases).

Previous studies have identified three distinct families of oxygen reductases (*6*) (the A-, B-, and C-families) and two families of nitric oxide reductases (cNOR and qNOR) (*4*, *60*). More recently, new sequences have been made available from microbial genome and metagenomic sequencing efforts. This has resulted in a much larger data set for the analysis of the heme−copper superfamily, the results of which will be published separately. There are enough sequences available to clearly define a number of subfamilies within the A-, B-, and C-families of oxygen reductases and also to define five additional families (D-, E-, F-, G-, and H-families). Similarly with the nitric oxide reductases, several additional families have been identified in addition to the cNOR and qNOR families (manuscript in preparation).

The A-family of oxygen reductases is by far the largest (72% of the 1170 sequences of heme−copper oxygen reductases included in the current analysis) and most studied group of enzymes in the heme−copper superfamily. Several X-ray structures have been determined and site-directed mutagenesis, along with biophysical methods, has been extensively used to elucidate the structural and mechanistic properties of this family. Two proton-conducting input channels, the D- and K-channels, are well defined by both X-ray crystallography and site-directed mutagenesis of prokaryotic members of the family (*27*−*31*). These two channels are used to provide protons from the bacterial cytoplasm (or mitochondrial matrix) to the active site for chemistry (4 $H^+$) and for proton pumping (4 $H^+$). The D-channel has been a focus of attention because it is clearly involved in the proton pump of the A-family of oxygen reductases. Some mutations within the D-channel can eliminate proton pumping (*30*, *61*, *62*) whereas other mutations completely block all catalytic function (*63*). It has been noted previously that the oxygen reductases in the C-family do not appear to have residues equivalent to those corresponding to the D-channel of the A-family of oxygen reductases (*6*, *12*, *47*). The current work examines this using the large data set now available.

The premise of the approach is that the proton-conducting channels will involve functionally important polar and/or ionizable residues. In principle, this need not necessarily be the case, as exemplified by the gramicidin channel, in which backbone carbonyls are utilized to form a hydrogen bond network with water (*64*). However, the gramicidin channel is different from the channels being considered in the current work because gramicidin conducts cations other than protons whereas the channels of interest in cytochrome oxidase only conduct protons. In channels that only conduct protons through membrane proteins, the participation of polar/ionizable side chains appears to be universal (*41*, *65*). This is clearly observed in the A-family of oxygen reductases, where the pattern of conserved polar amino acids defines both the K- and D-channels (*6*).

In the A-type oxygen reductases, the K-channel leads from a surface-exposed glutamate at the cytoplasmic surface to the active site tyrosine, which is cross-linked to a histidine (*28*, *66*). Besides the active site tyrosine, there are three other conserved residues in the A-family of enzymes which line the K-channel: T/S359, K362, and E101$^{II}$ (subunit II) (numbering of the *R. sphaeroides* $aa_3$-type oxidase) (Figure 2A). Similarly, there is a pattern of highly conserved residues in the D-channel in the A-family of oxygen reductases: D132, N121, N139, N207, Y33, and E286.

Other examples of proteins in which ionizable or polar amino acids are critical components of proton-conducting pathways are the bacterial photosynthetic reaction center (*67*, *68*), the $F_1F_o$-ATP synthase (*69*, *70*), the $bc_1$ complex (*71*−*74*), bacteriorhodopsin (*75*), succinate dehydrogenase (*76*), and the recently discovered E-channel in the fumarate reductase from *Wolinella succinogenes* (*77*).

Of particular interest is the cNOR family of nitric oxide reductases. Sequence analysis shows no pattern of conserved polar residues that could define a proton input channel from the bacterial cytoplasm (Hemp and Gennis, in preparation) but does reveal the presence of two conserved glutamates that could be involved in forming a proton input channel from the periplasmic side of the membrane. Experimental data confirm that protons are, indeed, taken from the periplasm and that the two glutamates do appear to be important for this function (*14*, *59*). Hence, within the heme−copper superfamily, sequence alignments predicting the presence or absence of proton-conducting channels have been validated experimentally for the D- and K-channels of the A-family of oxygen reductases and the cNOR family of nitric oxide reductases.

In light of these correlations, it is meaningful that the pattern of conserved residues identified only one proton input channel for the C-family of oxygen reductases, spatially equivalent to the K-channel in the A-family of oxygen reductases, though largely comprised of different residues. The only residue in common between the K-channels in the A- and C-families of enzymes corresponds to T359 in the *R. sphaeroides* $aa_3$-type oxidase. The expectation tested in this work is that mutations of conserved, polar residues in the putative K-channel will block proton translocation required for both chemistry and proton pumping by the C-family oxygen reductases.

*The K-Channel*. Five of the nine residues (apart from the active site tyrosine) that were proposed to be part of the K-channel in the C-family of oxygen reductases were implicated as being functionally important for catalytic function by site-directed mutagenesis of the *V. cholerae* and *R. sphaeroides* $cbb_3$-type oxidases: T219, Y227 and/or Y228, N293, and Y321 (*V. cholerae* numbers). The location of these residues in a model of the CcoN subunit of the *V. cholerae* enzyme is shown in Figure 2B. In addition, it was previously shown that the active site tyrosine (Y255), located at the terminus of the K-channel is essential for function (*26*). In the A-family of oxygen reductases, there is a conserved glutamic acid in subunit II which is located at the entrance of the K-channel, E101$^{II}$ in the *R. sphaeroides* $aa_3$-type oxidase (*78*). In the C-family of enzymes, a homologue of subunit II is not present, and there are no conserved residues in any of the accessory subunits of the C-family of oxygen reductases (CcoO, CcoP, or CcoQ) that could be identified as equivalent to the glutamate in the A-family of enzymes.

It is noted that the K-channel analogue in the C-type oxygen reductases contains no protonatable residues, in contrast to both the D- and K-channels in the A-type oxygen reductases. It has been suggested previously that a histidine residue (H303 in the *R. sphaeroides* C-type oxygen reductase) near N293 might be an important component of the K-channel in the C-type oxygen reductases (*47*). Mutation of this histidine to valine resulted in reduced oxidase activity (*79*, *80*). However, this residue is not conserved in the C-family (Supporting Information Figure S1), and mutation to alanine (H303A) in the *R. sphaeroides* $cbb_3$-type oxidase has been reported to have no effect on enzyme activity (*56*). The current work (Table 2) shows that a glycine is not tolerated at this location, which does not imply that H303 is a required for proton translocation.

The three conserved residues for which mutation had no effect on activity (S240, S244, and T297) (see Figure 2B) are all located just below N293 near the entrance to the channel. The N293 residue is estimated in structural models to be 7−10 Å from the cytoplasmic surface and unlikely to have direct contact with protons from the cytoplasm. A proton-conducting pathway should be required for the transfer of protons from the cytoplasm to the level of N293. The S240, S244, and T297 residues could participate in this, but it may be that there are multiple pathways possible so that no single residue is essential for the function. A similar situation is proposed for the highly conserved serines and threonines (S142, S200, and S201; *R. sphaeroides* $aa_3$-type numbering) within the D-channel of the A-family of oxygen reductases (*81*). Multiple mutations might resolve this matter.

*Absence of a Second Proton Input Channel in the C-Family of Oxygen Reductases*. Analysis of the C-family shows that no residues are conserved that could reasonably be postulated to be components of a proton input channel apart from the K-channel. Earlier work noted that there is a conserved tyrosine in the C-family that is structurally analogous to the tyrosine at the top of the D-channel in some subfamilies of the A-type oxygen reductases (*6*, *47*). It was previously reported that mutation of this residue to phenylalanine in the *R. sphaeroides* C-type oxygen reductase (Y265F) resulted in an enzyme with low activity; however, there was no effect on pumping (*47*). Sequence analysis shows that this residue is not conserved and is a phenylalanine in many C-type sequences (Supporting Information Figure S1). Therefore, it is unlikely that this tyrosine plays the same role as in the A-type oxygen reductases. If the C-family has a channel analogous to the D-channel, it is not formed by conserved residues.

An alternative proton input channel has been suggested between D364 (*R. sphaeroides* $cbb_3$ oxidase numbering) on the cytoplasmic side and E383 (*R. sphaeroides*) near the high-spin heme (*47*). In support of this, E383 was mutated to glutamine, resulting in complete loss of activity (*47*). However, sequence analysis shows that neither D364 nor E383 are conserved within the C-family of oxygen reductases or within any subfamily (Supporting Information Figure S1). In many sequences the residue equivalent to E383 is a glutamine. Structural modeling (not shown) predicts that E383 could be as close as ~4 Å from the histidine ligand to the high-spin heme in the active site. Hence, mutation of this residue might modify the midpoint potential of the high-

spin heme and could explain the experimental results (*47*). There are no other conserved residues between D364 and E383 which could form the rest of a channel. There are also no conserved residues anywhere else in the protein which could form a potential alternative proton channel in any of the subfamilies comprising the C-family of oxygen reductases.

If a second proton input channel is present in the C-family of oxygen reductases, it does not contain conserved polar residues.

*A Possible Proton Exit Channel*. The C-type oxygen reductase family is closely related to the cNOR and qNOR nitric oxide reductase families (*6*, *82*) and shares a number of conserved sequence features with them that are not found in the other oxygen reductase families (Table 1). One feature is the presence of the two conserved glutamate residues (E126 and E129, *V. cholerae* numbering) located in the loop region between helix III and helix IV on the periplasmic side of the protein (Supporting Information Figure S2). Modeling (Figure 4) shows that the conserved glutamates in the III−IV loop region of the C-family of oxygen reductases could be in a similar location as the glutamates in models of the cNOR nitric oxide reductases (*5*, *14*). It is therefore reasonable to propose that this pair of glutamates may share a common role in both the cNOR's and the C-type oxygen reductases. Mutations were made to test the functional importance of these glutamates in the C-family. Activity measurements with the *V. cholerae* oxidase clearly demonstrate that these two glutamates are functionally important (Table 2). These results are very similar to those obtained in the cNOR family (*59*) in which it was proposed that the two glutamates are involved in a proton-conducting pathway from the periplasm to the enzyme active site. Possibly, the equivalent glutamates in the C-type oxygen reductases form an exit pathway for protons to the periplasm. This proposal must be further examined by analysis of proton pumping in these mutants.

The III−IV loop region has been previously modeled in a manner that places the two glutamates within transmembrane helix IV (*47*). It was proposed that residue Y181 (*R. sphaeroides* C-type oxidase numbering) in the III−IV loop forms a critical association with W263 in helix VI and that K179 in the III−IV loop interacts with the heme propionates along with R471 in the X−XII loop. These residues were all conserved in the data set of Sharma et al. (*47*). However, the current sequence analysis with the larger data set shows that K179, Y181, W263, and R471 are not conserved within the C-family of oxygen reductases.

## CONCLUSIONS

(1) Sequence analyses, structural modeling, and site-directed mutagenesis demonstrate that the C-type oxygen reductases have a conserved proton channel spatially analogous to the K-channel leading from the cytoplasmic surface to the novel cross-linked histidine−tyrosine cofactor in the active site.

(2) Extensive sequence and structural analyses have also shown that the C-type oxygen reductases do not have a conserved channel analogous to the D-channel and that no other residues are conserved that could form an alternative channel from the cytoplasmic surface of the protein to the active site. These results strongly suggest that the C-type oxygen reductases have only one proton input channel and that the K-channel is responsible for the uptake of all protons, both for catalysis and pumping. Although the principles behind the mechanism of proton pumping are very likely to be the same, it is evident that the functions of residues in the D-channel shown to be critical in the A-family of oxygen reductases must be performed differently in the C-family of oxygen reductases. The invariant structural features shared by all of the heme−copper oxygen reductases are the metal ligands and the heme propionates. These may play specific roles in the proton pump mechanism, as has been proposed in numerous models (*83*−*86*).

(3) Two totally conserved glutamates in the C-family of oxygen reductases may be part of a proton exit pathway.

Further experiments, including the structural determination of a C-type oxygen reductase, will be necessary to clarify how these enzymes function.

## SUPPORTING INFORMATION AVAILABLE

Two figures and two tables as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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