Two active site residues, Asp-98 and His-255, of copper-containing nitrite reductase (NIR) from *Alcaligenes fae\-calis* have been mutated to probe the catalytic mechanism. Three mutations at these two sites (D98N, H255D, and H255N) result in large reductions in activity relative to native NIR, suggesting that both residues are involved intimately in the reaction mechanism. Crystal structures of these mutants have been determined using data collected to better than 1.9-Å resolution. In the native structure, His-255 Ne2 forms a hydrogen bond through a bridging water molecule to the side chain of Asp-98, which also forms a hydrogen bond to a water or nitrite oxygen ligated to the active site copper. In the D98N mutant, reorientation of the Asn-98 side chain results in the loss of the hydrogen bond to the copper ligand water, consistent with a negatively charged Asp-98 directing the binding and protonation of nitrite in the native enzyme. An additional solvent molecule is situated between residues 255 and the bridging water in the H255N and H255D mutants and likely inhibits nitrite binding. The interaction of His-255 with the bridging water appears to be necessary for catalysis and may donate a proton to reaction intermediates in addition to Asp-98.

The copper-containing nitrite reductase (NIR) from *Alcaligenes fae\-calis* strain S-6 belongs to a group of enzymes in the dissimilatory denitrification pathway, the process whereby nitrogen oxides compounds are reduced to dinitrogen gas (1). NIR is a key enzyme that catalyzes the first committed step in this pathway (2). The catalytic function of NIR is to catalyze the one electron reduction of nitrite (NO$_2^-$) to nitric oxide (NO) under anaerobic conditions (3). In *vitro*, N$_2$O is also produced from the close homologue *Achromobacter cycloclastes* NIR (AcNIR), if NO is not removed from the reaction (4). The *in vivo* electron donor to *A. fae\-calis* S-6 NIR (AfNIR) is pseudoazurin, a small blue copper protein that belongs to the cupredoxin family of electron transfer proteins (3, 5).

Crystallographic analysis of NIR has revealed a structure of three identical 37-kDa subunits, each of which contains 343 amino acids folded into two $\beta$ barrel domains (6, 7). Each NIR monomer contains one type I and one type II copper site that are differentiated by their respective spectroscopic signatures. The type I blue copper atoms are buried within the N-terminal domains, and each one is coordinated by four amino acid residues (His-95,2 Cys-136, His-145, and Met-150). The histidine and cysteine residues coordinate the type I copper in a distorted trigonal planar geometry with the methionine forming a weaker interaction at the axial position. Three mononuclear type II coppers are located at the interface between the subunits and are ligated by a water and three histidines (His-100, His-135, and His-306). His-100 and His-135 are provided by the N-terminal domain of one subunit, whereas His-306 is derived from the C-terminal domain of an adjacent subunit. The type I and II copper centers are approximately 12 Å apart and are connected covalently through Cys-136, a type I ligand, and His-135, a type II ligand.

Crystallographic, spectroscopic, and functional studies of NIR from several species have revealed that the type I copper center is the site of electron transfer and the type II copper is the site of nitrite reduction (7–9). Nitrite displaces the ligand water at the type II site as shown by crystallographic structures (10, 11) and electron nuclear double resonance spectroscopy (12, 13). Steady-state kinetics (14) are consistent with a proposed catalytic mechanism that proceeds by an ordered process where nitrite binds to the oxidized type II copper center before an electron is transferred from the type I copper (10, 11).

Analysis of the crystal structure of AcNIR suggested that the active sites, Asp-98 and His-255, which are observed to be close but not ligating to the type II copper, participate in the reaction mechanism (6). Structures derived from nitrite-soaked oxidized crystals of both native AcNIR and AfNIR reveal that nitrite bound asymmetrically via the oxygen atoms (10, 11). Structures using ascorbate-reduced crystals of native AfNIR revealed a loss of the ligand water, and the subsequent addition of nitrite at low temperature showed weaker nitrite binding (11). These crystallographic observations are consistent with nitrite binding preferably to the oxidized enzyme. In the ni-
trite-bound structures, the side-chain carboxylate of Asp-98 is observed to form a direct hydrogen bond to the oxygen atom of the nitrite ligand, suggesting a direct role in protonation of intermediates in the reaction pathway.

The role of His-255 in the reaction is more controversial. Early modeling by Strange et al. (15) suggested that bound nitrite may be hydrogen-bonded to His-255, enabling a direct proton transfer by this residue during catalysis. His-255 has been suggested to also donate a proton indirectly to reaction intermediates via a proton shuffling pathway using the bridging water to Asp-98 (10). A mutation to glutamate of the residue equivalent to His-255 in RnNIR suggested that this residue may be important in electrostatic stabilization of nitrite binding (16).

Additional insight into the mechanism has come from structural comparisons of the active sites of copper-containing A. xylosoxidans NIR (AxNIR) and AfnNIR to the zinc sites in superoxide dismutase (SOD), thermolysin, astacin, and carbonic anhydrase (11, 14, 15, 17). The common structural motif of carboxylic anhydrase, astacin, and NIR is a metal site with three protein ligands, a fourth distal ligand water, and a proton-abstracting group (a carboxyl or hydroxyl group) forming a hydrogen bond to the ligand water (15). Asp-98 in AfnNIR is structurally analogous to the proton-abstracting residues found in the zinc-containing enzymes, suggesting a related role for this residue. A catalytic role for His-255 has been suggested from a comparison to the SOD copper center that is ligated by four histidine ligands in a plane and a water molecule in the apical position (18). The recent crystal structure of the reduced yeast SOD (19) demonstrates a structural rearrangement following protonation of a histidine ligand (His-63) resulting in a more tetrahedral coordination of the copper similar to that found in AxNIR (14). As a result, His-63 adopts a position structurally analogous to His-249 in AxNIR and His-255 in AfnNIR. Interestingly, SOD activity has been observed previously in NIR from Rhodopsseudomonas sphaeroides (20) and is localized at the type II copper center in AxNIR (14). Together, these findings show an important catalytic role for His-63 in AfnNIR and are suggestive of a similar role for the His-255 analogue in AfnNIR.

Here we report the crystallographic, spectroscopic, and functional characterization of mutants of two key residues, Asp-98 and His-255, in the active site of NIR from A. faecalis S-6. Nitrite reduction activity assays of these mutants show that both Asp-98 and His-255 are essential for high level NIR activity. Furthermore, analysis of the high resolution crystal structures of these mutants demonstrates a direct proton donation role for Asp-98, whereas His-255 is likely responsible for indirectly providing a proton during catalysis and directing the binding of nitrite to the active site. The new information gained from investigation of these mutants has led to a revision of the mechanistic model of NIR catalysis.

EXPERIMENTAL PROCEDURES

Construction of NIR Mutants—Site-directed mutagenesis of the AfNIR gene (21) was carried out using synthetic oligonucleotides as described by Kukimoto et al. (7). The mutagenic primer used for the D98N mutant was 5’ CCG ATG ATC AAC TAT TTC CAT GCG 3’ and incorporates an EcoT22I restriction endonuclease site. The primer for the H255D mutant was 5’ GCC GAT ACG CTA GCA CAT GCG 3’ and encodes an internal MluI cleavage site. A similar primer was used for the H255N mutant with the exception that the GAT codon for His-255 was changed to AAT. Mutations were confirmed with M13 dye-deoxy nucleotide sequencing (22). To increase expression levels, mutants were amplified by the polymerase chain reaction and cloned downstream of a T7 promoter in-frame with a C-terminal His tag. Nickel affinity chromatography was used to isolate the protein to a purity of >95% as determined by SDS-polyacrylamide gel electrophoresis. The histidine tag was removed with a Factor Xa cleavage sequence followed by passing the digested sample over a High Q anion exchange column (Bio-Rad, Hercules, CA). A final dialysis step against Tris buffer, pH 7.0, supplemented with 10–50 mM copper resulted in full occupation of the copper sites as determined by atomic absorption spectroscopy. Yields of purified AfnNIR mutants were approximately 20–30% of the culture. A high level of AfnNIR protein was observed in the culture medium, and this procedure begins at residue Ala-4 and includes an extra four residues at the C terminus (Ile-Glu-Gly-Arg) that are part of the Factor Xa cleavage sequence.

Spectral Analysis—Samples of native AfnNIR and the D98N, H255N, and H255D mutants in 10 mM MES buffer, pH 6.0, were prepared to a concentration of 2 µM, prior to injection onto a reverse-phase column interfaced to an electrospray mass spectrometer. The protein was eluted with increasing concentrations of acetonitrile, pH 2.0, and injected directly into the carrier stream (90% acetonitrile/water) at a flow rate of 50 µl/min into a PE-Sciex API 300 quadrupole mass spectrometer. The copper content of native AfnNIR and the three mutant forms was measured by graphite furnace atomic absorption spectroscopy with a copper detection lamp at a wavelength of 327.4 nm. A standard curve was generated using 12.5, 25, and 50 parts per billion copper standards made up in 2% nitric acid. AfnNIR samples were diluted in 2% nitric acid, and duplicate 15-µl samples were injected into the furnace via an autosampler. The concentration of copper in the AfnNIR samples was interpolated from the standard curve, and the molar ratio of copper to protein was calculated using protein concentration values determined by the method of Bradford (23) using a bovine serum albumin standard. Optical scanning spectra were recorded at 25 °C on a Cary 3E UV-visible spectrophotometer fitted with a thermostat circulating water bath. Protein samples were analyzed in 10 mM Tris buffer, pH 7.0. EPR spectra were recorded at X-band frequencies on a Bruker ESP 300E electron paramagnetic resonance spectrometer equipped with a Hewlett-Packard Model 532B frequency counter. For liquid helium spectra (19.5 K) the EPR instrument was fitted with an Oxford Instruments Model 900 continuous flow helium cryostat and an ITC4 temperature controller. Liquid nitrogen spectra were collected at 87 K. AfnNIR samples were concentrated to approximately 1 mM, and buffer was exchanged with 10 mM MES and 40 mM KPO4, pH 7.2. Parameters used for data collection at 19.5 K were: modulation frequency 100,000 kHz, modulation amplitude 4.428 G, microwave frequency 9.450 GHz, and microwave power 0.3975 milliwatt (mW). A modulation amplitude of 0.708 G and a microwave power of 1.992 mW were used for data collected at 87 K.

Activity Measurements—Nitrite reductase activity was measured at 30 °C as described by Rakutani et al. (8). Methylviologen reduced with sodium nitrite as a redox acceptor was used as the electron donor to NIR, which in turn catalyzes the one-electron reduction of nitrite to nitric oxide. The starting solution contains 2 mM sodium nitrite, 0.1 mM methylviologen, 5 mM dithionite, and 20 mM KPO4 buffer (pH 7.0). NIR was added such that the final nitrite concentration was between 0.5 and 1.4 mM after 20 min. Residual nitrite was determined using the Griess reagents, and for the control, 1.8 mM nitrite was added. Units are defined as the amount of NIR required to reduce 1 µmol of NO2− per minute.

Crystallographic Data Collection—All three mutants crystallized in similar conditions: 0.1 M sodium cacodylate, 10–15% polyethylene glycol 4000, 6000, or 8000, and either zinc salt (D98N, H255N: 50–75 mM) or cadmium salt (H255D: 50 mM) at pH 4.7. The protein concentration used was 10–15 mg/ml. X-ray diffraction data from the mutants were collected on a Rigaku R-AXIS Iic image plate system using CuKα radiation generated by a Rigaku RU 300 rotating anode generator and a graphite monochromator. Data sets were collected at a temperature of 15–20 °C. The diffraction data frames were exposed for 35–40 min with a crystal oscillation angle of 0.75° and were processed with the program DENZO (24). Data collection statistics are summarized in Table 1.

Structure Solution and Refinement—The starting model used in refinement of the D98N mutant was chain A of the 1.8 Å resolution nitrite-soaked native AfnNIR structure (11) after removal of the solvent atoms, nitrite, and the side chain of residue 98. The D98N mutant structure was solved by molecular replacement using the program AMORE (25) and refined to a resolution of 1.9 A by the maximum likelihood method using the program CNS (26). The D98N structure was used as the starting model for the H255N structure. Subsequently, refinement of the H255N and H255D structures did not yield a free R-factor below 30%. A self-rotation function showed that 2-fold symmetry was present perpendicular to the crystallographic 3-fold axis. Submission of the reflection data to the Merohedral Crystal Twinning web server (27) confirmed that these crystals are indeed twinned. The H255N and
H255D data sets were corrected using the program DETWIN from the CCP4 suite (28), and subsequent refinement reduced the free R-factor to below 22%. Manual intervention was accomplished using the visualization program O (29), and the program PROCHECK (30) was used to identify regions of the structure requiring manual fitting. Refinement statistics for the final coordinate sets are presented in Table I.

**RESULTS**

Amplification of the genes carrying native and mutant forms of AfNIR followed by cloning into a T7 promoter expression vector produced stable genetic constructs. Recombinant native AfNIR isolated from this expression construct is within 5 Da of the calculated mass following cleavage of the N-terminal methionine residue as measured by mass spectrometry (Table II). The observed mass difference for each mutant from native AfNIR is within 3 Da of the expected mass difference.

Copper content was measured by atomic absorption spectroscopy to ensure that the mutations did not affect occupation of copper. The copper content of the samples was found to be 1.8 ± 0.1 atoms of copper per AfNIR monomer except for the H255N mutant, which had a lower copper occupation of 1.5 atoms of copper per monomer (Table II). The variation in the copper content is likely due to small differences in copper occupation between preparations and errors involved in the determination of protein concentrations using the Bradford assay.

UV-visible spectroscopy was used to probe changes in the electronic structure of the type I copper site. The visible absorbance maxima of native AfNIR and the three mutants are at the same three characteristic wavelengths of 458, 585, and 680 nm as seen in previous preparations of native AfNIR (3, 7). Also, the ratio of the 458- and 585-nm bands (1.3 ± 0.05) is well conserved among the three mutants and native AfNIR (Table II). Extinction coefficients for the mutants were calculated to be 3.0 × 10^3 M^-1 cm^-1 for the trimer using absorbance at 458 nm and protein concentrations determined by the method of Bradford (23).

A more rigorous analysis of both the type I and type II copper sites was performed with EPR spectroscopy, which showed a slight perturbation in the type II copper sites in the D98N and H255N mutants, but not in the H255D mutant (Fig. 1). Overall, the EPR parameters are similar to previously published values (7, 16, 31) with the exception of a slightly diminished type II copper coupling (A) of 100 ± 5 G in the D98N mutant and an increased g value of 2.53 for the type II signal from the H255N mutant (Table III). These perturbations may be the result of small shifts in the positions of the ligand waters observed in the crystal structures.

**Activity Assays**—The specific activity measured for native AfNIR is within experimental error of that reported for native AfNIR from a previous periplasmic recombinant expression system (7). The specific activities of the mutants are much lower than the native enzyme (Table II). Of the three mutants,
D98N AfNIR is the most active with a specific activity 94-fold less than that of the native AfNIR. The H255D and H255N mutants are a further 5- and 18-fold less active, respectively.

Crystallography—All three mutants crystallized in space group R3 with one monomer in the asymmetric unit resulting in the functional trimer being generated by the crystallographic 3-fold. These crystals are isomorphous with those obtained previously for M150E AfNIR; however, previous crystals contained previously for M150E AfNIR; however, previous crystals are isomorphous with those obtained previously for M150E AfNIR; however, previous crystals are in space group P2_1,2_1, and contain the complete trimer in the asymmetric unit (11, 17). The residues modeled begin at Ala-4 and end at Gly-339 (D98N structure) or Glu-342 (H255N and H255D structures). Ramachandran plots of the mutant structures shows that over 90% of the residues are in the most favored conformation with the remaining residues occupying allowed conformations. In each of the mutants there is a short region of poor density that correlates to a surface-exposed disordered loop starting at residue 187 and ending at residue 192. A least-squares fit of carboxic anhydrase atoms (root mean square deviation < 0.2 Å for main-chain atoms) showed that overall folds of the three mutant structures are similar to the previously determined native AfNIR structure (17).

The models include additional metal binding sites that are occupied by either zinc ions (D98N, H255N) or cadmium ions (H255D). Interestingly, one of these sites is located at the trimer axis near residue Asp-275 and may function to stabilize subunit interactions. The other two metal sites are located at crystal contacts. Although unlikely, the presence of zinc in the crystallization buffer may result in substitution of the copper sites. The green color of the crystals indicates that the type I site contains primarily copper. Crystal structures of preparations of type II-depleted AxNIR do not show the presence of zinc in the active site despite EXAFS evidence consistent with zinc binding to surface residues (14). Furthermore, anomalous scattering experiments of M150E AfNIR showed the presence of zinc bound to the mutated type I site with negligible binding at the type II site (17). Analysis of the crystallographic structures shows that the type I and II copper ligand distances and bond geometries are in reasonable agreement with previously published values of native AfNIR structures (11, 17) except for a small increase in the type II copper ligand distances observed in the H255D structure. The longer ligand bond lengths may be due to partial occupation of the copper site by cadmium, an additive in the crystallization mix.

Structure of the D98N Mutant—The type II copper in the active site of native NIR is situated at the interface between two monomers and is coordinated in a tetrahedral arrangement by the imidazole rings of three histidine residues and a solvent water molecule (Wat-503). In the native AfNIR structure, the Asp-98 side chain forms a well-defined hydrogen bond to this ligand water via atom O1. A second hydrogen bond is formed from atom O82 to His-255 Nε2 via a bridging water molecule (Fig. 2). In the D98N mutant, the Asn-98 side-chain amide is rotated by approximately 40° about the χ2 torsion angle resulting in the N82 atom being displaced by about 1.00 Å relative to the O81 atom of Asp-98 in the native AfNIR structure. Furthermore, Asn-98 N82 is located 3.42 Å away from the ligand water and has poor geometry to form a hydrogen bond (Fig. 2). The ligand water has also shifted (~0.8 Å) from the location of the equivalent water in the native AfNIR structure (11).

In contrast, the amide oxygen (O61) of Asn-98 is located in the same approximate position as the carboxylate oxygen (O82) atom of Asp-98 (Δ0.40 Å) such that the hydrogen bond (3.15 Å) to the bridging water is retained. Analogous to the native AfNIR structure, Asn-98 O61 also participates in a hydrogen bond (3.14 Å) with the backbone amide of Phe-99. As a result of these extensive hydrogen bond interactions, the Asn-98 side chain is well defined in the structure and has an average B-factor of 25.7 Å². The orientation of the Asn-98 side-chain amide was assigned based on crystallographic B-values, 26.8 Å² for N82 and 25.7 Å² for O81, and on the hydrogen bond donor and acceptor properties of the amide nitrogen and oxygen atoms.

Structure of the H255N Mutant—In native AfNIR, His-255, like the copper ligand His-306, is situated on the adjacent monomer on the opposite side of the active site from His-100, His-135, and Asp-98. The Ne2 atom of His-255 is linked through a water-bridged water hydrogen bond to the side chain of Asp-98 (Fig. 2). The most obvious effect of replacing His-255 with an Asn is an opening of the active site that allows room for an extra water molecule (B-factor of 42.0 Å²) that is not observed in the native AfNIR structure (Fig. 2). This novel solvent molecule (Wat-1099) is located between Asn-255 Oε1 (2.86 Å) and the bridging water (Wat-1098, 2.58 Å) and maintains a rigid hydrogen bond network and proton shuttling pathway between residue 255 and Asp-98. Water 1099 is also situated 2.33 Å from the ligand water (Wat-503) and 3.20 Å from the type II copper. The short distance between Wat-1099 and the ligand water is due to a shift in the position of the latter water of about 1.4 Å such that the hydrogen bond to Asp-98 O61 is retained. The conformation of Asp-98 is unaffected by the mutation.

In the native AfNIR structure, the ND1 atom of His-255 is involved in a bifurcated hydrogen bond to the backbone carboxyl of Gly-279 and Thr-280 Oγ1. As a result of a small rotation about the χ2 torsion angle of Asn-255, the N82 atom is directed toward only the side-chain hydroxyl of Thr-280. The orientation of the Asn-255 side chain was chosen to maintain this hydrogen bond interaction and to minimize the difference in the B-factors of the O61 and N82 atoms. The average B-factor of the Asn-255 side chain is 23.3 Å².

Structure of the H255D Mutant—The conformation of the Asp-255 side chain is almost identical to that of Asn-255 (Fig. 2). The O61 atom of Asp-255 was directed toward Thr-280 Oγ1, which in turn forms a hydrogen bond to the main-chain carboxyl of Gly-286. As observed in H255N AfNIR, an additional solvent water molecule (Wat-1099) is situated close (~2.5 Å) to the type II copper atom (Fig. 2). This water is clearly defined in omit difference maps and has a B-factor of 29.8 Å². Wat-1099 is located 3.14 Å from Asp-255 Oε2, 2.87 Å from the bridging water, and 2.87 Å from the ligand water. Unlike the H255N structure, the ligand water does not shift significantly from the position found in the native structure. The interaction of Asp-98 with the ligand water (2.8 Å) and the bridging water (3.3 Å) are largely unchanged from that observed in native AfNIR.

DISCUSSION

Active Site Hydrogen Bond Network—In the NIR structures determined to date, a bridging water molecule links the side chains of Asp-98 and His-255 (6, 7, 10, 17). This water appears

| Table III | EPR parameters of native and mutant NIRs |
|-----------|----------------------------------------|
| NIR       | Type I       | Type II      |
|           | g.  A1 (G)   | g.  A1 (G)   |
| Native    | 2.19  72 ± 10| 2.33  135 ± 5|
| D98N      | 2.21  70 ± 5 | 2.36  100 ± 5|
| H255N     | 2.21  82 ± 10| 2.53  160 ± 10|
| H255D     | 2.19  70 ± 5 | 2.33  140 ± 15|

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to assist in orientating Asp-98 and has been suggested to act as part of a proton shuttling pathway to this residue (10). As shown in Fig. 3, the likely location of the hydrogens of the bridging water can be inferred based on the available protein hydrogen bond donors and acceptors. The bridging water donates a proton to Asp-98 O\textsubscript{d2} and is a proton acceptor from His-255 N\textsubscript{e2}. Gly-259 N (not shown) and another water occupy the remaining hydrogen bond capacity of the bridging water completing a rigid hydrogen bond network (Fig. 3). Surprisingly, the D98N, H255N, and H255D mutant structures show little perturbation in the position of this water molecule despite mutations of these residues that alter hydrogen bond interactions.

**Role of Asp-98**—The positioning of protons in the active site as shown in Fig. 3 assumes that Asp-98 is negatively charged and His-255 is positively charged as would be expected at pH \textasciitilde 6, the optimum for activity, and at pH \textasciitilde 5 under which crystals were obtained. In native AfNIR, Asp-98 serves as a proton acceptor from both the ligand and bridging waters. The D98N mutation results in a side chain that acts as both a hydrogen bond acceptor (atom O\textsubscript{d1}) and donor (atom N\textsubscript{e2}) resulting in the loss of one of these hydrogen bonds to residue 98. From the crystal structure of this mutant, the Asn-98 side chain is reoriented such that N\textsubscript{e2} is further away from the ligand water (Fig. 2). Thus Asn-98 retains the hydrogen bond to the bridging water at the expense of losing the hydrogen bond to the water bound to the type II copper site, suggesting that the former interaction is stronger in the native enzyme. In the absence of a stabilizing interaction with residue 98 in the D98N mutant, a shift in the ligand water results in a small perturbation in the electronic environment of the type II copper site as detected by EPR (Table III). The lack of interaction between Asn-98 and the ligand water is consistent with a model of Asp-98 not being protonated, favoring the binding of substrates that can act as proton donors. A key feature of a proposed mechanism for nitrite reduction by AfNIR is the direct role played by Asp-98 as the proton donor to the nitrite (10, 11). The loss of this critical interaction between residue 98 and the ligand water and the inability of Asn-98 to act as a proton donor are the likely causes of the low activity of the D98N mutant.

The proposed protonation state of Asp-98 is supported by the examination of carbon monoxide bound to reduced native and D98N AfNIR by Fourier transform infrared spectroscopy (32).
The vibrational band observed for CO bound to native AfNIR is unaffected by changes in pH or the presence of deuterated solvent, presumably because Asp-98 is not protonated and thus cannot form a hydrogen bond with CO. In contrast, similar spectra of CO bound to D98N AfNIR showed strong pH dependence and are consistent with a protein group, likely the side-chain amide of Asn-98, forming a hydrogen bond to the CO.

Role of His-255—In the hydrogen bond model (Fig. 3), His-255 is protonated with the Ne2 atom acting as a proton donor to the bridging water. The mutation of His-255 to Asn or Asp would be expected to eliminate this interaction; however, for each mutant an additional water molecule is introduced into the active site and acts as an adapter by donating protons to either Asn-255 or Asp-255 and the bridging water (Fig. 2). This additional water overlaps with the location of nitrite bound to the type II copper in the native enzyme (11) and would need to be displaced for substrate to bind. In addition, the hydrogen bond between this additional active site water (Wat-1099) and the ligand water may further limit or alter the binding of nitrite. Not surprisingly, these mutations of AfNIR at His-255 are more than 450-fold less active than native AfNIR and are even less active than D98N AfNIR. Furthermore, the higher activity of D98N AfNIR may be due to His-255 donating protons via the bridging water and stabilizing intermediates in the reaction mechanism as an alternative to Asp-98. The copper content of H255N NIR is slightly lower than that observed for the other mutants; however, the difference is not likely to change the specific activity by more than 2-fold.

Recently, the analogous residue to His-255 in Rhodobacter sphaeroides 2.4.3 NIR (RsNIR) (His-287) has been mutated to a glutamate and characterized functionally and spectroscopically (13, 16). The H287E mutant is 100-fold less active than native RsNIR as measured by the ability to oxidize cytochrome c. Electron nuclear double resonance and EPR spectroscopy show greatly reduced nitrite binding to the type II site in the H287E mutant leading to the suggestion of a charge repulsion effect between the negatively charged Glu-287 and the nitrite molecule (13, 16).

To correlate the RsNIR mutation (16) with the crystal structures of the His-255 AfNIR mutants, a structural model of the H255E mutation was generated from the nitrite-soaked native AfNIR structure (11). This model shows that the side-chain carboxylate of Glu-255 may be positioned within hydrogen bonding distance of both the bridging water and the bound nitrite. Nitrite bound to the active site copper is likely protonated; however, the proximity of a second negative charge in addition to that of Asp-98 could be expected to alter nitrite binding. Comparison of the H255E model with the H255D and H255N AfNIR crystal structures reveals that a steric clash exists between the position of the Glu-255 side chain and the additional active site water (Wat-1099).

Revised Mechanism—Fig. 4 presents a revised catalytic mechanism for NIR incorporating roles for the bridging water and His-255. The resting oxidized enzyme has water bound to the type II copper forming a hydrogen bond with the negatively charged Asp-98 side chain. The nitrite substrate displaces the ligand water and is protonated when bound to the type II copper and also serves as a proton donor in the hydrogen bond with Asp-98. Reduction of the type II copper is followed by reduction and decomposition of the nitrite resulting in the formation of a transient complex with a hydroxyl and NO bound to the copper. Protonation of the hydroxyl ion by His-255 via the bridging water and release of NO returns the enzyme to the resting state.

An attempt to model His-255 in the nitrite-soaked structure (11) in an orientation such that a geometrically favorable hy-
hydrogen bond could be formed with the nitrite substrate was unsuccessful. An alternative role for His-255 may be to provide a positive charge in a location that assists in the reduction step leading to the formation of the transient complex of NO and water bound to the copper (Fig. 4). Thus, the loss of this positive charge rather than direct donation of a proton may contribute to the large reduction in activity of the H255N and H255D mutants. Analysis of the previously determined nitrite-soaked structure (11) shows that the bridging water is too distant (3.7 Å) to form a hydrogen bond to the oxygen of the bound nitrite that interacts with Asp-98. The position of this nitrite oxygen in the transient complex may be such that the bridging water could act to donate the second proton in conjunction with His-255.

Conclusions—The large loss of activity shows clearly that both Asp-98 and His-255 play a critical role in the catalytic mechanism of NIR. A stabilizing hydrogen bond from Asp-98, absent in the D98N mutant and donated from the ligand water and later from the nitrite substrate, is required for full activity of the enzyme and is consistent with a role in donating protons during the reaction. The structural conservation of the bridging water in all three mutants and the proximity to the substrate binding site suggest an important catalytic role, likely by acting as a conduit through which protons are shuttled and in orientating Asp-98. The dramatic reduction in activity in the H255N and H255D mutants, suggests that residue 255 has a catalytic role, possibly through donation of a proton via the bridging water and the positioning of a positive charge to stabilize electrostatically reaction intermediates.

Very recently, steady-state kinetic parameters reported for mutants of NIR from A. xylosidans at both Asp-98 and His-255 are consistent with these residues being involved in both catalysis and substrate binding (33). Further experimental work is needed to refine and resolve inconsistencies in the catalytic models proposed to date.

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*Active Site Mutants of Nitrite Reductase*