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Role of a Conserved Glutamine Residue in Tuning the Catalytic Activity of *Escherichia coli* Cytochrome *c* Nitrite Reductase[†].

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ABBREVIATIONS

NrfA, cytochrome *c* nitrite reductase; e^-, electron; r.m.s.d., root mean square deviation; EPR, Electron Paramagnetic Resonance; MCD, Magnetic Circular Dichroism; PFV, Protein Film Voltammetry; i_{cat}, experimentally determined catalytic current; E_{cat} , the potential at the steepest part of the catalytic wave associated with the onset of catalysis.

ABSTRACT.

The pentaheme cytochrome *c* nitrite reductase (NrfA) of *Escherichia coli* is responsible for nitrite reduction during anaerobic respiration when nitrate is scarce. The NrfA active site consists of a hexacoordinate high-spin heme with a lysine ligand on the proximal side and water/hydroxide or substrate on the distal side. There are four further highly conserved active site residues including a glutamine (Q263) positioned 8 Å from the heme iron for which the sidechain, unusually, coordinates a conserved, essential calcium ion. Mutation of this glutamine to the more usual calcium ligand, glutamate, results in an increase in the K_m for nitrite by around 10-fold, while V_{max} is unaltered. Protein film voltammetry showed that lower potentials were required to detect activity from NrfA Q263E when compared with native enzyme, consistent with the introduction of a negative charge into the vicinity of the active site heme. EPR and MCD spectroscopic studies revealed the high spin state of the active site to be preserved, indicating that a water/hydroxide molecule is still coordinated to the heme in the resting state of the enzyme. Comparison of the native and modified enzyme X-ray crystal structures showed an increased bond distance between the active site heme iron and the distal ligand in the latter as well as changes to the structure and mobility of the active site water molecule network. These results suggest that an important function of the unusual Q263-calcium ion pair is to increase substrate affinity through its role in supporting a network of hydrogen bonded water molecules stabilizing the active site heme distal ligand.

A broad range of enteric bacteria express a cytochrome *c* nitrite reductase (NrfA) that is synthesized under anaerobic conditions in the presence of low nitrate concentrations and a carbon source such as glycerol (*1,2*). This periplasmic enzyme catalyses the 6 e^- reduction of nitrite to ammonia. It can also convert the potential intermediates of nitrite reduction, nitric oxide (NO) and hydroxylamine (NH_2OH), to ammonia and has been proposed to have a role in enteric NO detoxification (*3*).

In γ-proteobacteria such as *Escherichia coli* the *nrf* operon encodes membrane proteins NrfD and NrfC together with a periplasmic soluble protein NrfB to act as a transient electron donor chain to the terminal reductase, NrfA. In ε-proteobacteria such as *Wolinella succinogenes* and *Sulfurospirillum deleyianum* and in δ-proteobacteria such as *Desulfovibrio desulfuricans,* however, the reductase exists as a stable NrfA:NrfH complex (*2, 4*). Crystal structures of representative electron donors of both types revealed significant structural differences between the tightly-bound electron donor, NrfH (*5*), and the transient electron donor, NrfB (*6, 7*).

Although the method of electron input to NrfA differs between the two bacterial systems, the active site is highly conserved. The X-ray crystal structures of NrfA from *E. coli* (*8*), *W. succinogenes* (*9*), *S. deleyianum* (*10*), *D. desulfuricans* (*11*) and the NrfHA complex from *Desulfovibrio vulgaris* (*5*) have been solved and show NrfA to be a homodimeric enzyme with each monomer containing two calcium ions and five covalently-attached *c*-type hemes that are named hemes 1 to 5 according to their position in the amino acid sequence. Four hemes are attached by conventional CXXCH sequence motifs and have bis-histidine ligands while the fifth, active site heme (heme 1) is attached through a unique CXXCK motif that provides a lysine residue to the proximal side of the heme. The distal ligand is provided by a water/hydroxide that is displaced in the

presence of substrate such as nitrite. In addition to the lysine ligand, Lys 126 (all residue numbering refers to the *E. coli* NrfA), there are four further conserved active site residues, Tyr 216, Arg 106, Gln 263, and His 264 that are proposed to contribute to a positive environment within the active site pocket that allows access to the negatively charged substrate, nitrite (NO_2^-). Some of these residues are also believed to act as proton donors during enzyme turnover (*12*). The active site QH dipeptide sequence motif comprising Gln 263 and His 264 is highly conserved in NrfA sequences and in Ramachandran plots His 264 is the only residue to fall in the generously allowed regions, suggesting an important structural or catalytic role for this residue.

In all available crystal structures of NrfA enzymes (*5, 8-11*) an octahedrally-coordinated calcium atom (calcium I) is present ~ 11 Å from the active site heme iron. This calcium ion appears to play a role in maintaining active site structure, most notably by preventing His 264 from occupying the distal coordination site of heme 1 (*11*), and in contributing positive charge to the surface of the substrate access channel (*10*). The coordination sphere of this ion comprises both oxygen atoms of the carboxyl group of Glu 215, the main chain carbonyl groups of Lys 261 and Tyr 216 as well as two water molecules. The final ligand to this calcium ion is the sidechain amide oxygen atom of Gln 263. Analysis of the 3368 calcium-containing structures in the Protein Databank reveals that whilst aspartate, glutamate and asparagine contribute a total of 67 % of the 36175 calcium ion ligands, glutamine is responsible for only 3.2 %. In fact, where glutamine provides a calcium ligand, the glutamine sidechain amide oxygen atom comprises only 1.6 % of the total calcium ligand interactions. In the 269 structures where these ligands occur, the majority are at the surface of the protein and, in the case of enzymes, away from the active site. The NrfA enzymes represent the only family where this conserved

glutamine – calcium ion pair is close enough to the active site to be involved in enzyme activity. Hence, the adoption of glutamine for this role in the active site of NrfA may be important to function. Calcium ions positioned close to the active sites of enzymes often fulfill functional roles. For example, in horseradish peroxidase calcium ions are present on both the proximal and distal sides of the active site heme, where they play a role in stabilizing the structure of the functional enzyme (*13*). The structural similarities between the location of the calcium sites on the distal side of the catalytic hemes in NrfA and in class II and III peroxidases has previously led to the suggestion that they may share similar physiological roles (*11*).

To help provide insights into the roles that the active site calcium ion and coordinating glutamine residue may have in nitrite reduction to ammonia by cytochrome *c* nitrite reductase, we have mutated the glutamine at position 263 in *E. coli* NrfA to the more usual calcium ligand, glutamate. The changes that occur to the structure and activity of the enzyme revealed by a combination of X-ray crystallography, electron paramagnetic resonance (EPR), magnetic circular dichroism (MCD), enzyme kinetics and protein film voltammetry (PFV) show that this residue modulates the operating potential of the enzyme and also increases affinity for the substrate nitrite by anchoring a network of water molecules at the active site.

MATERIALS AND METHODS.

Mutation, expression and purification of NrfA

The template used to introduce the Q263E substitution in *nrfA* was plasmid pJG1.2, which is a derivative of pJG1 (*14*) from which the 4 kb fragment from the *Hin*dIII site in *nrfC* to the *Eco*RI site downstream of *nrfG* had been deleted. Plasmid pJG1.2 therefore

encodes only NrfA and NrfB. The Q263E substitution was introduced into the *nrfA* gene on plasmid pJG1 using the Quikchange Site-Directed Mutagenesis kit (Stratagene) following the manufacturer's instructions. The forward primer was JR Q263E FOR, 5` CGCCAATGCTGAAAGCG***GAA***CACCCGGAATATGAAACC 3` ; the reverse primer was JR Q263 REV, 5` GGTTTCATATTCCGGGTG***TTC***CGCTTTCAGCATTGGCG 3` (the three bases of codon 263 are underlined; bases in italics show changes from the original sequence). Plasmids isolated from transformants after mutagenesis were confirmed by sequencing the complete coding region of the *nrfA* gene. No unexpected substitutions were found.

Plasmid pJG1.9aQ263E was transformed into the *E. coli* strain JCB4083a cells that have the genotype Δ*narZ::ω* Δ*narL::Tn10* Δ*napGH* Δ*nrfAB* Δ*nirBDC*::Kan^R.and also contained the cytochrome *c* maturation plasmid pEC86 (*15*). Transformed cells were grown aerobically overnight at 37 °C in 10 L of Terrific Broth with 100 μg/mL carbenicillin and 30 μg/mL chloramphenicol. Cells were harvested by centrifugation and proteins purified according to published procedures (*16*).

Methyl viologen assays

The rate of methyl viologen oxidation was used to measure the rate of electron input to NrfA as described previously (*16*). Electrons transferred to NrfA from methyl viologen previously reduced with sodium dithionite caused a change in the absorbance of methyl viologen that could be measured at 600 nm. A molar extinction coefficient of 13.7 mM^{-1} cm^{-1} was used to convert the change in absorbance to the number of electrons transferred to the enzyme.

The amount of ammonium formed during turnover of both enzymes was measured according to published procedures (*17*). An incubation mixture containing 50 mM HEPES pH 7.0, 2 mM $CaCl_2$, 5 mM $NaNO_2$, 1 mM methyl viologen and 3 nM NrfA was sparged with N_2 and incubated at 25 °C before addition of 2 mM $Na_2S_2O_4$ to start the reaction. After 5 minutes the reaction was stopped by vortexing the assay mixture in air to oxidize the excess reductant. The amount of ammonia formed was measured using the NADH dependent formation of glutamate from α-ketoglutarate and ammonia (*17*).

Protein Film Voltammetry of NrfA

Cyclic voltammetry was performed using a three-electrode cell configuration as described previously (*18*, *19*). The reference electrode employed was Ag/AgCl (saturated KCl). All potentials are quoted versus the standard hydrogen electrode, achieved via the addition of 0.197 V to experimental data. The electrochemical cell was housed in a Faraday cage within a nitrogen-filled anaerobic chamber maintained at <5 ppm O_2. Immediately prior to each experiment the pyrolytic graphite edge working electrode was polished with an aqueous slurry of 0.3 μm alumina, rinsed and dried with a tissue. Protein films were formed by placing1 μL of ice-cold NrfA sample (0.15 μM Q263E or 0.17 μM native in 50 mM HEPES, 2 mM $CaCl_2$, pH 7.0) directly onto the electrode surface for approximately 10 seconds, then removing the excess. The electrode was immediately placed into an electrochemical cell containing 50 mM HEPES, 2 mM $CaCl_2$ at pH 7.0 and 20 °C. Stock solutions of $NaNO_2$ were prepared fresh daily in ice-cold buffer and thoroughly purged with argon prior to their addition to the electrochemical cell. To quantify the catalytic activity, the current measured at -0.6 V in the absence of nitrite was subtracted from each voltammogram in the presence of nitrite to give the catalytic current. Signal loss was noted during the course of experiments and found to be a first

order process. Prior to analysis catalytic currents were corrected for this effect using rate constants of 1.7 x 10^{-4} s^{-1} for Q263E and 4 x 10^{-4} s^{-1} for native NrfA. Data were fitted to the Michaelis-Menten equation using Origin (Microcal Software Inc.).

EPR and MCD spectroscopies

EPR spectra were measured with a Bruker ER300D spectrometer fitted with a dual mode cavity type ER4116DM interfaced to an ELEXSYS computer control system (Bruker Analytische Messtechnik GmBH) and equipped with a variable temperature cryostat and liquid helium transfer line (Oxford Instruments). MCD spectra were recorded on JASCO circular dichrographs models J810 and J730 for the UV-visible and near-infrared (NIR) regions respectively, used in conjunction with an Oxford Instruments superconducting solenoid 6 Tesla SM1 magnet with an ambient temperature sample bore. The intensities of spectra presented are referred to concentrations based on an extinction coefficient of 497,650 $M^{-1}cm^{-1}$ for the Soret absorption bands near 409 nm. Samples were prepared in deuterium oxide solutions containing 50 mM HEPES and 2 mM $CaCl_2$. pH* is the apparent pH of the D_2O-based solutions measured using a standard glass pH electrode.

Crystallisation and X-ray data collection

Native NrfA was crystallized under conditions different to those described previously (*8*). Briefly, purified enzyme was concentrated to 10 mg ml^{-1} and centrifuged at 16,000 g for 10 min at 4 °C before crystallization. Crystals were obtained under aerobic conditions by the vapour diffusion hanging drop method using 20 % (v/v) PEG 10K in 100 mM Na-HEPES pH 7.5. For data collection, single crystals were soaked with a solution of reservoir

buffer containing 20 % (v/v) ethylene glycol as a cryoprotectant. X-ray diffraction from single crystals was measured using an ADSC detector on beamline ID-29 at the E.S.R.F. (Grenoble). The native NrfA crystal chosen for data collection was of space group $P2_1$ with cell dimensions a = 90.4 Å, b = 79.3 Å, c = 137.6 Å, α = β = 90°, γ = 101.5°. These cell dimensions are highly similar to those reported previously in the determination of the structure of the native *E. coli* enzyme at 2.5 Å resolution (*8*). The recombinant NrfA Q263E crystals were of space group $P2_12_12_1$ with cell dimensions a = 82.3 Å, b = 91.2 Å, c = 295.5 Å, α = β = γ = 90 °. In both crystal forms the solvent content was approximately 45 % (v/v) with four molecules of NrfA in the crystallographic asymmetric unit. Diffraction datasets for both native NrfA and NrfA Q263E were obtained from single crystals to resolutions of 1.74 Å and 2.04 Å, respectively, using X-ray wavelengths of 0.931 and 0.992 Å. Datasets were processed using MOSFLM (*20*) and SCALA (*21*) as part of the CCP4 package (*22)*. Data collection statistics are summarized in Table 1.

Crystal structure solution and refinement

A high resolution structure of oxidised native NrfA at 1.74 Å resolution was determined by molecular replacement using a single monomer from the 2.5 Å resolution structure of the native enzyme available in the Protein DataBank (PDB entry 1GU6) (*8*) as a search model. Molecular replacement using MOLREP as part of the CCP4 package (*22*) was used to generate an initial structural model that contained four NrfA monomers in the asymmetric unit. This model was improved by alternating rounds of manual model building using COOT (*23*) and automatic refinement using REFMAC (*24*). Addition of 2149 water molecules using ARP (*25*), four sulfate ions and 32 ethylene glycol molecules gave a final structure with R_{cryst} (R_{free}) of 15.4 % (18.8 %) for all data in the resolution range 31.3 - 1.74

Å. When analyzed for stereochemical quality using PROCHECK (*26*) the structure has 99.7 % of residues in favored regions of the Ramachandran plot. The sole outlier is the active site residue His 264 which falls in the generously allowed region as previously reported (*8*). No residues fall in disallowed regions.

The structure of NrfA Q263E was determined by molecular replacement using as a search model the refined 1.74 Å resolution native NrfA structure from which sulfate ions, water and cryoprotectant molecules had been removed. This was corrected by cycles of manual model building and automatic refinement in the same fashion as used for the structure of the native protein. After addition of 1782 water molecules and 15 ethylene glycol molecules the refined structure had an R_{cryst} (R_{free}) of 17.1 % (22.6 %) for data in the resolution range 79.0 – 2.04 Å. Stereochemical quality analysis using PROCHECK revealed the structure has the same Ramachandran distribution as the 1.74 Å native structure. Average r. m. s. d. values were calculated using SUPERPOSE as part of the CCP4 package (*22*) and use both main chain and side chain atoms.

The refined coordinates have been deposited to the Protein DataBank with accession numbers 2RDZ and 2RF7 for the native and Q263E enzymes, respectively.

RESULTS

NrfA Q263E was purified and observed to run as a single band on Coomassie-stained SDS polyacrylamide gels. The coordination environment of the hemes of the purified Q263E NrfA enzyme in solution was assessed by a combination of MCD and EPR spectroscopies. The MCD of Q263E NrfA is indistinguishable from that of the native enzyme (Supporting information, Figure 1). The near-infrared MCD has a charge transfer band at ~1500 nm (Supporting information, Figure 1B, 1D) consistent with the four bis-histidine coordinated low-spin hemes resolved in the crystal structures. A high-spin charge transfer derivative feature, centered at ~ 630 nm, has previously been assigned to the active site heme with a proximal lysine and a distal water/hydroxide ligand (*8*). Significantly, this peak has a similar extinction coefficient in both the WT and Q263E enzymes, indicating that the active site water remains coordinated to the distal position of the heme. The EPR spectroscopy of the WT and Q263E enzymes is also similar with respect to the low spin features (Supporting information, Figure 2), consistent with the active site heme in both enzymes having coordination from a water/hydroxide on the distal side and a lysine ligand on the proximal side.

Catalytic properties of native and Q263E NrfA

The catalytic properties of NrfA Q263E were assessed in solution using methyl viologen, reduced with sodium dithionite, as an electron donor (*8*) and by cyclic voltammetry with the enzyme adsorbed on graphite electrodes (*19*). Methyl viologen transfers electrons to NrfA through hemes exposed at the enzyme surface. Electrons are then transported through a chain of hemes to the active site, where they are used to

reduce nitrite directly through the lysine-coordinated heme 1. Figure 1 shows the rate of nitrite reduction catalyzed by NrfA in the presence of increasing amounts of nitrite for both the native and Q263E NrfA enzymes. The activity of the native NrfA is significantly higher at 50 μM nitrite when compared with NrfA Q263E, while the activity of both enzymes is similar at concentrations greater than 1 mM. The data were fitted using a Hanes plot to determine K_m and V_{max} values for the rate of nitrite reduction by both forms of NrfA. The K_m of the native NrfA was 33 ± 15 μM, and the V_{max} was 629 ± 25 NO_2^- s^{-1} at 25 °C, similar to the values previously determined (*8*). The K_m of NrfA Q263E was 413 ± 63 μM, more than 10-fold greater than the native NrfA K_m, while the V_{max} was 641 ± 41 NO_2^- s^{-1}, similar to the native NrfA and indicating that Gln 263 has a role in nitrite recognition but not in nitrite reduction.

The rate of ammonium formation at 5 mM NO_2^- by NrfA Q263E was measured as 533 ± 42 NH_4^+ s^{-1} while the rate of methyl viologen oxidation under identical conditions was 3126 ± 144 e^- s^{-1}. The ratio of the electrons consumed to ammonia produced is 5.9 ± 0.7, indicating that NrfA Q263E, like the native enzyme (*27*), reduces nitrite stoichiometrically to ammonia.

Protein film voltammetry (PFV) of NrfA has been well characterized and provides a sensitive probe of catalytic properties of the enzyme (*18,19,28)*. Previous titration of the native enzyme with nitrite yielded a K_m of around 25 μM, comparable to that found in methyl viologen activity assays (*8, 19*). NrfA Q263E is also amenable to study by PFV, with a reductive (negative) catalytic current observed in the presence of nitrite (Figure 2A). The catalytic response is more clearly viewed after subtraction of the voltammogram in 0 μM nitrite which also allows ready quantitation of catalytic current magnitudes (i_{cat}) (Figure 2B). After baseline subtraction the catalytic currents of the

forward and reverse scans overlay, and the signal was found to be rotation rate independent indicating a steady state response. Plotting i_{cat} at -0.6 V versus nitrite concentration enabled calculation of a K_m of 290 ± 50 μM for NrfA Q263E (Figure 2C). This is a value 10-fold greater than that for the native enzyme determined by PFV and the behavior is in good agreement with that displayed in methyl viologen assays.

Protein film voltammetry provides additional information to methyl viologen based assays since the enzyme activity is defined across the electrochemical potential domain. For native NrfA the activity versus potential profile exhibits a 'peaked' response at nitrite concentrations below the K_m (Figure 2D). The peak reflects an increase in activity as the electrode potential is lowered followed by an attenuation of the activity to approach a constant, non-zero value. The two parts of the response are highlighted in plots of the first derivative of catalytic current with respect to potential, where they appear as a positive and negative peak, respectively (Figure 3A, 3B); as the nitrite concentration is increased and the catalytic rate approaches V_{max}, the attenuation becomes less apparent and a boost in activity starts to appear over a similar potential range (Figure 2D). Eventually the enzyme-limited, i.e. maximal, catalytic response is achieved where the attenuation is no longer present and the profile shows the onset of catalysis is followed by an additional boost in activity as the potential is lowered. Again, this is clearly illustrated in the first derivative plot where two positive peaks are apparent (Figure 3C).

For NrfA Q263E, when the nitrite concentration is less than the K_m the catalytic profile is similar to that of native NrfA with a clear peak of activity (Figure 2B, 3A). However, as the nitrite concentration is raised and the catalytic rate approaches its maximal value, the waveshape deviates from that of the native enzyme. The attenuation

is no longer visible but no boost of activity is apparent as the wave describes an essentially sigmoidal increase in activity as the applied potential is lowered. Reflecting this waveshape, the current derivatives for Q263E at high nitrite concentrations show a single positive feature, in contrast to the two positive features displayed by native NrfA under equivalent conditions (Figure 3C).

Significantly, the overlaid first derivative plots also illustrate shifts in the potential dependence of the Q263E response relative to that of native NrfA (Figure 3). For NrfA Q263E the nitrite reductase activity is turned on below ca. -150 mV, while less driving force is required to activate native enzyme with activity initiated below ca. -50 mV. The potentials of the peaks in the first derivative plots, E_{cat} for the positive feature at higher applied potential and E_{switch} for the negative feature, quantitate the positions of the corresponding features in the catalytic wave (Figure 3A). The negative displacement of Q263E activity compared to native enzyme is reflected in the relative values of E_{cat}, which are ca. 100 mV more negative for Q263E (-230 mV) than native NrfA (-130 mV). E_{switch} is also slightly more negative (ca. 35 mV) for NrfA Q263E when compared to the native enzyme.

High resolution crystal structures of native and Q263E NrfA

Native NrfA from *E. coli* crystallized in space group $P2_1$ with two dimers in the crystallographic asymmetric unit. The crystal structure was solved by molecular replacement and refined at high resolution (1.74 Å). The most significant difference between the structures of the two dimers in the asymmetric unit was a small rotation of monomers at the dimer interface; the *E. coli* NrfA dimer has a dissociation constant of 4 μM (*7*) which may reflect the flexibility of the dimer interface. The existing 2.5 Å

resolution crystal structure of the enzyme (PDB entry 1GU6) was solved in space group $P2_12_12_1$ (*8*). Thus, small changes in the packing at the dimer interface allow purified *E. coli* NrfA to produce two different crystal forms from similar crystallization conditions. The average pairwise r.m.s.d. of the four NrfA monomers in the high resolution structure is only 0.63 Å and the monomers are thus essentially identical. The arrangement of the five conserved active site residues is very similar to that found in the high resolution crystal structures of the *W. succinogenes* and *S. deleyianum* NrfA enzymes (solved at 1.6 Å and 1.9 Å resolution, respectively) differing by an r.m.s.d. of only 0.28 and 0.26 Å. However, the new high resolution structure of *E. coli* NrfA has allowed the definition of ordered water molecules in the active site. The positions of these active site water molecules are highly conserved across the four monomers in the asymmetric unit as well as in the structures from *W. succinogenes, S. deleyianum* and *D. desulfuricans.* The water network around the active site has been proposed to provide protons to the active site and allow product access and egress (*10*). Five of the conserved water molecules in the active site of NrfA are shown in Figure 4A. The first of these (labeled W1) is the distal water molecule bound to heme 1 and hydrogen bonded to the side chain of His264. Two further water molecules form direct hydrogen bonding interactions with W1: one water molecule bridging W1 and the phenolic hydroxyl group of Tyr 216 (W2) and the other coordinated by the carboxyls of the two propionate groups of heme 1 (W3). Two further conserved water molecules hydrogen bond to the side chains of active site residues. These are a water molecule (labeled W4) bridging the guanidinium group of Arg 106 and W2 and a final water hydrogen bonded to Tyr 216 and Gln 263 (labeled W5). The water distal ligand to the active-site iron has a coordination distance of 2.26 ± 0.03 Å (Figure 4A, Table 2). This is similar to the distance (2.17 ± 0.02 Å) observed in the previously-

determined 2.5 Å resolution structure of the *E.coli* enzyme (8) and only marginally longer than those observed in the structures of the *W. succinogenes* (2.03 Å) and *D. desulfuricans* enzymes (2.09 ± 0.01 Å).

In both the native and NrfA Q263E structures, the atomic temperature factors (B-factors) in the region around heme 2, including several α-helices at the surface of the molecule, are typically higher than other regions of the enzyme. This is most evident in the crystal structure of the NrfA Q263E enzyme where one chain has significantly higher B-factors in the region of heme 2 than the other three chains, suggesting that this region is more mobile than the corresponding regions in analogous domains. Polypeptide chains that have more extensive surface contacts in the crystal lattice have lower B-factors (Table 2). We attribute the variance in B-factor to the differences in interface and symmetry-related contacts between the monomers (Table 2) and propose that a decrease in surface contacts around the region of heme 2 allows this region more freedom. The area around heme 2 is the binding site of the electron donor NrfB (*7, 8*), and in the NrfHA structure the electron donor causes significant movement of the chains around heme 2 (*5*). It is therefore likely that the high B-factors observed in these regions of the chain reflect an intrinsic flexibility that allows NrfA to adjust its structure locally to form a tight complex with NrfB.

The NrfA Q263E protein crystallized in space group $P2_12_12_1$ with cell dimensions similar to those found in the original 2.5 Å native structure (*8*). The structure of NrfA Q263E is very similar to that of the native enzyme with an overall average r.m.s.d. of 0.63 Å between the monomers. The positions of the active site amino acids were not significantly different with an average r.m.s.d. of only 0.15 Å. The crystal structure of NrfA Q263E revealed that Glu 263 is coordinated to the calcium I ion by only one of its

oxygen atoms and in a similar conformation to Gln 263, rather than acting as a bidentate ligand as typically occurs between a carboxyl group and a calcium ion (Figure 4B). However, by comparing omit maps calculated in the region of the active site for native NrfA and NrfA Q263E structures (Figures 4A and 4B) it is evident that the networks of hydrogen bonded water molecules in the active sites of native NrfA and NrfA Q263E differ. The electron density corresponding to the water molecule that is hydrogen bonded to Tyr 216 in the native structure (W5) is diminished and appears to have been elongated. It has also shifted in position so that the hydrogen bond to Tyr 216 is lost and instead bridges Glu 263 and Tyr 82, thus breaking the extended hydrogen bond network involving the distal water molecule. The remaining conserved water molecules, including W2 and W3, are retained but show a similar diminution in their electron density levels. In the refined structure, the length of the bond between the distal water (W1) and the heme iron has increased to 2.58 ± 0.06 Å (Table 2). This increase in the heme-water ligand distance mirrors an increase in the B-factor of the distal water molecule; the active site water in the native enzyme structure has an average B-factor of 13.2 ± 1 $Å^2$ while in NrfA Q263E it rises to 31.6 ± 2 $Å^2$. Similar increases in atomic temperature factors are observed for other conserved active site water molecules (W2-W3) forming the hydrogen bonded network at the active centre (Figure 4, Table 3). That it is only this complex of hydrogen bonded water molecules that is affected by the mutation is evidenced by the fact that the average B-factor calculated for all water molecules is almost unchanged following the mutation. In addition, the B-factors of other conserved active site water molecules remain similar. For example, W6, the water molecule buried in a pocket on the proximal side of heme 1 and adjacent to Lys 126 has a B-factor of 10.4 ± 1 $Å^2$ in the native structure and 13.8 ± 2 $Å^2$ in NrfA Q263E. The atomic temperature factors of Lys

126, the proximal ligand to the heme, also remains relatively unchanged (Table 2), consistent with the suggestion that the reason for the delocalization of the water molecules at the active site is caused by changes near the distal side of the heme.

Both native NrfA and NrfA Q263E were crystallized under identical conditions at pH 7.5 but were shown to have different space groups. We do not consider the differences at the active site to be an artifact of either resolution or change in space group as the previously published *E. coli* NrfA structure refined at the much lower resolution of 2.5 Å was revealed to have a water coordinated to the active site at a similar distance to the native NrfA structure, despite being in the same space group as the Q263E NrfA structure. The water molecules in the active sites were conserved and had correspondingly low temperature factors.

DISCUSSION

The means by which enzymes tune their activities to the availability of their substrates is a question of continuing interest. This is of particular relevance for enzymes operating on simple substrates such as nitrite where the extended interacting surfaces offered by larger substrates are absent. In the case of *E. coli* cytochrome *c* nitrite reductase, the low micromolar K_m for nitrite confers on the enzyme the capacity to effectively respire nitrite in the physiological concentration range found in the gut (*29*). This work has illustrated the importance of a conserved active site glutamine residue coordinated to a calcium ion in defining this enzyme's high affinity for the physiological substrate, nitrite.

Comparison of both native NrfA and NrfA Q263E using EPR and MCD spectroscopy indicated that the active sites of both native NrfA and NrfA Q263E were similar, with a water/hydroxide molecule still coordinated to the active site heme iron. A structural comparison of both NrfA enzymes using X-ray crystallography revealed that their structures were almost identical and there were no significant changes in the positions of active site residues. However, examination of the water molecules within the active site revealed that, on mutation of Gln 263 to glutamate, a switch in the coordination of a water molecule bound to Gln 263 ultimately breaks a highly-coordinated arrangement of hydrogen bonded water molecules at the active site.

In contrast to the apparently subtle changes in the structure of the active site, the kinetic properties of NrfA were dramatically altered through this mutation. Although the V_{max} of nitrite reduction does not significantly change, the K_m is observed to increase by a factor of more than 10-fold. That similar K_m values arose in methyl viologen assays and protein film voltammetry indicates that the increased K_m of the mutant enzyme is independent of the nature of the electron donor and most likely associated with changes induced at the active site. Thus, these results provide compelling evidence that Gln 263 does not contribute significantly to the catalytic turnover of nitrite to ammonia. Instead the glutamine appears to play an important role in increasing the affinity of NrfA for nitrite at the active site of the enzyme. Certainly the choice of glutamine over the more usual glutamate for ligation of the essential calcium ion will result in a less-negatively charged substrate access channel that should promote binding of the negatively charged substrate, nitrite, in the active site.

Protein film voltammetry reveals that the Q263E mutation has a further consequence on NrfA activity in that more negative potentials are required to activate nitrite reduction.

We have previously proposed that E_{cat} reflects in some part the reduction potential of the active site lysine-coordinated heme (*19*). The finding of a lower E_{cat} for NrfA Q263E than native enzyme suggests that the reduction potential of the lysine-coordinated heme is lower in the Q263E variant consistent with the introduction of additional negative charge into the active site. The lower potentials required to drive catalysis by NrfA Q263E may explain why this enzyme does not display an obvious 'boost' in activity at maximum turnover rates despite a V_{max} predicted to be equal to that of native NrfA from the results of the methyl viologen assays. We note that absolute catalytic rates are not available from the PFV since the amount of electrocatalytically active enzyme is not known. The redox potential of methyl viologen has been measured as -450 mV, significantly lower than the catalytic potential of both native NrfA and NrfA Q263E, and so, in methyl viologen assays, no difference is observed between the maximum activities of the native or Q263E forms of NrfA.

E_{switch} is slightly lower in NrfA Q263E than the native enzyme. We previously suggested that when an attenuation of activity is observed on accessing lower potentials this is driven by reduction of one (or both) of the bis-histidine coordinated hemes lying near the dimer interface (*19*). When E_{switch} is sufficiently separated from E_{cat} each potential may be correlated with the reduction potentials of the centres that modulate enzyme activity. However, for peaked catalytic wave shapes E_{switch} and E_{cat} will to some extent reflect the consequences of overlapping the contributions from each process. As a result, the potential at the foot of the attenuation process, i.e. where catalysis achieves a constant value at lower potentials, is likely to be a clearer reflection of the redox properties of the centre responsible for the reductive attenuation than E_{switch} itself (Figure 3A and 3B). For NrfA Q263E the foot of the attenuation occurs at potentials similar to

that of native NrfA. Thus, we consider that the Q263E mutation has little effect on the properties of the centre that when reduced, attenuates activity.

Taken together, the structural and kinetic data demonstrate that, in addition to modulating the redox potential, Gln 263 also stabilizes the water network of the active site which ultimately allows NrfA to bind nitrite specifically to the active site heme at micromolar concentrations. These findings are also in agreement with the EPR and MCD spectroscopy, which indicate that the active site is still a hexa-coordinate high spin heme capable of binding water/hydroxide and therefore able to bind and reduce nitrite.

These findings now allow us to propose a mechanism of binding of nitrite to the oxidized *E. coli* NrfA. In the X-ray structure of the *W. succinogenes* NrfA-nitrite complex, the binding of nitrite to the active site results in complete displacement of W1 and movement of W2 by 0.8 Å to a position 2.8 Å from either oxygen of the nitrite substrate and 2.7 Å from the active site tyrosine (*12)*. As these waters are highly conserved in both the *W. succinogenes* and *E. coli* NrfA structures it is likely that binding of nitrite to the active site of *E. coli* NrfA would result in a similar displacement of W1 and W2. In the NrfA Q263E crystal structure both of these water molecules have increased temperature factors and likely correspondingly lower occupancies (*30*) and so their participation in the active site hydrogen bonding network has also been disrupted, suggesting that the binding of nitrite to the active site will also be affected and that the main role of W2 is to stabilize nitrite at the active site during substrate turnover.

Surprisingly, in the crystal structure of *D. vulgaris* NrfA complexed with NrfH (5), the active site water has an average bond length of 2.80 ± 0.1 Å, much higher than the bond length observed in other published NrfA structures; the reasons for this are unclear but heme Fe – water coordination distances of 2.7 Å have been identified in horseradish

peroxidase crystal structures where the water forms a ligand to a hexa-coordinate high spin heme (*31*). While we cannot readily reason why the *D. vulgaris* NrfA active site water network is so different from that seen in other NrfA structures, our results show that a NrfA active site with disordered waters can reduce nitrite but will have a decreased affinity for nitrite. While *D. vulgaris* NrfA has been shown to reduce nitrite, no K_m has been published. However, the *D. vulgaris* NrfA enzyme has been shown to be constitutively expressed in the absence of nitrite and to effectively reduce sulfite (*32*), thus the active site of this NrfA may not be optimized for nitrite.

Despite being highly conserved among cytochrome *c* nitrite reductases, the calcium I ion of NrfA is too far from the catalytic site to play a direct part in the reaction cycle. Previous suggestions have included its participation via a structural role in helping to preclude His 264 from displacing water to occupy the distal heme binding site, thus inactivating the enzyme (*11*). We are now in a position to propose a further role for this ion in ensuring that the sidechain amide nitrogen of Gln 263 is positioned towards the substrate channel in such a way as to participate in stabilizing the network of hydrogen bonded water molecules extending to the active site. The simple mutation of this glutamine residue to a glutamate introduces a hydrogen bond acceptor group in place of a donor and leads to a disruption of the hydrogen bonding integral to this network. This has significant knock-on effects at the active site, causing the heme–ligand bond to lengthen, the catalytic potential of the heme to decrease, and decreasing the affinity of the active site for nitrite whilst still maintaining hexa-coordination of the high-spin catalytic heme. In the NrfA enzyme this region marks the boundary between the disordered waters of the solvent access channel and the highly ordered active site water molecules. Perturbation of

this boundary causes a subset of the active site water molecules to increase in disorder and attenuate the affinity of the active site for the distal ligand.

SUPPORTING INFORMATION AVAILABLE

Comparison of the EPR and MCD spectra of native NrfA and Q263E NrfA reveals that the high spin state of the active site is preserved. This material is available free of charge via the Internet at http://pubs.acs.org

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Table 1. X-ray data collection and refinement statistics

	NrfA native	NrfA Q263E
Data collection [a]		
Wavelength (Å)	0.931 Å	0.992 Å
Resolution (Å)	31.3 - 1.74 (1.82 –1.74)	79 – 2.04 (2.15 – 2.04)
Unique reflections	193,651 (26,427)	137,863 (19,032)
Completeness (%)	99.0 (92.9)	97.1 (92.9)
R_{sym} (%)	7.3 (35.1)	9.6 (32.7)
I/σ	7.7 (2.1)	3.6 (0.9)
Multiplicity	3.9 (3.1)	3.7 (3.6)
Average atomic B-factor (Å^2)	16.3	18.8
Refinement [a,b]		
R_{cryst}	15.4 (28.0)	17.1 (21.8)
R_{free}	18.8 (34.5)	22.6 (26.3)
Model		
Protein atoms[c]	14026	13986
Water	2149	1782
Heme atoms	860	860
Other	140	68
Bond length rmsd (Å)	0.013	0.018
Bond angle rmsd (°)	1.3	1.5
Average atomic B-factor (Å^2)	16.8	22.0

[a] Values in parentheses indicate the highest resolution shell.

[b] $R = \Sigma| F_o - F_c | / \Sigma F_o$. R_{cryst} is calculated with the 95 % of data used during refinement. R_{free} is calculated with a 5 % subset of data not used during refinement.

[c] The difference in protein atoms is due to an increased number of dual conformations observable at increased resolutions. Both structures contain the same number of amino acids.

Table 2: Variation in the coordination distances of the active site axial heme ligands and overall amino acid B-factor of the polypeptide chains in the four monomers of NrfA

NrfA	Chain	Distal ligand (Å)	Proximal ligand (Å)	B-factor ($Å^2$)	Contacts[a]
Native	A	2.28	2.15	14.9	95
	B	2.17	2.19	13.4	100
	C	2.27	2.24	17.3	79
	D	2.32	2.18	13.4	123
	Mean[b]	2.26 ± 0.03	2.26 ± 0.02		
Q263E	A	2.52	2.03	20.8	72
	B	2.49	2.40	18.0	71
	C	2.55	2.12	17.4	77
	D	2.77	2.00	28.1	59
	Mean[b]	2.58 ± 0.06	2.14 ± 0.08		

[a]Contacts refers to the number of residues involved in interactions between neighbouring polypeptide chains in the crystal lattice and were determined using MSD PISA (*33*) at the European bioinformatics institute *(http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html).*

[b]values are presented ± standard error.

Table 3. Temperature factors (B-factors) of active site water molecules in the crystal structures of native NrfA and NrfA Q263E[a].

Water Molecule	Native NrfA ($Å^2$)	NrfA Q263E ($Å^2$)
W1	13.2 ± 0.6	31.6 ± 1.7
W2	21.2 ± 0.2	34.8 ± 1.7
W3	12.1 ± 0.9	17.1 ± 0.9
W4	21.3 ± 1.2	18.9 ± 2.9
W5	36.5 ± 3.3	30.3 ± 2.2
W6	10.4 ± 0.7	13.8 ±2.0
Average B-factor for all waters in structure.	31.4	31.6

[a]Values for the active site waters W1 to W6 are given ± standard error.

FIGURE LEGENDS

Figure 1: Comparison of nitrite reduction rates from native NrfA (♦) and NrfA Q263E (■) as measured by methyl viologen assays. Lines show Michaelis-Menten behavior with K_m values of 33 ± 15 μM and 413 ± 63 μM, and V_{max} values of 629 ± 25 and 641 ± 41 NO_2^- reduced s^{-1} for native NrfA and NrfA Q263E, respectively. Values are presented ± standard error. Experiments performed in 50 mM HEPES pH 7.0, 2 mM $CaCl_2$.

Figure 2: Protein film voltammetry of NrfA Q263E and native NrfA when titrated with nitrite. (**A**) Cyclic voltammograms of NrfA Q263E in nitrite concentrations of 0, 20, 50, 99, 197, 499, 1020, 1950 and 2840 μM. (**B**) Baseline-subtracted voltammetry of NrfA Q263E from panel A. Nitrite concentrations are as shown. Catalytic current magnitude (i_{cat}) at -0.6 V indicated for response at 20 μM nitrite. (**C**) Plot of catalytic current magnitude at -0.6 V versus nitrite concentration. Catalytic currents are presented after time correction of data in panel B. Line shows Michaelis-Menten behaviour with K_m = 290 μM and i_{max} (V_{max}) = 1.15 μA. (**D**) Baseline-subtracted voltammetric response of native NrfA in nitrite concentrations as shown. Experimental conditions are scan rate 30 mV/s, electrode rotation rate 3000 rpm, and buffer/electrolyte of 50 mM HEPES, 2 mM $CaCl_2$, pH 7.0, 20 °C.

Figure 3: Comparison of the catalytic current-potential profiles of Q263E and native NrfA at nitrite concentrations below (**A**), near (**B**) and above (**C**) the K_m for nitrite**.** Profiles are shown as the first derivatives of catalytic current with respect to potential to emphasize their multiple features (see text for details). (solid line) Q263E NrfA and (broken line) native NrfA. Nitrite concentrations are as indicated where K_m = 290 μM for

Q263E and K_m = 25 μM for native NrfA. In panel A the features E_{cat}, E_{switch} and the region of the foot of the attenuation wave (*) are indicated for the Q263E derivative. Experimental conditions are as Figure 2.

Figure 4: Stereoviews of the active sites of native and Q263 mutant NrfA enzymes (A) Active site of native NrfA at 1.7 Å resolution. An $|F_o - F_c|$ omit map (calculated using experimentally determined structure factor amplitudes and with phases calculated for the final refined structure minus the active site water molecules) contoured at 5 σ is shown in green. The structure shown is the final experimentally determined structure with the five conserved active site residues and Tyr 82 shown in stick representation. The dashed lines indicate hydrogen bonds with distances less than 3.1 Å. The calcium I ion is shown as a green sphere. (B) Active site of NrfA Q263E refined at a resolution of 2.0 Å. An $|F_o - F_c|$ omit map calculated as for the native enzyme structure in (A) and contoured at 4 σ is shown in green. The dashed lines indicate hydrogen bonds between waters with distances less than 3.1 Å.

FIGURE 1

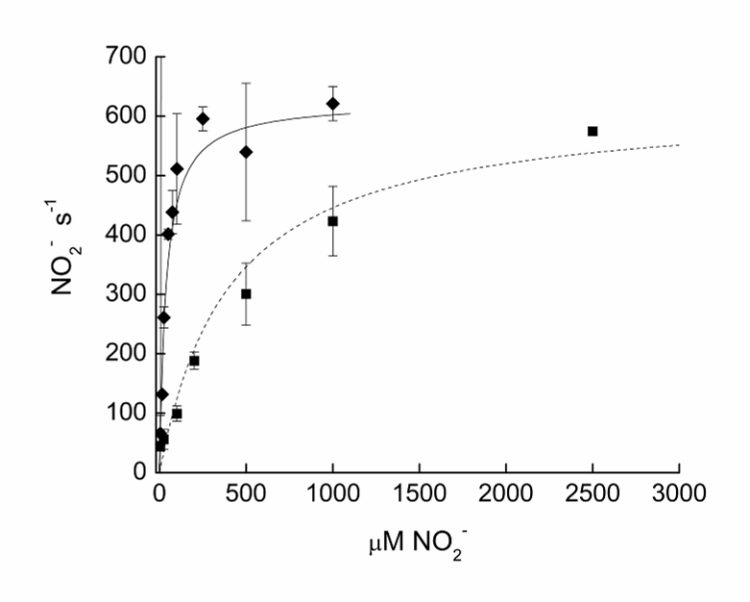

700
600
500
400
300
200
100
0
0
500
1000
1500
2000
2500
3000
NO_2^- s^{-1}
$\mu M\ NO_2^-$

FIGURE 2

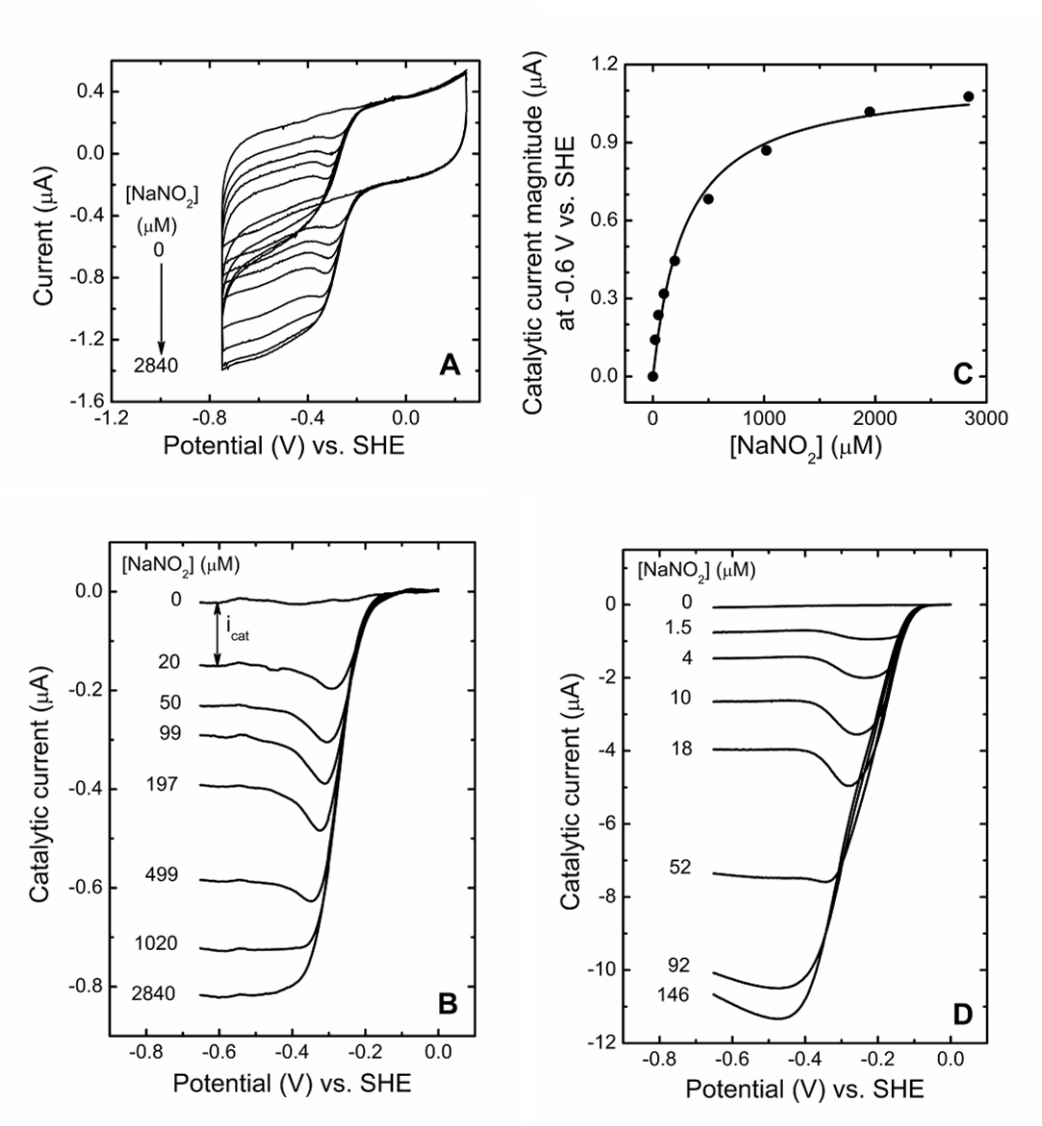

A
Current (μA)
Potential (V) vs. SHE
[NaNO2] (μM)
0
2840
C
Catalytic current magnitude (μA) at -0.6 V vs. SHE
[NaNO2] (μM)
B
Catalytic current (μA)
Potential (V) vs. SHE
[NaNO2] (μM)
0
20
50
99
197
499
1020
2840
icat
D
Catalytic current (μA)
Potential (V) vs. SHE
[NaNO2] (μM)
0
1.5
4
10
18
52
92
146

FIGURE 3

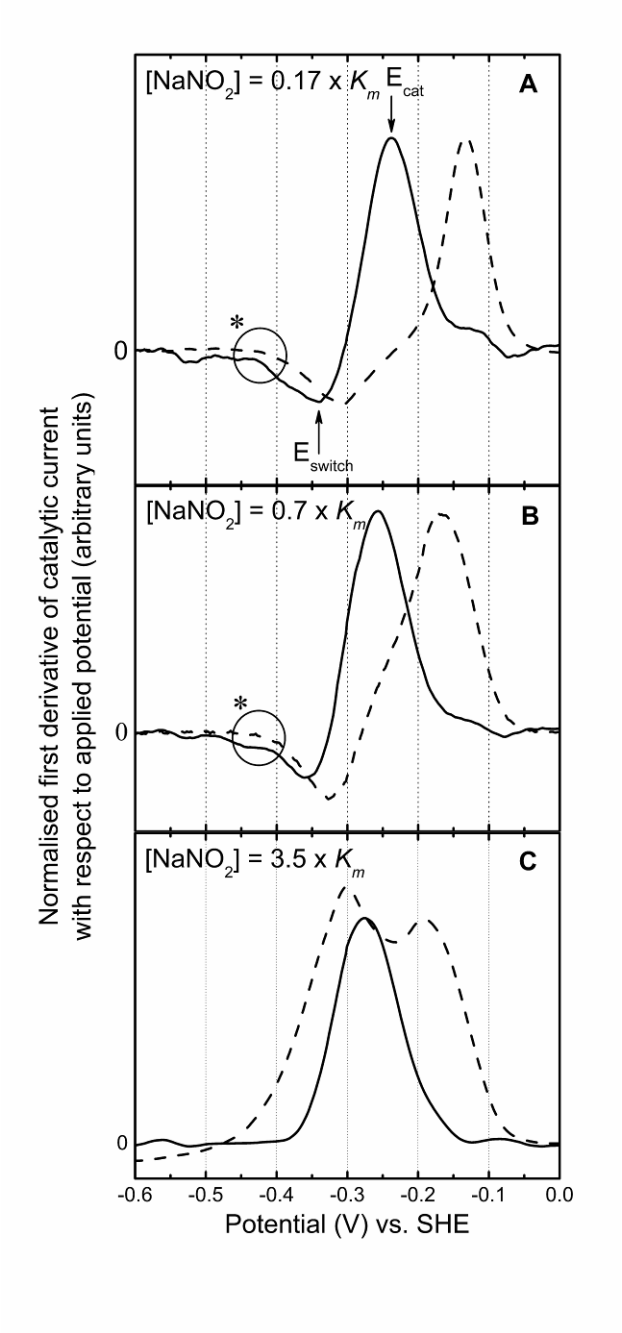
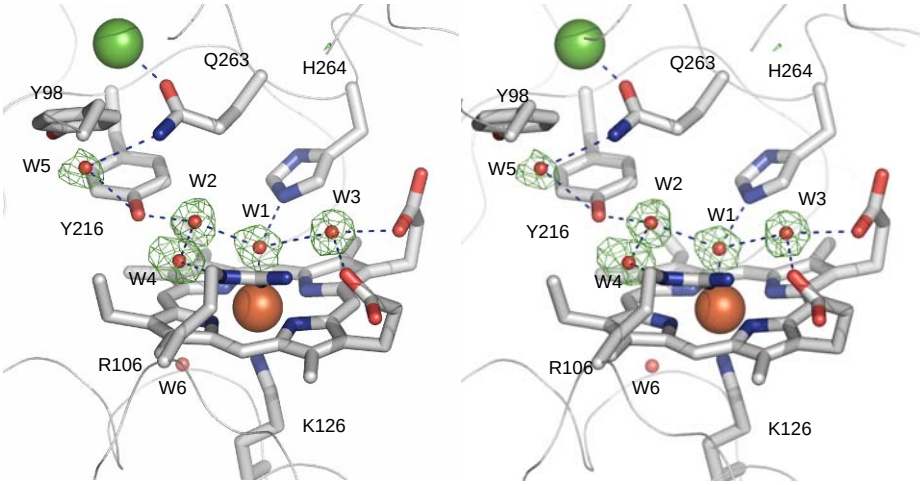

[NaNO2] = 0.17 x Km Ecat
A
*
0
Eswitch
[NaNO2] = 0.7 x Km
B
*
0
[NaNO2] = 3.5 x Km
C
0
Normalised first derivative of catalytic current
with respect to applied potential (arbitrary units)
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
Potential (V) vs. SHE

FIGURE 4

A


Q263
H264
Y98
W5
W2
W1
W3
Y216
W4
R106
W6
K126
Q263
H264
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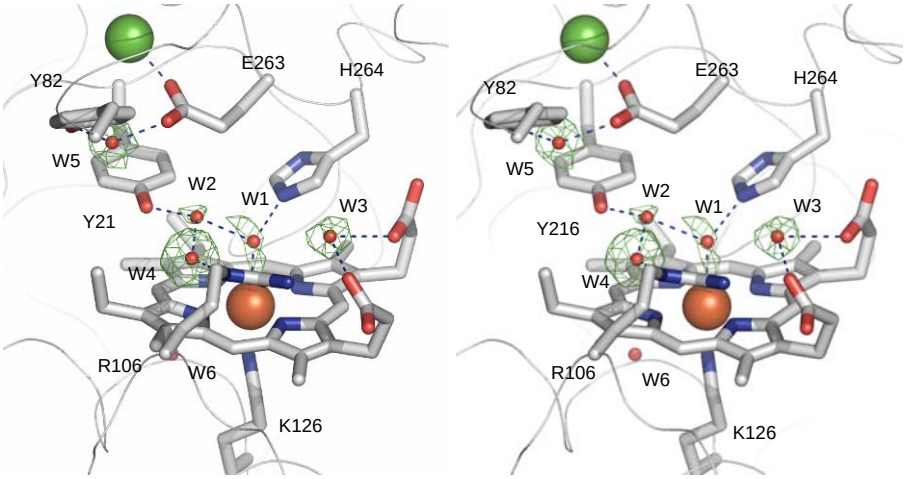
W3
Y216
W4
R106
W6
K126

B


E263
H264
Y82
W5
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Y21
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R106
W6
K126
E263
H264
Y82
W5
W2
W1
W3
Y216
W4
R106
W6
K126

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Role of a Conserved Glutamine Residue in Tuning the Catalytic Activity of *Escherichia coli* Cytochrome *c* Nitrite Reductase.

Thomas A. Clarke, Gemma L. Kemp, Jessica H. Van Wonderen, Rose-Marie A. S. Doyle, Jeffrey A. Cole, Nick Tovell, Myles R. Cheesman, Julea N. Butt, David J. Richardson and Andrew M. Hemmings.