CHARACTERIZATION OF THE QUINOL NITRIC OXIDE REDUCTASE FROM
PERESEPHONELLA MARINA

BY

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THESIS

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ABSTRACT

qNOR is a nitric oxide reductase in the heme-copper oxidoreductase superfamily that catalyzes the two electron reduction of NO to N$_2$O using electrons derived from quinol. In this study, we heterologously expressed and characterized the qNOR from the thermophilic, denitrifying bacteria *Persephonella marina*. The reaction rate of this qNOR varied linearly from $0.22\pm0.05 \frac{\mu M\ NO}{\mu M\ protein+s}$ at 42°C to $2.3\pm0.3 \frac{\mu M\ NO}{\mu M\ protein+s}$ at 60°C. We also report the first $K_m$ for a purified qNOR, and first activation energy of any NOR, 3.1±0.6 μM NO and 114 kJ/mol respectively. qNORs have three highly conserved glutamate residues in their active sites. Mutagenesis studies demonstrate that E476 is likely the Fe$_B$ ligand, E480 helps maintain the electronegative environment of the active site, and that E545 is involved in the proton entry pathway. A binding site for the inhibitor HQNO was identified in the recently reported qNOR structure (Matsumoto et al. 2012). Using sequence analysis and mutagenesis we were able to confirm that the quinol binding site is comprised of residues H295, R705, and D709, which are the same residues that bind HQNO. Also in this same crystal structure, a hydrophilic channel connecting the cytoplasm to the active site was resolved and hypothesized to be a proton entry channel, similar to the K-channel of heme-copper oxidases. However, the channel was not confirmed for active protein. In this study, we also investigated proton entry pathways in qNOR. We found that the residues lining the proposed cytoplasmic proton entry channel were not conserved and mutations of the polar residues to leucine had little effect on activity. These findings indicate that this channel is not important for active enzyme. We also attempted to elucidate a complete periplasmic proton entry pathway, but as most mutations were made near the active site heme b$_3$, a total lack of activity in most mutants was difficult to interpret. However, the near complete conservation of several polar residues between the heme b$_3$
propionate and the active site, allows us to hypothesize their importance in delivering protons to
the active site once they reach the level of the heme propionate.
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CHAPTER ONE: GENERAL INTRODUCTION

Nitrogen is a fundamental element for all life on Earth, therefore an understanding of the complex network of biological nitrogen transformations is essential. In recent history the nitrogen cycle has been drastically altered due to the agricultural application of nitrogen in the form of fertilizers (Tilman 1999). Only 55% of the applied nitrogen is actually utilized by crops (Liu et al. 2010), and the majority of the remaining nitrogen is returned to the atmospheric N\(_2\) pool via microbial nitrification and denitrification. Denitrifying organisms reduce nitrate and nitrite through a series of reactions:

\[
\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2
\]

While N\(_2\) is the ultimate product of denitrification, N\(_2\text{O}\) is a common final product in many microorganisms (Bonin et al. 2002). The increased flux through denitrification has led to increased atmospheric N\(_2\text{O}\), an extremely potent greenhouse gas and known contributor to ozone depletion (Ravishankara et al. 2009). Due to the potential impact on the global climate resulting from increased atmospheric N\(_2\text{O}\), it is very important to understand the key enzyme in its production, nitric oxide reductase.

Nitric oxide reductases (NORs) belong to the heme-copper oxidoreductase superfamily, which is a diverse family of integral membrane enzymes that play a major role in biological energy transformation. This superfamily is divided into two classes based on the chemistry they perform: oxygen reductases and nitric oxide reductases. Nitric oxide reductase members catalyze the two electron reduction of NO:

\[
2\text{NO} + 2e^- + 2H^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}
\]
CHAPTER TWO: THE CONSERVED ACTIVE SITE GLUTAMATES AND QUINOL BINDING SITE

Introduction

Within the NOR families, there are two closely related families: cNOR and qNOR. They are named to indicate the electron donors they use: cytochrome c and quinones, respectively. cNOR is a two subunit complex (Hino et al. 2010), while qNOR is a single subunit, resembling a fusion of the two cNOR subunits (Matsumoto et al. 2012). cNOR has been isolated from many organisms, and several mutational studies have been completed, notably from Paracoccus denitrificans (Flock et al. 2009 and Thorndycroft et al. 2007) and recently, Thermus thermophilus (Lici A. Schurig-Briccio, et al. manuscript in preparation). qNOR has been studied in less detail, and has only been isolated from Ralstonia eutropha (Cramm et al. 1999), Pyrobaculum aerophilum (de Vries et al. 2003), and Geobacillus stearothermophilus (Matsumoto et al. 2012). The crystal structure of qNOR from G. stearothermophilus was recently published. Unfortunately, the published qNOR structure is of an inactive enzyme variant, likely due to the selective crystallization of enzymes with the incorrect metal assembled into the active site.

qNOR is known to be an integral membrane enzyme that contains a low-spin heme b and a binuclear center containing a high-spin heme b₃ and an active site metal (Matsumoto et al. 2012), likely an iron (Fe₈) as was found in the qNOR from Ralstonia eutropha (Cramm et al. 1999). qNOR receives electrons from quinol, and a putative quinol binding site was identified in the crystal structure using the quinol mimic, HQNO (Matsumoto et al. 2012). While most cNORs are part of a chain of denitrification enzymes in environmental microbes, several qNORs have been found with or without other denitrification enzymes in human pathogens (Barth et al. 2009).
These organisms are able to use qNOR to detoxify the NO produced by host macrophages (Sevanin et al 2005).

In this study, we have expressed and characterized a qNOR from the thermophilic, denitrifying bacteria *Persephonella marina*. The organism was originally isolated from a deep sea thermal vent, where it is known to survive over a temperature range of 55 to 80°C, but lives optimally at 73°C (Götz et al. 2002). With the isolated qNOR from this thermophilic organism, we completed the first comprehensive mutational study of a qNOR. This work also represents the first study of a thermophilic NOR over a range of elevated temperatures. Furthermore, we were able to measure the activation energy and $K_m$ for NO for the first time in a purified qNOR, elucidate the function of the conserved glutamates within the active site, and confirm the quinol binding site.

**Experimental Procedures**

**Construction of the NOR expression plasmid**

Using *P. marina* genomic DNA, the 2,200 base-pair region encoding the nitric oxide reductase was amplified using PCR. The fragment was then ligated into a pET-22b vector (Novagen). Codons for six histidine residues were added to the C-terminal end of the protein during amplification with specific primers. All mutants were created with site directed mutagenesis using the Quick Change Kit (Stratagene).

**Expression and Purification of Recombinant qNOR**

The C43 strain of *E. coli* (DE3) carrying the pET-22b expression vector along with a plasmid for the expression of rare codons, pRARE, was grown in LB media at 37°C shaking at 200 rpm. Once the cultures reached an OD$_{600}$ of 0.7, the expression vector was induced by adding
isopropylthio-β-galactoside (IPTG) to a final concentration of 1 mM. The cultures were grown under the same conditions for four hours post induction, and then the cells were harvested by centrifugation at 8,000 rpm for 10 minutes at 4°C. The cell pellet was resuspended in buffer containing 100 mM TrisHCl pH 8, 100 mM NaCl, and 5 mM MgCl₂ along with DNase (Sigma) and protease inhibitor cocktail (Sigma). The cells were broken by passing the cell suspension through a Microfluidizer three to four times at a pressure of 80,000 psi. The membrane fraction was collected by centrifugation of the disrupted cell membranes at 40,000 rpm for 4 hours at 4°C. The isolated membranes were then resuspended in buffer containing 100 mM TrisHCl pH 8 and 100 mM NaCl. n-Dodecyl-β-D-maltopyranoside (DDM, Anatrace) was added slowly to a final concentration of 1% and then allowed to solubilize with slow stirring for 2 hours at 4°C. This solution was centrifuged at 40,000 rpm for 30 minutes at 4°C and then the supernatant was applied to a nickel-NTA agarose resin (Qiagen) that had been pre-equilibrated with buffer containing 100 mM TrisHCl pH 8, 100 mM NaCl, 0.05% DDM, and 25 mM imidazole. The resin was washed with the same buffer containing up to 50 mM imidazole, and then the protein was eluted in the same buffer with 100 mM imidazole. The protein was concentrated using 100 kDa molecular weight cutoff centrifugal filter units (Millapore) and dialyzed in buffer of 100 mM TrisHCl pH 8, 100 mM NaCl, 0.05% DDM, and 10% glycerol. Small aliquots of the protein sample were flash frozen in liquid nitrogen and then stored at -80°C until use.

UV-visible spectra were taken using an Agilent Technologies 8453 UV-visible spectrophotometer combined with ChemStation software. Concentration of the samples were determined using the pyridine hemochrome method, with an extinction coefficient of $\varepsilon_{412} = 0.25 \mu M^{-1} \text{cm}^{-1}$ (of the oxidized spectrum). While this number was calculated experimentally for this enzyme, it is in good agreement with the value found for *G. stearothermophilus* qNOR, 0.204
µM⁻¹ cm⁻¹ (Matsumoto 2012 Nature). Metal Analysis was performed using a Spectro Genesis inductively coupled plasma optical emission spectrometer (Thompson et al 2010).

**Enzymatic Activity Measurements**

Nitric oxide reduction activity was measured using the Microrespiration System and multimeter (Unisense) equipped with a miniaturized Clark-type nitric oxide sensor (Unisense) and monitored by SensorTrace software. The sensor was polarized with +1250 mV and allowed to stabilize for at least 18 hours. Once a stable signal was obtained, the sensor was calibrated in a solution of 0.1 M H₂SO₄ and 0.1 M KI with a standard of nitrite (Ricca Chemical Company) at the temperature required for the activity assays. For the enzymatic activity measurements, the 1 mL reaction contained 50 mM citrate buffer pH 6, 100 mM D-glucose, 10 µg ml⁻¹ glucose oxidase and 10 µg ml⁻¹ catalase, 0.01% DDM, 1 mM dithiothreitol (DTT), and 100 µM menadione. The sensor tip was lowered into the reaction chamber and allowed to stabilize (approximately five minutes) with gentle stirring (100 rpm) and then a nitric oxide (NO) solution (prepared by injecting nitric oxide gas into an anoxic vial of H₂SO₄ at pH 3) was added to a concentration of approximately 30-40 µM NO. The degradation of NO without enzyme was recorded. The reaction was started with the addition of 0.1 µM purified enzyme, and the degradation of NO was recorded and used to calculate activity. Activity units are expressed as µM NO µM protein⁻¹ s⁻¹. Kₘ values were calculated with Origin software using a fit to a Michaelis-Menten curve.

**Sequence Analysis**

Genes encoding cNOR and qNOR proteins were retrieved from over three thousand sequenced genomes. The sequences were aligned using Muscle v.3.8 and manually edited to match the
structure alignment of qNOR and cNOR when necessary. Conservation was observed and calculated using BioEdit software.

Results

**Characterization of the qNOR from Persephonella marina**

The qNOR from *P. marina* was heterologously expressed in *E. coli* then purified, and the sample had a single band at approximately 55 kilodaltons (kDa) on a SDS-PAGE gel (Figure 2.1a). Although the calculated molecular weight of the protein is 83 kDa, it migrates faster in the gel due to its high hydrophobicity. The optical spectra has a Soret band at 412 nm and 424 nm in the oxidized and reduced protein respectively, and peaks at 527 nm and 558 nm only in the reduced protein spectra (Figure 2.1b), characteristic of heme b (Berry and Trumpower 1987). In cNOR, the active site contains a non-heme Fe (Hino et al. 2010), however Zn was found in the analogous position in the qNOR structure. The Zn containing qNOR is enzymatically inactive, suggesting that another metal is required for correct assembly of the active site (Matsumoto et al. 2012). Metal analysis of the isolated *P. marina* qNOR found that the sample contained significant quantities of Fe and only a trace amount of Zn, confirming Fe as the correct active site metal.

The qNOR had activity reaction rate of $0.74 \pm 0.07 \frac{\mu M \ NO}{\mu M \ protein \times s}$ at 50°C, which was sensitive to several inhibitors. The quinol site inhibitors, aurachin and 2-heptyl-4-hydroxy quinoline-N-oxide (HQNO), were found to completely abolish activity at 250 nM and 20 µM respectively. These concentrations are comparable to typical inhibition in other enzymes (Yap et al. 2010). Cyanide (KCN), known to bind to the active site of heme-copper oxygen reductases and cause complete inhibition (Nůsková et al. 2010), has also been shown to inhibit cNOR with a
concentration of 100 µM (Lici A. Schurig-Briccio, et al. manuscript in preparation). 200 µM KCN caused a complete loss of activity in qNOR as well.

The measured $K_m$ for NO in isolated *P. marina* qNOR is 3.1±0.6 µM at 50°C (Figure 2.2). There are no other reported $K_m$ values for an isolated qNOR, however in whole cells of *Neisseria gonorrhoeae* the apparent $K_m$ for NO is 1.2 µM (Cardinale and Clark 2005). A $K_m$ of 0.25 µM has been reported for the cNOR from *P. denitrificans* (Fujiwara and Fukumori 1996), which is lower than the values calculated for the qNORs. It is possible that there is a real difference in $K_m$ between cNOR and qNOR, which may be functionally important, as has been seen in the oxygen reductase families (Ekici et al. 2012, Ouyang et al. 2012).

**Temperature Dependence of Activity and Activation Energy**

*P. marina* is a thermophilic bacterium known to grow optimally at 73°C (Götz et al. 2002). However due to experimental constraints, our assays could only be performed long-term at 50°C, with short-term experiments possible up to 60°C. Our experiments over the temperature range of 42°C to 60°C demonstrated a very clear temperature dependence for enzymatic activity. At 42°C, the reaction rate was 0.22±0.05 $\frac{\mu M \text{ NO}}{\mu M \text{ protein} \cdot s}$; three fold lower than the rate at 50°C. At 60°C, the rate was 2.3±0.3 $\frac{\mu M \text{ NO}}{\mu M \text{ protein} \cdot s}$, another three fold higher than the rate at 50°C. Because the temperature dependence is linear within this temperature range, we can extrapolate a reaction rate at the ideal growth temperature of 73°C. As shown in Figure 2.3a, this number is approximately 3.7 $\frac{\mu M \text{ NO}}{\mu M \text{ protein} \cdot s}$, which is in good agreement with the reported value for the qNOR from *Ralstonia eutropha*, of 3.6 s⁻¹ (Cramm et al. 1999). The qNOR from the thermophile *Geobacillus stearothermophilus* has activity rate of 0.7 $\frac{\mu M \text{ NO}}{\mu M \text{ protein} \cdot s}$ at 40°C (Matsumoto et al. 2002).
2012), while this organisms’ optimal temperature is 55°C (Novotny et al. 2008). Therefore the measured reaction rate of P. marina at 50°C is also in good agreement with other qNORs at suboptimal temperatures.

We were able to use the Arrhenius equation \( k = Ae^{-Ea/RT} \) to calculate the activation energy for the reaction. The Arrhenius plot in Figure 2.3b also displays a linear relationship between the natural log of rate and reciprocal temperature, meaning that the activation energy does not appear to change with temperature. Using the slope of this line, we calculated that the activation energy as +114 kJ/mol. As this is such a large energy, it could potentially explain why the NORs tend to have lower reaction rates.

**Active Site Glutamates**

In the active site of cNOR and qNOR there are three conserved glutamate residues (Matsumoto et al. 2012) (Figure 2.4). These residues have been investigated in cNOR (Lici A. Schurig-Briccio, et al. manuscript in preparation, Flock et al. 2009 and Thorndycroft et al. 2007) however no comprehensive study has been completed for a qNOR. We mutated each conserved glutamate to both glutamine and aspartate to attempt to elucidate the roles that the functional group and side chain length play in activity.

E476 is near the active site metal, and it is conserved in all qNORs. The structure of cNOR (Hino et al. 2010) shows that the homologous residue (E211) is an Fe_B ligand, while in the qNOR crystal structure the homologous residue (E512) is in the same spatial location, but is not a ligand to the active site metal (Matsumoto et al. 2012). We hypothesize that this difference is a result of incorrect metal incorporation into the crystal structure qNOR active site, with Zn replacing Fe, as there is no known reason why the Fe_B coordination should be different between
these two closely related enzymes. Therefore as we have shown that the *P. marina* qNOR active site metal is Fe, we will assume that E476 is a Fe\(_B\) ligand.

All mutant activities were measured at both 42°C and 50°C. E476Q is the only mutation of this residue that preserved activity (Table 2.1), and this activity was only detected at 50°C. The enzyme was unstable at 50°C, and the activity was very low, with only 7% of wild type activity. E476D did not have detectable activity at any measured temperatures. The same results were seen with the E476A mutant, which is comparable to what was seen in the *G. stearothermophilus* qNOR (Matsumoto et al. 2012), as they report that the equivalent mutant (E512A) had no detectable activity. Similar results were also seen with the cNOR from *P. denitrificans* where the equivalent of E476A (E198A) had an activity ranging from 1.5-5% of wild type activity (Flock et al. 2009 and Thorndycroft et al. 2007). Taken together, this strongly suggests that the E476 residue is extremely important, and furthermore our results demonstrate that it is the size of the residue that is more important than the functional group. Conversely, these results differ to what was recently determined with the *T. thermophilus* cNOR. Mutations to D, Q, and A in the equivalent residue (E211) all retain significant activity (Lici A. Schurig-Briccio, et al. manuscript in preparation). While this differs from our results and from previously published results, it tells us that this residue, while extremely important, may not be entirely essential, at least in the cNOR family.

E480 has been proposed to be important for creating an electronegative environment near the active site in the cNOR family (Hino et al. 2010), and it is conserved in 92% of qNORs as well. To investigate the function of E480 in qNOR, we made the mutations E480D and E480Q. E480Q had no detectable activity, while E480D maintained significant activity. In fact, this mutation led to a dramatic increase in activity at 42°C and 50°C, and also displayed a different
inhibition profile than wild type qNOR at both temperatures. Inhibition required at least double the amount of KCN, aurachin, and HQNO as wild type qNOR. Future studies to investigate the activity rates of qNOR with naturally occurring non-conserved glutamates in the corresponding position would be interesting and potentially help elucidate the function.

NO reduction requires protons, therefore qNOR must have a pathway for protons to get to the active site. While the proton entry pathway has been disputed, most proposals terminate the proton entry pathway at the equivalent residue of E545 (Salomonsson et al. 2012, Matsumoto et al. 2012, Hino et al. 2010, Lici A. Schurig-Briccio, et al. manuscript in preparation), which is conserved in all qNORs. It has been proposed that E545 delivers protons to the active site, and our mutagenesis studies support this hypothesis. E545D retains significant activity at both temperatures, while E545Q shows a very low activity at 50°C and no activity at 42°C (Table 2.1). E545 must be very important for active site stability because both mutations led to unstable enzymes. To further investigate the effect of residue size, we tested E545M which had no detectable activity, confirming that the functional group is much more important at this site than the size of the residue.

The temperature dependence seen in the active site mutations show some very interesting traits. When the protein was first introduced to the reaction chamber, the activity progressed as normal, but after a few minutes (2-3 minutes) in most mutants, the activity slowed to a stop. We interpreted this phenomenon as instability at elevated temperatures and attributed it to a disturbance in some sort of interaction critical to protein stability. We never saw a similar behavior in the wild type qNOR protein. Based on our results, we hypothesize that the conserved glutamates within the active site of qNOR are important for both activity and structural stability.
Quinol Binding Site

The structure of qNOR was solved with a quinol mimic, HQNO, bound to the protein (Matsumoto et al. 2012) (Figure 2.5a). To verify that this location is the binding site of the native menaquinol, we made mutations to the conserved residues found within hydrogen bonding distance of the functional groups on the quinol mimic. Based on spatial location within the 3-dimensional structure and residue conservation, we propose that H295, R705, and D709 make up the menaquinol binding site. Within the qNOR family, R705 and D709 are both 100% conserved. H295 is 96% conserved, and in the remaining 4%, there is always another amino acid in the same location that could form a hydrogen bond. Within the cNOR family, we do not see any conservation of H295 or D709, but we do see conservation of R705 (Figure 2.5b). However, as qNOR evolved from cNOR, it is possible that qNOR used this already present arginine for the new function of quinol binding.

The crystal structure has several conserved, hydrophobic residues that are close enough to interact with the nonpolar tail of the quinone mimic, however we were unable to investigate the role of these residues due to the insolubility of natural quinones in our experimental setup. Since we used the menaquinone analog, menadione, which has no hydrophobic tail, we were only able to investigate the residues of the quinol binding site that interact with the functional region of the quinone.

To test our hypothesis that H295, R705, and D709 make up the quinol binding site, we made mutations of these three residues to residues that should not be able to participate in hydrogen bond formation. All mutations, even a fairly conservative mutation, D709N, resulted in mutants with no detectable activity at both 42°C and 50°C (Table 2.2). Taken together with the crystal
structure, and conservation data, this strongly supports the hypothesis that H295, R705, and D709 make up the functional residues of the quinol binding site.

**Discussion**

Denitrification is an integral part of the nitrogen cycle so it is imperative that we understand the enzymology of this process. In this work the qNOR from *Persephonella marina* was heterologously expressed in *E. coli* and biochemically characterized. The purified enzyme showed the characteristic heme b peaks. Only minimal zinc was found in the metal analysis, confirming that iron is the correct active site metal of qNOR enzymes. The reaction rate of qNOR varies linearly from 42°C (0.22±0.05 $\frac{\mu M \ NO}{\mu M \ protein \cdot s}$) to 60°C (2.3±0.3 $\frac{\mu M \ NO}{\mu M \ protein \cdot s}$). This allowed us to extrapolate an activity of 3.7 $\frac{\mu M \ NO}{\mu M \ protein \cdot s}$ at 73°C, which is the ideal growth temperature for *Persephonella marina*. It also allowed us to calculate the activation energy of the catalyzed reaction to be +114 kJ/mol, the first reported activation energy value for a NOR.

Oxygen reductase members of the heme-copper oxidoreductase superfamily have much larger reaction rates than all of the NORs currently characterized. For example, both the B-family oxygen reductase (ba3 oxidase) and cNOR from *T. thermophilus* have been characterized. The B-family oxygen reductase has a reaction rate of 125 $\frac{\mu M \ O_2}{\mu M \ protein \cdot s}$ (or 500 e−/s) at 25°C (Chang et al. 2012), while the cNOR has a drastically lower rate of 0.1 $\frac{\mu M \ NO}{\mu M \ protein \cdot s}$ at 42°C (Lici Schurig-Briccio et al., manuscript in preparation) To date, we have no clear explanation for this observation, as the NOR chemistry is also extremely favorable ($E'_0=+1.177 \, V$, Zumft 2005). The high activation energy that we report here provides at least some insight as to why the NOR reaction might be slower than oxygen reductase activity.
NO is an obligate intermediate in denitrification and is known to be quite toxic, therefore denitrifying bacteria, such as *Persephonella marina*, must fine tune NOR activity to prevent accumulation of NO. Steady-state concentrations during denitrification are typically in the nanomolar range (Goretski et al. 1990), while the concentration needed for NO toxicity is much higher, in the millimolar range (Brunelli et al. 1995). In denitrifying bacteria the NOR might not only meet the bioenergetics needs of the organism, it may also serve to maintain the steady-state concentration below toxic levels. This hypothesis has also been proposed for pathogenic bacteria that have qNORs. It is thought that the qNOR of *Neisseria gonorrhoeae* may function to maintain NO levels low enough to avoid a pro-inflammatory response in the host (Cardinale and Clark 2005).

Due to the importance of NO concentration *in vivo*, the $K_m$ value for NOR is very interesting. For the first time, we report a $K_m$ value for a purified qNOR, which is $3.1 \pm 0.6$ µM at 50°C. It is interesting to compare this $K_m$ value for qNOR with the known values for other members of the heme-copper oxygen reductases. qNOR is most closely related to the C-family of oxygen reductases. The C-family oxygen reductase from *Bradyrhizobium japonicum* is known have a $K_m$ of 7 nM for oxygen (Preisig et al. 1996). This family of oxygen reductases are common in microaerophilic bacteria, and their $K_m$ values are much lower than A-family oxygen reductases. qNOR is more distantly related to the A-family oxygen reductases, but the *P. marina* $K_m$ for NO is quite similar to the $K_m$ for $O_2$ in the A-family oxygen reductase (Merle and Kadenbach 1982, Ekici et al. 2012).

Due to structural and sequence similarities, many heme-copper oxidases have been found to be capable of nitric oxide reduction, as well as oxygen reduction. However, the nitric oxide activity is much lower than oxidase activity. For example, the cbb$_3$ from *Pseudomonas stutzeri* has been
shown to have a $V_{\text{max}}$ of $1.6 \frac{M\text{NO}}{M\text{protein}s}$ and a $K_{\text{m}}$ of $12 \mu\text{M NO}$, while the $V_{\text{max}}$ for oxidase activity is $140 \frac{M\text{O}_2}{M\text{protein}s}$ (Forte et al. 2001). Obviously the reaction rate for NO is drastically slower in this case, but interestingly, it is quite comparable with the $V_{\text{max}}$ of the qNOR from *Persephonella marina*. However, the $K_{\text{m}}$ value for NO in cbb$_3$ is still approximately four fold higher than the Km in the *P. marina* enzyme.

We also completed a mutational study of both the active site conserved glutamates and the quinol binding site. Our results found that E476, the Fe$_B$ ligand, is relatively intolerant to mutations. Only E476Q had any detectable activity, indicating that the size of this residue is more important than the functional group. E480 has been proposed to contribute to the electronegative environment of the active site (Hino et al. 2010), and our results support this hypothesis. E480Q is not active, while E480D is much more active than wild type. It is interesting to speculate why the enzyme has not evolved to have D480 in place of E480 if the activity is so dramatically increased. This result may be specific to the *P. marina* sequence and will have to verified in other qNORs. Our results indicate it is possible that E545 delivers protons to the active site. E545Q is only marginally active at higher temperatures, but E545D maintains significant activity over the temperature range tested.

Finally, we were able to confirm that the site binding the quinol mimic in the qNOR crystal structure is the functional menaquinol binding site. The binding site consists of H295, R705, and D709. Even conservative mutations to this region have no detectable activity using menadione as the electron donor. The qNOR quinol binding site is extremely similar to the site in another member of the heme-copper oxidoreductase superfamily, the A-family bo$_3$ quinol oxygen reductase. While the quinol binding sites in these two enzymes evolved independently, the three
conserved residues that we propose for quinol binding in qNOR are also found in the bo₃ quinol oxygen reductase, along with an additional Q. Similar to our results with qNOR, mutation of any single residue in bo₃ oxygen reductase drastically reduces activity. Mutations to the R, D and H retain only 1-3% of wild type activity (Abramson et al. 2000). We did not find a conserved Q near the quinol binding site in the qNORs. It is possible that this is due to the different binding affinity for quinol needed in these two enzymes. The bo₃ oxygen reductase has two quinol binding sites, a Q₉ and Q₁₀ site. The Q₉ site, which is discussed above, is thought to bind quinol tightly as a cofactor. Instead of binding and releasing quinol at the Q₉ site with each turnover, a new quinol, binds at the Q₁₀ site, which is then used to re-reduce the quinone in the Q₉ site (Yap et al. 2010). Because qNOR is thought to have only one site that binds and releases quinol with each turnover, it is possible that the enzyme is more efficient with looser binding, requiring only three residues instead of four.
CHAPTER THREE: THE PROTON ENTRY CHANNELS

The heme-copper oxidoreductase superfamily is a diverse family of integral membrane enzymes consisting of oxidases and nitric oxide reductases (NOR) (van der Oost et al. 1994). Heme-copper oxidases perform the four electron reduction of oxygen to water:

\[ \text{O}_2 + 4e^- + 4\text{H}_\text{in}^+ + n\text{H}_\text{in}^+ \rightarrow 2\text{H}_2\text{O} + n\text{H}_\text{out}^+ \]

These enzymes conserve energy by using protons from the cytoplasm for chemistry and by pumping protons across the membrane to create a charge separation (Wikström 1977). Therefore, oxidases must have proton pathways spanning the length of the membrane. Conserved, hydrophilic pathways, called the D- and K-pathways, connect the cytosol/mitochondrial matrix to the active site for proton delivery. In the crystal structures, these pathways are filled with water ordered by conserved, polar residues (Iwata et al. 1995, Soulimane et al. 2000, Buschmann et al. 2010). Proton exit pathways for pumped protons must connect the active site to the periplasm/inner membrane space. No pathway is clear in any crystal structure, and no proposed pathways have been confirmed through mutagenesis (Popovic and Stuchebrukhov 2005). However, many studies have indicated that the proton exit pathway extends via the heme propionates (Wikström et al. 2003, Chang et al. 2012) through multiple pathways (Brzezinski and Gennis 2008).

The other heme copper oxidoreductase superfamily members, NOR, are used during denitrification. Denitrification is a common form of anaerobic respiration that reduces nitrate (or nitrite) to nitrogen through the series of reactions:

\[ \text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2 \]
NOR are responsible for a single step in this pathway, the two electron reduction of nitric oxide to nitrous oxide, which is accomplished by the following reaction:

$$2\text{NO} + 2e^- + 2\text{H}^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$$

Within the NOR superfamily, there are two closely related families - cNOR and qNOR. NOR chemistry also requires protons, and as the active site is buried in the membrane, there must be a pathway for proton entry. cNOR is known to be non-electrogenic (Shapleigh and Payne 1985, Bell et al. 1992, Hendriks et al. 2002), but the reason for its electroneutrality is unknown. The lack of energy conservation is particularly interesting due to the highly favorable reaction catalyzed by NOR ($E'_0 = +1.2$ V), which is actually more favorable than oxidase chemistry ($E'_0 = +0.8$ V). Electrons are donated from periplasmic donors, so protons must come from the periplasm as well. Similarly to the oxidases, there is no clear channel from the active site to the periplasm in the crystal structure. No pathway similar to a D- or K-channel was evident in the X-ray structure either (Hino et al. 2010). Several proton entry pathways have been hypothesized for cNOR (Reimann et al. 2007, Hino et al. 2010, Pisliakov et al. 2012, Lici A. Schurig-Briccio, et al. manuscript in preparation), but to date, none have been confirmed.

The crystal structure of qNOR was recently solved (Matsumoto et al. 2012). Unfortunately, the structure was of inactive enzyme due to incorrect active site metal, Zn$_B$ instead of Fe$_B$.

Surprisingly, unlike cNOR, a hydrophilic channel connecting the cytoplasm to the active site was discovered in the structure of qNOR. This channel was lined with polar residues that coordinated many ordered waters. Near the active site, this channel was also found in a similar location as the K-channel of the closely related cbb$_3$–type oxidase (Buschmann et al. 2010). Because of its hyrophilicity and similarity to oxidase channels, it was proposed to be a
cytoplasmic proton entry channel for qNOR. A few mutants of the qNOR proton entry channel were presented and shown to be inactive. However, these mutations were either directly in the active site or eliminated a salt bridge (Matsumoto et al. 2012), confusing their interpretation. No mutations in the bulk of the channel were reported. While the qNOR proton channel is a very intriguing idea, further studies are needed to validate this channel for active enzyme.

For this study, the previously characterized qNOR from the thermophilic bacteria *Persephonella marina* was used. This qNOR was found to have a turnover number of 0.74±0.07 s⁻¹ at 50°C using DTT reduced menaquinone analog, menadione, as electron donor. The turnover number varied linearly between 42°C and 60°C, allowing for extrapolation to the *P. marina*’s ideal temperature - approximately 3.7 s⁻¹ at 73°C. Mutagenesis studies were also used to investigate the roles of conserved active site glutamate residues and to identify the conserved residues involved in the quinol binding site. In this study, we used sequence alignments combined with mutagenesis to investigate the roles of the residues implicated in previously proposed proton entry pathways both from the cytoplasm and the periplasm (Matsumoto et al. 2012, Hino et al. 2010, Lici A. Schurig-Briccio, et al. manuscript in preparation, Salomonsson et al. 2012).

**Materials and Methods**

**Expression and Purification of Recombinant qNOR**

Plasmid construction, expression, and protein purification were completed as described in Chapter 2. The qNOR gene was cloned into a pET-22b expression vector (Novagen), and a histidine tag was added to the C-terminal end of the protein. The C43 strain of *E. coli* carrying a pRARE plasmid for the expression of rare codons was used for expression. qNOR was purified from the membrane fraction using a Ni-NTA resin (Qiagen). The UV-visible spectra were taken
using an Agilent Technologies 8453 UV-visible spectrophotometer combined with ChemStation software. The concentration of samples was determined using the extinction coefficient $\varepsilon_{412} = 0.25 \, \mu M^{-1} \, cm^{-1}$ (of the oxidized spectrum).

**Enzymatic Activity Measurements**

Nitric oxide reduction activity was measured using the Microrespiration System and multimeter (Unisense) equipped a miniaturized Clark-type nitric oxide sensor (Unisense) and monitored by SensorTrace software, exactly as described in Chapter 2. All activity assays were carried out using DTT and menadione as electron donors at 50°C.

**Sequence Analysis**

Genes encoding cNOR and qNOR proteins were retrieved from over three thousand sequenced genomes. The sequences were aligned using Muscle v.3.8 and manually edited to match the structure alignment of qNOR and cNOR when necessary. Conservation was observed and calculated using BioEdit software.

**Results**

**Cytoplasmic Proton Entry Pathway**

In the heme-copper oxidases, the residues lining the D- and K-pathways are nearly completely conserved (Hemp et al. 2007, Chang et al. 2009). If the qNOR channel is important for active enzyme, we would expect to see similar conservation for the polar residues involved in the channel (Figure 3.1a). Using sequence alignments with over 400 sequences from a diverse selection of organisms, we found little to no conservation (Table 3.1). Only Glu 591, Asn 622, and Tyr 600 (G. stearothermophilus numbering) showed significant conservation.
In heme-copper oxidases, mutation to channel residues causes drastic reduction to activity (Hemp et al. 2007, Chang et al. 2009), so similar results are expected for qNOR. The *G. stearothermophilus* channel residues conserved in *P. marina* (Figure 3.1b) were mutated to leucine (Table 3.1). Of the four mutants, three showed very high activity compared to wild type (0.74±0.07 s⁻¹). It is interesting to note that although E555 (*P. marina* numbering) is conserved, the activity of E555L is hardly significantly different than wild type. N586L had no detectible activity. However, as this residue is extremely close to the heme, we cannot differentiate between the mutation perturbing the putative proton channel or the heme. Taken together, the lack of conservation and the high activity remaining in most mutants provide very strong evidence that the cytoplasmic proton entry channel is not important for active enzyme.

**Periplasmic Proton Entry Pathway**

Because our data does not support the importance of the cytoplasmic proton entry pathway, we investigated other proposed proton entry pathways from the periplasm. While no pathway has been confirmed for cNOR, one of the proposed pathways (Hino et al. 2010) has been supported by mutagenesis studies (Lici A. Schurig-Briccio, et al. manuscript in preparation). Many of the residues are conserved in qNOR as well, and the corresponding pathway is: Asp 83, His 607, Thr 521, Asn 598, and Ser 542 (Figure 3.2). All of these residues are very conserved in qNOR, with the exception of His 607 (Table 3.2). This residue shows little conservation, but is still worth consideration due to its location and role as a heme propionate ligand. Mutations were made to residues that should not interact with protons (Table 3.2). D86L is the only mutant that had a perturbed UV-visible spectra. All other mutants had normal spectra compared to wild type (data not shown), indicating the hemes are in similar environments in wild type and mutants. All
mutants for this pathway but T521L had no detectable activity. However, our results are difficult to interpret because these residues are so close to the heme.

A unique periplasmic proton entry pathway was recently proposed for qNOR (Salomonsson et al. 2012). This pathway is similar as the above described, but in this case, the pathway is Asp 464 to Glu 395 to Thr 521 to Asn 598 to Ser 542 (Figure 3.2). E395 is the calcium ligand. Both cNOR and qNOR, along with the related cbb₃–type oxidase, have a calcium ion bridging the heme b and heme b₃ propionates (Matsumoto et al. 2012, Hino et al. 2010, and Buschmann et al. 2010) that likely helps maintain heme orientation for effective electron transfer (Hino et al. 2010). The equivalent of E395 has been studied by mutagenesis in qNOR from G. stearothemophilus (E429), and E429A, E429Q, E429D drastically reduced the activity (Matsumoto et al. 2012). Similar results have been seen for corresponding residues in cNOR (Lici A. Schurig-Briccio, et al. manuscript in preparation, Butland et al. 2001, Flock et al. 2009, Thorndycroft et al. 2007).

In this case, there is an interesting increase in activity in E395L (Table 3.2). There is no clear explanation for this increase in activity because the UV-visible spectrum appeared normal and the inhibition profile was similar to wild type (data not shown). However, these results indicate that this residue is not likely involved in a proton entry channel if it remains highly active without a polar functional group. D464L showed no detectable activity, but again, these results are difficult to interpret due to this residue’s proximity to the heme b₃.

Discussion

In this study, we investigated the previously proposed proton entry pathways in qNOR. The most intriguing pathway proposed is the cytoplasmic proton entry channel resolved in the recent
crystal structure of inactive qNOR (Matsumoto et al. 2012). However, our sequence analysis studies revealed little conservation of the residues found lining this channel, and mutations to the corresponding residues in *P. marina* had little effect on activity. So, we conclude that the entire channel is not important for activity. In fact, it is possible that this channel is merely an effect of the perturbed active site and the random chance that the *G. stearothermophilus* enzyme contains polar residues in this region. Sequence alignments show that polar residues are not conserved or common in this region. Due to incorrect active site metal, the active site structure differs between cNOR and qNOR (Hino et al. 2010, Matsumoto et al. 2012), which could easily cause global conformational changes. When the enzyme is in its active configuration, the active site should be similar to cNOR—probably leading to a global conformation similar to cNOR. While this channel is clearly visible in the inactive enzyme, it may close or change conformation in active enzyme, making it nonessential. A crystal structure of active protein is needed to prove this hypothesis.

As our data did not support the importance of the proposed cytoplasmic proton entry channel and no other cytoplasmic pathways were seen in the crystal structure, we hypothesized that protons likely come from the periplasm, similarly to cNOR. Mutations in previously proposed pathways were very close to the active site heme b₃, so the total loss of activity in most mutants was difficult to interpret. We are unable to comment on the involvement of these conserved residues for activity.

In cNOR, qNOR, and cbb₃ oxidase a Ca^{2+} ion between the propionates of heme b and active site heme b₃ is stabilized by two conserved glutamate residues nearby (Buschmann et al. 2010, Hino et al. 2010, Matsumoto et al. 2012). Prior to the crystal structures, homology structural models predicted these two glutamates were on the periplasmic surface in cNOR (Flock et al. 2006).
Mutagenesis studies showed these residues were important to activity (especially the polar functional group), so they were predicted to be the entrance for the proton entry pathway (Flock et al. 2008). However, when the crystal structures were resolved, these residues were found near the calcium (Hino et al. 2010), confusing the mutagenesis data. Still, numerous studies have predicted the importance of these residues for proton entry in cNOR and qNOR (Flock et al. 2006, Flock et al. 2008, Hino et al. 2010, Salomonsson et al. 2012, Pisliakov et al. 2012), even in their now known positions. However, studies show the calcium ligands are most important for structural integrity. Studies on *G. stearothermophilus* qNOR and *R. sphaeroides* and *V. cholera* cbb3–type oxidases show that the loss of enzymatic activity in mutants of these residues coincides with a loss of calcium (Matsumoto et al. 2012 and Ouyang et al. 2012). This indicates that activity changes in these mutants correlates with the structural role of calcium, not proton entry (Hino et al. 2012, Ouyang et al. 2012). This point was further demonstrated with our data from E395, a calcium ligand for *P. marina* qNOR. This residue is likely not part of a proton entry pathway because E395L had a surprisingly large increase in activity. While we have no clear explanation for this activity, the large activity without a polar functional group in this location indicates E395 is not important for proton entry.

We cannot propose a specific proton entry pathway that stretches from the periplasm to the active site, but the conservation of polar residues (Thr 521, Asn 598, Ser 542) that connect the heme propionate to Glu 545 (the likely proton entry site in the active site (Hino et al. 2010) is very intriguing. These residues are extremely conserved in cNOR (Lici A. Schurig-Briccio, et al. manuscript in preparation) and qNOR both (Table 3.2), and due to their location, it seems probable that they are important for proton entry below the level of the heme propionate. Mutations of the corresponding residues in cNOR from *T. thermophilus* (N335, S277, T330) to
leucine are still active, unlike *P. marina*, but their activity is minimal Lici A. Schurig-Briccio, et al. manuscript in preparation).

However, how do protons get to the level of the heme propionate? Currently, we have no conclusive answers, just as there is no answer to the same question regarding pumped proton exit in oxidases. To date, in oxidases there is no defined pathway, but most agree that multiple pathways are most probable (Brzezinski and Gennis 2008). Therefore, the same could be true for both cNOR and qNOR. In fact, a recent computational study proposed several possible pathways in cNOR (Pisliakov et al. 2012), coinciding with our hypothesis. We propose that protons have multiple entry pathways that end at the heme b₃ propionate. From there, the very conserved Thr 521, Asn 598, and Ser 542 may help get the protons to Glu 545 and the active site. Further mutational and computational studies will be needed to investigate this hypothesis.

In order to find a proton entry pathway, we may first need to understand the conformational changes of the active site during catalysis. Currently, the specific catalytic mechanism for NOR is unknown. However, there is not enough room for two NO to bind at the binuclear center resolved in the crystal structure (Hino et al. 2010). During the catalytic cycle of NOR, conformational changes at the binuclear center must occur to allow proper positioning of two NO molecules suitable for N-N bond formation. These conformational changes at the binuclear center may have ramifications throughout the protein, allowing us more insight into proton entry pathways. Experimental, structural, and theoretical analyses of NOR in multiple oxidation and liganded states are essential for further investigation of proton entry pathways in these enzymes.
Figure 2.1 SDS-PAGE and the UV-Visible Spectra of Purified qNOR. (A) Coomassie Brilliant Blue staining on SDS-PAGE of the purified enzyme. (B) The UV-visible spectra of the oxidized (dotted line) and reduced (solid line) purified enzyme.
Figure 2.2 $K_m$ for NO of qNOR at 50°C. When fitted to a Michaelis-Menten curve, the $K_m$ is equal to 3.1±0.6 µM NO.
Figure 2.3 Temperature Dependence of the qNOR. (A) The linear relationship between rate and temperature between 42°C and 60°C. Extrapolating the line to the ideal temperature for this qNOR (73°C) estimates the rate to be 3.7 s\(^{-1}\). (B) The Arrhenius Plot of the linear relationship between rate and temperature. The calculated activation energy is equal to 114 kJ/mol.
Figure 2.4 The Conserved Glutamates of the qNOR Active Site. A model of the qNOR from *Persephonella marina* created using the structure of qNOR from *Geobacillus stearothermophilus* and the structure of the active site of cNOR from *Pseudomonas aeruginosa*. The active site of cNOR was used due to the incorrect active site metal (Zn\textsubscript{B}) in qNOR.
Figure 2.5 The Quinol Binding Site of qNOR. (A) The quinol binding site in *Geobacillus stearothermophilus* (PDB 3AYG) with the bound quinol mimic HQNO. The corresponding residues in *Persephonella marina* are H295, R705, and D709. (B) Sequence alignments displaying the conservation of these residues in qNOR but not in cNOR.
Figure 3.1 Putative Cytoplasmic Proton Entry Channels in *Persephonella marina* and *Geobacillus stearothermophilus*. (A) The proton entry channel proposed based on the *Geobacillus stearothermophilus* qNOR crystal structure. PDB 3AYG (B) The corresponding residues in the structural model of the qNOR from *Persephonella marina*. The model was created using the *Geobacillus stearothermophilus* structure and the *Pseudomonas aeruginosa* cNOR active site.
Figure 3.2. Proposed Periplasmic Proton Entry Channels. The previously proposed periplasmic proton entry pathways. The pathway proposed for cNOR (Hino et al. 2010, Lici A. Schurig-Briccio et al., manuscript in preparation) begins at Asp 83, and the unique pathway proposed for qNOR (Salomonsson et al. 2012) starts at Asp 464. Both pathways share a similar route from the heme b$_3$ propionate to the active site Glu 545.
Table 2.1 Nitric Oxide Reductase Activity of Active Site Glutamate Mutants.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Proposed Function</th>
<th>Mutation</th>
<th>Activity at 42°C</th>
<th>% Wild Type Activity at 42°C</th>
<th>Activity at 50°C</th>
<th>% Wild Type Activity at 50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Q</td>
<td>n.d.</td>
<td>0.052±0.07(^*)</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>n.d.</td>
<td>2.0±0.3(^†)</td>
<td>270.2</td>
<td></td>
</tr>
<tr>
<td>Glu 480</td>
<td>Electronegative Environment</td>
<td>D</td>
<td>0.43±0.06(^*)</td>
<td>195.5</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q</td>
<td>n.d.</td>
<td>0.39±0.05(^*)</td>
<td>52.7</td>
<td></td>
</tr>
<tr>
<td>Glu 545</td>
<td>Proton Entry to Active Site</td>
<td>D</td>
<td>0.042±0.011(^*)</td>
<td>19.1</td>
<td>0.040±0.002(^*)</td>
<td>5.4</td>
</tr>
</tbody>
</table>

*Activity is not stable. After 2-3 minutes, activity is lost.
†Activity shows different inhibition profile than wild type.

The units of activity are s\(^-1\).

n.d. not detected.
<table>
<thead>
<tr>
<th>Residue</th>
<th>Mutation</th>
<th>Activity at 42°C</th>
<th>Activity at 50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine 295</td>
<td>F</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Arginine 705</td>
<td>L</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Aspartate 709</td>
<td>N</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. not detected

The units of activity are s\(^{-1}\)

Table 2.2 Nitric Oxide Reductase Activity of Proposed Quinol Binding Site Mutants
<table>
<thead>
<tr>
<th>Residue in <em>G. stearothermophilus</em></th>
<th>Residue in <em>P. marina</em></th>
<th>Conservation %</th>
<th>Mutation</th>
<th>Activity</th>
<th>% Wild Type Activity$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu 281</td>
<td>-</td>
<td>10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys 597</td>
<td>Leu 561</td>
<td>40% (Arg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu 594</td>
<td>Lys 558</td>
<td>38% (Glu)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu 591</td>
<td>Glu 555</td>
<td>80%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gln 545</td>
<td>Glu 509</td>
<td>10% (Q)/23% (E)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asn 622</td>
<td>Asn 586</td>
<td>91%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr 660</td>
<td>Phe 624</td>
<td>75% (Tyr)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$ Wild type activity is 0.74±0.07 s$^{-1}$

- No corresponding residue
(b) No mutation done
n.d. Not detected

Table 3.1 **The Residues in the Proposed Cytoplasmic Proton Entry Pathway**
<table>
<thead>
<tr>
<th>Residue</th>
<th>Conservation %</th>
<th>Mutation</th>
<th>Activity</th>
<th>% Wild Type Activity ($^1$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp 83</td>
<td>93%</td>
<td>D83L*</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>His 607$^+$</td>
<td>25%</td>
<td>H607F</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Thr 521</td>
<td>84%</td>
<td>T521L</td>
<td>0.38±.02</td>
<td>51.3%</td>
</tr>
<tr>
<td>Asn 598</td>
<td>97%</td>
<td>N598L</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ser 542</td>
<td>99%</td>
<td>S542L</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Asp 464</td>
<td>25% (D)/72% E</td>
<td>D464L</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Glu 395$^+$</td>
<td>84%</td>
<td>E395L</td>
<td>2.00±0.09</td>
<td>270.3%</td>
</tr>
</tbody>
</table>

$^1$ Wild type activity is 0.74±0.07 s$^{-1}$  
$^*$ Perturbed UV-visible spectra  
$^+$ Structural Importance  
n.d. Not detected

Table 3.2 The Residues of Proposed Periplasmic Proton Entry Channels
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