Structure of Nitrite Bound to Copper-containing Nitrite Reductase from Alcaligenes faecalis

MECHANISTIC IMPLICATIONS*

(Received for publication, July 22, 1996, and in revised form, August 21, 1997)

Michael E. P. Murphy‡, Stewart Turley, and Elinor T. Adman§

From the Department of Biological Structure, School of Medicine, University of Washington, Seattle, Washington 98195-7420

The structures of oxidized, reduced, nitrite-soaked oxidized and nitrite-soaked reduced nitrite reductase from Alcaligenes faecalis have been determined at 1.8–2.0 Å resolution using data collected at −160 °C. The active site at cryogenic temperature, as at room temperature, contains a tetrahedral type II copper site liganded by three histidines and a water molecule. The solvent site is empty when crystals are reduced with ascorbate. A fully occupied oxygen-coordinate nitrite occupies the solvent site in crystals soaked in nitrite. Ascorbate-reduced crystals soaked in a glycerol-methanol solution and nitrite at −40 °C remain colorless at −160 °C but turn amber-brown when warmed, suggesting that NO is released. Nitrite is found at one-half occupancy. Five new solvent sites in the oxidized nitrite bound form exhibit defined but different occupancies in the other three forms. These results support a previously proposed mechanism by which nitrite is bound primarily by a single oxygen atom that is protonable, and after reduction and cleavage of that N−O bond, NO is released leaving the oxygen atom bound to the Cu site as hydroxide or water. Copper-containing proteins are important mediators of denitrification, the reduction of nitrate and nitrite to gaseous nitric oxide, nitrous oxide and dinitrogen. Denitrification plays a role in determining the amount of biologically available nitrogen in soil and aqueous systems. Some of the gaseous products (NO and N2O) are greenhouse gases or react with ozone. Nitrite reductase (EC 1.7.99.3) is the best studied of the copper denitrification enzymes. Copper-containing nitrite reductase has been isolated from Alcaligenes faecalis strain S-6 (1) and found to receive electrons from pseudoazurin, a cupredoxin (2). The structure of the related enzyme from Achromobacter cycloclastes (3) revealed that NIR is a trimer of 37-kDa subunits. The trimer has four blue type I copper sites (Cys, His, His, and Met ligands), one buried within each subunit, and three mononuclear type II copper sites (three His ligands) found at the subunit interfaces. Investigation of copper NIRs from several species has revealed that the type I copper is the site of electron transfer with pseudoazurin and the type II copper is the site of nitrite reduction (4–6). Pulse radiolysis studies on A. cycloclastes NIR have demonstrated a first order rate constant for internal electron transfer between the type I and type II copper of 1.4 × 103 s−1 (7). The primary product of NIR is nitric oxide (NO); however, the enzyme can produce nitrous oxide (N2O) if nitric oxide is allowed to accumulate (8).

Many organisms make use of a heme cd1 nitrite reductase instead of the copper protein (9). The structure of this NIR from Thiosphaera pantotropha reveals a two domain structure, with heme c bound in a cytochrome c like domain, and heme d1 bound in a β-propeller domain, liganded by a tyrosine from the other domain (10). A heme iron nitrosyl intermediate (Fe⁺–NO⁺) is proposed in the mechanism for NO production (11). A similar mechanism has been proposed for copper NIR whereby nitrite is N-coordinated to a reduced copper followed by protonation, dehydroylation and production of a copper nitrosyl (Cu⁻–NO⁻) intermediate (8). Model compounds of NIR with both N-coordinate bound to Cu(I) and O-coordinate nitrite (12–14) bound to Cu(I) and Cu(II) have been characterized. Protonation of the nitrite adduct of the N-bound Cu(I) compound in non-aqueous solution results in the production of NO (12).

Direct evidence for intermediates of the proposed mechanism is lacking. Using isotopically labeled nitrite one can show that NIR can catalyze the nitrosylation of azide, hydroxylamine and nitrite to yield nitrous oxide (8, 15). Azide was shown to inhibit production of NO, and to yield decreasing 15NNO and small amounts of 14N15NO. On the other hand, hydroxylamine did not inhibit NO production and stimulated 14N15NO production, suggesting that it was more effective at trapping a bound NO species. In either case, a Cu(−NO)− species was hypothesized, not directly detected. When nitrite binds to oxidized type II copper in the related A. xylosoxidans NIR, electron nuclear double resonance spectroscopy suggests that nitrite displaces the water ligand (16). No change in the electron nuclear double resonance spectrum is seen when 15N nitrite is used, so that it is tempting to interpret the binding to Cu as O bound, although Howes et al. (16) point out that weak coupling might preclude this interpretation (16). In the cytochrome cd1 system, 18O-labeled water can be incorporated into nitrite (17); this observation is interpreted as the rehydration of a Fe2⁺–NO− species. As pointed out by Averill (11), no such 18O exchange has been seen in the copper nitrite reductases, and the trapping experiments with 13N15N were done with cell-free extracts, where NO reductase could have confounded the interpretation. Soaking nitrite into crystals of oxidized A. cycloclastes, NIR results in a partially occupied nitrite bound asymmetrically via the oxygen atoms (18). Finally, extended x-ray absorption fine...
structure experiments on A. xylosoxidans NIR shows O-bound nitrite (19).

Toward understanding the productive mode of ligation of nitrite to the active site copper, we report here high resolution structures of A. faecalis NIR in both the reduced and oxidized states in the presence and absence of nitrite to determine how the protein structure influences the binding mode of nitrite.

**EXPERIMENTAL PROCEDURES**

*Preparation of Crystals and Data Collection—Recombinant A. faecalis NIR was expressed in Escherichia coli and purified as described elsewhere (20). Green crystals were obtained using the hanging drop method with a reservoir solution containing 10% polyethylene glycol 4000 and 0.1 M sodium acetate, pH 4.0–4.8 (21). All crystals used in this study are in the space group P2₁2₁2₁, and are isomorphous. Data was collected at −160 °C using crystals from four different conditions: air oxidized, ascorbate reduced, nitrite soaked, and ascorbate reduced and then nitrite soaked at low temperature.

Oxidized crystals were soaked in freshly prepared reservoir solution. Reduced crystals were prepared by soaking in 10 mM ascorbate at 30 °C for 1 h, followed by soaking in a freshly prepared reservoir solution plus 10% glycerol over 30 min and remained colorless. These crystals were quickly transferred onto the end of a −40 °C air stream to allow transport of the crystal from the slush bath to a −160 °C N₂ cryostream. Once frozen, crystals were a faint amber color. It was not possible to carry out the converse reaction (that is, to reduce nitrite-soaked crystals with ascorbate at −40 °C) nor was it possible to reduce crystals at all at −40 °C. A Rigaku R-AXIS IIC image plate system was used to collect x-ray diffraction data from each of the four cryocooled crystals. Copper Kα radiation was produced from a rotating anode generator operated at 50 kV and 100 mA and focused with mirrors. Data from the reduced nitrite-soaked crystals were processed with the program DENZO (22). All other data sets were processed using software provided by the manufacturer (23). The details of each data collection are summarized in Table I.

**Structure Solution and Refinement—**Each of the crystals for the four data sets is isomorphous with crystals of the original 2.0 Å resolution refined structure (24) and contains the NIR trimer in the asymmetric unit. This structure was used as the starting point for refinement of the higher resolution (1.8 Å) oxidized nitrite soaked data. A randomly selected portion (8%) of the data was reserved for computation of the free R factor (25). Refinement was carried out with the program X-PLOR (26) using the parameter set parhcsdx.pro (27). In the final stages, all data were included in the refinement. The copper geometry, including the relative position of the nitrite molecules, was not restrained. B-factors were refined using data from 5 Å to the resolution limit (Table II) for 25–40 steps per refinement cycle. Manual adjustment and modeling was done using the program O (28). Additional solvents were identified by searching for peaks in F₁–F₀ maps using the CCP4 suite of programs (29).

The refined model from nitrite-soaked crystals (from data with the highest resolution) was used as the starting point for the other three data sets: oxidized, reduced, and nitrite-soaked reduced. The same refinement methods were applied to each of these. Final refinement statistics for all four structures are presented in Table II.

### TABLE I

| Crystal | Oxidized | Reduced | Nitrite-soaked oxidized | Nitrite-soaked reduced |
|---------|----------|---------|-------------------------|------------------------|
| Cell Dimensions (Å) | | | | |
| a | 61.86 | 61.79 | 61.65 | 62.1 |
| b | 102.8 | 102.4 | 102.4 | 102.8 |
| c | 146.3 | 146.2 | 146.1 | 146.6 |
| Resolution (Å) | 2.0 (2.25–2.0) | 2.0 (2.25–2.0) | 1.8 (2.0–1.8) | 1.85 (2.0–1.85) |
| R–merge<sup>a</sup> | 0.062 (0.136) | 0.068 (0.165) | 0.054 (0.108) | 0.049 (0.262) |
| Completeness | 90.0 (3.4) | 90.1 (3.1) | 90.1 (3.9) | 39.5 (2.6) |
| Unique reflections | 46591 (8200) | 51866 (10841) | 70076 (10128) | 67706 (7173) |
| Redundancy | 2 (1.2) | 3.4 (2.0) | 2.9 (1.5) | 2.3 (1.7) |

<sup>a</sup> Values in parentheses are for the highest resolution shell.

<sup>b</sup> When scaled together, the agreement between oxidized and reduced data sets was 0.14; between oxidized and nitrite-soaked oxidized, 0.17; between oxidized and reduced plus nitrite, 0.17; and between oxidized plus nitrite and reduced plus nitrite, 0.19.

<sup>c</sup> (I/I₀) is the average intensity divided by the average estimated error in the intensity.

\[
R = \frac{\sum_{hkl} |I(h, k, l) - \langle I(h, k, l) \rangle|}{\sum_{hkl} |I(h, k, l)|}\]

* TABLE II

| Parameter | Oxidized | Reduced | Nitrite-soaked oxidized | Nitrite-soaked reduced |
|-----------|----------|---------|-------------------------|------------------------|
| Resolution range (Å) | 10–2.0 | 10–2.0 | 10–1.8 | 10–1.85 |
| R-factor | 0.191 | 0.188 | 0.181 | 0.194 |
| Root mean square deviations: Bond length (Å) | 0.011 | 0.009 | 0.010 | 0.010 |
| Bond angles (°) | 1.7 | 1.5 | 1.7 | 1.8 |
| B-factor (Å<sup>2</sup>) (all) | 16.1, 17.4 | 19.0, 20.8 | 17.1, 19.1 | 17.6, 19.2 |
| Avg. solvent B-factor (Å<sup>2</sup>) | 21.8 | 31.4 | 27.6 | 28.6 |
| Type 1 copper B-factor (Å<sup>2</sup>) | 16.4 | 21.2 | 18.3 | 18.8 |
| Type 2 copper B-factor (Å<sup>2</sup>) | 15.2 | 18.7 | 16.4 | 15.7 |
| No. of reflections | 45955 | 51276 | 69446 | 67171 |
| No. of solvent atoms | 498 | 667 | 794 | 723 |

* TABLE II

| Parameter | Oxidized | Reduced | Nitrite-soaked oxidized | Nitrite-soaked reduced |
|-----------|----------|---------|-------------------------|------------------------|
| Protein Data Bank entry code | 1A57 | 1A58 | 1A56 | 1A58 |
RESULTS

The structure of oxidized NIR at cryogenic temperature is similar to the higher resolution room temperature structure reported previously (24). The nitrite-soaked and reduced crystals of NIR diffracted to greater than 2.0 Å resolution, and the overall quality of each structure is similar to or better than that of the room temperature structure (24). For each structure reported, the conventional R-factor is 19% or better (Table II). More than 90% of the residues are within the most favorable regions of a Ramachandran plot as defined by PROCHECK (30) and display reasonable stereochemistry (Table II).

Although the cell dimensions of the original crystal are larger than those from the low temperature data (63.0 × 103.6 × 146.9 Å), compression of the molecules into the smaller cell results in a shift of 0.8 Å overall, mostly in the direction of the largest cell dimension difference. There is one short stretch of residues 228–232 of the C chain that, through small adjustments in conformational angles and larger side chain rotations, moves about 3 Å. This short loop comprises a small helical bulge and simply rolls to one side to accommodate the tighter packing. Interestingly, this loop packs up against residues 228–232 of the A chain of a symmetry-related molecule and against residues 51–55 of that molecule. However the conformation of that loop in both the A and B chains remains as it was in the room temperature structure. The B values of the room temperature structure (PDB entry 2afn) are on the average 6 Å² higher for main chain atoms, 7 Å² higher for side chain atoms, 6–8 Å² for the copper atoms. Solvent B values are nearly 15 Å² higher.

Oxidized and Reduced NIR—The active site of NIR is located at the interface between two domains. The type II copper at the active site of oxidized NIR has a near perfect tetrahedral geometry (Fig. 1 A, Table III). Three histidine residues provide three ligands via N* atoms of the imidazole rings. The fourth

**TABLE III**

| Metal ligand geometry | oxidized | reduced | Nitrite soaked | Nitrite soaked |
|-----------------------|----------|---------|---------------|---------------|
| **Type I Copper Site (CuA)** |          |         |               |               |
| Ligand bond length (Å) |          |         |               |               |
| 95N*–Cu–136S*         | 2.06 (3) | 2.07 (2) | 2.09 (2)      | 2.10 (6)      |
| 145N*–Cu–150S*        | 1.98 (7) | 2.08 (4) | 2.00 (8)      | 2.03 (4)      |
| 150S*                 | 2.64 (5) | 2.58 (1) | 2.61 (6)      | 2.59 (6)      |
| Ligand bond angles (°) |          |         |               |               |
| 95N*–Cu–136S*         | 133 (2)  | 126 (4)  | 131 (1)       | 127 (2)       |
| 145N*–Cu–150S*        | 102 (1)  | 104 (2)  | 106 (2)       | 99 (4)        |
| 150S*–Cu–95N*         | 101 (3)  | 104 (3)  | 103 (1)       | 106 (2)       |
| Type II Copper Site (CuB) |          |         |               |               |
| Ligand bond length (Å) |          |         |               |               |
| 100N*–Cu–135N*        | 1.91 (7) | 1.94 (4) | 1.91 (5)      | 1.92 (8)      |
| 135N*–Cu–306N*        | 2.07 (1) | 2.01 (8) | 2.02 (5)      | 2.01 (13)     |
| 306N*                 | 2.09 (3) | 2.06 (2) | 2.15 (3)      | 2.11 (1)      |
| 503O (O1)             | 2.00 (23)| 2.3 (1)  | 2.1 (2)       | 2.4 (2)       |
| 503O (O2)             | 2.2 (1)  | 2.3 (2)  |              |              |
| Ligand bond angles (°) |          |         |               |               |
| 100N*–Cu–135N*        | 99 (3)   | 108 (1)  | 99 (3)        | 106 (1)       |
| 100N*–Cu–306N*        | 99 (2)   | 106 (4)  | 104 (2)       | 105 (3)       |
| 150S*–Cu–95N*         | 111 (2)  | 125 (3)  | 125 (3)       | 122 (3)       |
| 100N*–Cu–503O (O1,O2) | 148 (11) | 15, 893 (1) | 15, 490 (6)  |
| 150S*–Cu–503O (O1,O2) | 97 (8)   | 90, 127 (3) | 87, 123 (4)  |
| 306N*–Cu–503O (O1,O2) | 89 (8)   | 87, 102 (3) | 85, 105 (6)  |

**Metal Binding to A. faecalis Copper-containing NIR**

Oxidized and reduced NIR crystal structures are shown in Figure 1. The three histidine residues provide three ligands via N* atoms of the imidazole rings. The fourth ligand is an oxygen from either water or a hydroxyl ion. Two of these histidines are from domain 1, and his-306 is from domain 2 of another monomer in the trimer. All three histidines are located on β-sheet residues that form an integral part of the conserved β-barrel fold of each domain (33). The imidazole groups form hydrogen bonds to the remainder of...
the protein structure via the N\(^\circ\) atoms. His-135 and His-306 are hydrogen bonded to the main chain carbonyls of Cys-136 and His-306, respectively. His-100 forms a salt bridge with the side chain carboxylate of Glu-279.

The solvent copper ligand is hydrogen bonded to the side chain of Asp-98, which in turn forms a water-bridged hydrogen bond to the side chain His-255. Access to the copper site at the solvent ligand position is sterically limited by Ile-257, which is within 3.3 Å of the oxygen ligand.

Ascorbate-reduced crystals of NIR are colorless, indicating that the type I copper site is reduced to Cu(I). Inspection of the x-ray structure of these reduced crystals reveals that the active site copper solvent ligand is no longer present (Fig. 1B, and Table III). The conformation of other ligands does not change from the oxidized structure, resulting in a tri-coordinate tetrahedral copper site. The conformation of residues Asp-98 and His-255 are not perturbed by the absence of the solvent ligand.

In two of the three subunits C\(^1\)d of Ile-257 may be less ordered, adopting a conformation in which it becomes closer to the copper.

Although difference maps between the oxidized and reduced data showed shifts in atom positions at the type I site, consistent with differences seen between oxidized and reduced forms of pseudoazurin (34), the shifts are quite small (Table III). Differences in B values and poorer resolution preclude detailed analysis of the shifts.

Nitrite Soaked Oxidized and Reduced NIR—The environment around the active site copper of nitrite-soaked crystals is depicted in Figs. 1C and 2C. A nitrite molecule can be fit to electron density by replacing the active site solvent ligand such that the copper remains oxygen coordinate. The nitrite nitrogen lies at an approximate 45° angle to the plane defined by the copper and nitrite oxygens and is closely packed against the side chain of Ile-257. Attempts to fit nitrite in an N coordinate mode failed due to a poor fit to the density and a steric clash with Ile-257. Nitrite O2 forms a hydrogen bond to Asp-98 O\(^{\prime}\) (3.0 Å), which approaches the oxygen from the opposite side of the Cu–O plane to nitrite N. The other nitrite oxygen, O1, is hydrogen bonded to two water molecules. The average crystallographic B factor of the side chain of Asp-98 is 15 Å\(^2\) in the nitrite-soaked crystals, compared with 20 Å\(^2\) for reduced and oxidized crystals indicating that this residue is more ordered in the presence of nitrite. The conformations of His-255 and Ile-257 do not change upon nitrite binding.

The most notable difference, in addition to the nitrite, is the presence of five solvent molecules in the vicinity of the nitrite, the copper, and Asp-98 (Fig. 2). Only two of these are present in the oxidized structure, and a different pair is present in the reduced structure. The presence of these solvent molecules is also consistent with a more ordered Asp-98.

Reduced crystals soaked in 10 mM nitrite remained colorless unless allowed to warm to room temperature, upon which crystals became amber-brown. Data were collected from a slightly colored crystal at -160 °C. Difference density shows nitrite bound in an orientation similar to that found in the oxidized nitrite-soaked structure. The difference density is not as strong as in the oxidized structure, suggesting that the nitrite binding site is not fully occupied in these crystals (Fig. 1D). There is again less ordered solvent near Asp-98, and the average B value for Asp-98 is 17 Å\(^2\), intermediate to the oxidized and nitrite-soaked forms.

Fig. 2. Location of solvent sites in oxidized (oc) (A), reduced (rc) (B), nitrite-soaked (nc) (C), and reduced nitrite-soaked (nrc) (D) NIR. A, B, C, D, and E are five sites found in the nitrite-soaked oxidized form. C is common to all four, and A and E are unique to the nitrite-soaked oxidized form, whereas only sites B and C are in the oxidized form, and only C and D are in the reduced and nitrite-soaked reduced forms. Figure was created using Molscript (31) and Raster3D (35).
Evidence for O-coordinate binding of nitrite to oxidized type II copper of nitrite reductase has been observed crystallographically for *A. cycloclastes* (18) and by electron nuclear double resonance and extended x-ray absorption fine structure spectroscopy for *A. xylosoxidans* (16, 19). The present work differs from the *A. cycloclastes* NIR structure, in that the nitrite is bent such that the nitrogen points away from Asp-98 instead of toward Asp-98. This difference is likely due to the partial occupancy of nitrite in the *A. cycloclastes* NIR structure limiting the accuracy of the nitrite orientation. The electron density for nitrite bound to reduced NIR is also weaker than the oxidized structure. The Cu-O distances found in this study (Table III) are intermediate between those found in a dinitrito-O-O’ model complex, where the axial Cu-O distances are 2.5 Å and the basal Cu-O distances are 2.0 Å (37), and those found in a mono-nitrito complex, where the Cu-O distances are 2.2 and 2.0 Å (14). The other dimensions of the copper sites (Table III) are in reasonable agreement with those found in our earlier studies and those from extended x-ray absorption fine structure on the related *A. xylosoxidans* NIR (19).

An attempt to model an N-coordinate nitrite in the same location as the O-coordinate nitrate failed. Not only are there steric clashes with the side chain Ile-257, but difference maps computed after refining N-coordinate nitrate show negative density at the oxygen positions and positive density where the oxygens should be located. The space restriction and the stabilizing hydrogen bond to the carboxylate of Asp-98 likely limits nitrite binding to the O-coordinate mode. Restricting the volume of the ligand bound at the active site may be advantageous in limiting access to larger nonsubstrates, such as nitrate.

**Support for Proposed Mechanism**—The binding mode of nitrite to the copper site of *A. cycloclastes* NIR suggested a possible mechanism for nitrite reduction and dehydration (18). As shown in Fig. 3, we hypothesized (a) that nitrite displaces a water (or hydroxyl) bond to the copper and hydrogen bonds to Asp-98; (b) that the proton in this hydrogen bond remains with Asp-98 until the nitrite is reduced via the copper; (c) that the N-O bond at that oxygen breaks; and (d) that NO is released, restoring the active site. In this proposed scheme, the oxidized enzyme is in an intermediate protonation state, where the proton may formally reside on either the water (18) or Asp-98. Nitrite displaces the hydroxyl (top right), followed by reduction of the copper (bottom right). Nitrite decomposes after reduction and protonation to give an intermediate with hydroxyl and NO transiently bound to the copper. The native enzyme is restored when NO leaves. NO may rebind to copper, displacing hydroxyl (top left) as a possible first step in the production of N₂O.

**Solvent Structure at the Active Site**—There are five solvent sites (labeled as A, B, C, D, and E in Fig. 2, *nc*) seen in the oxidized nitrite-soaked crystals (Fig. 2, *nc*) in addition to the copper bound site in the oxidized native enzyme. Site C is found in all four structures; A and E are unique to the oxidized nitrite bound form; D is occupied only in nitrite bound or reduced forms; and B is occupied only in the oxidized forms. A plausible hydrogen bonding pattern is shown for these sites.

**Solvent Structure at the Active Site**—There are five solvent sites (labeled as A, B, C, D, and E in Fig. 2, *nc*) seen in the oxidized nitrite-soaked crystals (Fig. 2, *nc*) in addition to the copper bound site in the oxidized native enzyme. Site C is found in all four structures; A and E are unique to the oxidized nitrite bound form; D is occupied only in nitrite bound or reduced forms; and B is occupied only in the oxidized forms. A plausible hydrogen bonding pattern is shown for these sites.

Some of these water sites may play important roles in the mechanism. In the oxidized enzyme (Fig. 2, *oc*), the active site solvent is shown protonated and hydrogen bonded to Asp-98. Sites A and E, which hydrogen bond to the nitrite directly, may facilitate the reaction in two ways: both sites can hydrogen bond to nitrite O1, maintaining its orientation, and E can hydrogen bond to Asp-98, forcing its proton to go back to O2 of the nitrite. Site D, in turn, helps to position site E. All of this may help direct the product to a hydrophobic region above His-255 that is devoid of solvent and to modify the reduction potential of the nitrite to facilitate electron transfer. The role of His-255 and the solvent between it and Asp-98 is to ensure that Asp-98 remains well oriented and available for temporary bind-
Nitrite Binding to A. faecalis Copper-containing NIR

The amount of nitrous oxide produced by NIR from nitrite increases substantially when nitric oxide levels are high and is not detectable if nitric oxide is removed. Incubation of the copper NIR with $^{15}$NO$_2$ and $^{14}$NO, and yielding a mixed isotope N$_2$O, have been interpreted as the formation of a copper nitrosyl ($Cu^+-NO^-$) that reacts with a second nitrite (8). Assuming that these isotope studies on cell extracts actually reflect NIR activity (11) and not endogenous NO reductase activity, they can be interpreted in light of our present results. Our proposed scheme for production of NO does not include an N-coordinate copper nitrosyl intermediate. However, a copper nitrosyl may be formed by a rebound mechanism (8) such that released nitric oxide displaces the solvent ligand (Fig. 2), or it may be that NO remains near the active site so that when another nitrite is reduced, that species reacts with it to produce the observed N$_2$O without having to invoke an N-coordinate species.

Related Structures—Biomimetic models of NO and nitrite bound to copper have been synthesized and characterized. One model of Cu(I) coordinated with nitrite has been prepared with three t-butylpyrazol ligands in a distorted trigonal bipyramidal arrangement. In this complex, nitrite is asymmetrically O-coordinate (Cu–O = 1.975 and 2.169 Å) and has not been shown to yield NO (14). A similar Cu(I) complex with t-butylpyrazol ligands and NO is N-coordinate with a Cu–N distance of 1.759 Å and Cu–N–O angle of 163° (36). More recently, a Cu(I) nitrite complex using tri-isopropyltriazacyclononane to provide N ligands has been characterized in which nitrite is N coordinate to the copper (Cu–N = 1.903 Å) and that does quantitatively yield NO upon addition of protic acids (12). These biomimetic models provide precedence only for productive N coordinate nitrite binding to reduced copper but do not preclude a possible O-coordinate mechanism in NIR. Also, although there is no precedence for O-coordinate NO to copper, the proposed mechanism does not require the NO to be a stable copper ligand.

As noted previously (19), structural features of the type II site of NIR are paralleled in the zinc enzyme carbonic anhydrase, although in general the NIR active site is much less open to solvent. A zinc coordinated by a solvent atom near a proton-abstracting group, either a carboxylate or hydroxyl group (38), is typical of many zinc enzymes. The handedness of this site defined by the proton-abstracting group and substrate binding is typical of many zinc enzymes. The handedness of this site is defined by the proton-abstracting group and substrate binding is typical of many zinc enzymes. Nitrite reductase of A. xylosoxidans nitrite reductase (40) is consistent with our results, although they report a shorter nitrite O1-Cu distance and also propose an N-bound intermediate with which we disagree.

Acknowledgments—The recombination A. faecalis NIR was kindly provided by Musutuko Kimimoto and Makoto Nishiyama of the Department of Biotechnology, University of Tokyo, Tokyo, Japan.

REFERENCES

1. Kakutani, T., Watanabe, H., Arima, K., and Beppu, T. (1981) J. Biochem. 89, 483–472
2. Kakutani, T., Watanabe, H., Arima, K., and Beppu, T. (1981) J. Biochem. 89, 465–461
3. Godden, J. W., Turley, S., Teller, D. C., Adman, E. T., Liu, M. Y., Payne, W. J., and LeGall, J. (1991) Science 253, 438–442
4. Libby, E., and Averill, B. A. (1992) Biochem. Biophys. Res. Commun. 187, 1529–1535
5. Abraham, B. E., and Beppu, T. (1983) J. Biochem. 95, 587–593
6. Kimimoto, M., Nishiyama, M., Murphy, M. E. P., Turley, S., Adman, E. T., Horinouchi, S., and Beppu, T. (1994) Biochemistry 33, 5246–5252
7. Suzuki, S., Kohzawa, T., Deligear, Yamaguchi, K., Nakamura, N., Shidara, S., Kohyashiki, K., and Takagawa, S. (1994) J. Am. Chem. Soc. 116, 11145–11146
8. Halse, C. L., and Averill, B. A. (1989) J. Am. Chem. Soc. 111, 7222–7233
9. Coyne, M. S., Arakamukkani, A., Averill, B. A., and Tiede, J. M. (1989) Appl. Environ. Microbiol. 55, 2924–2931
10. Pulop, V., Muir, J. B. W., Ferguson, S. J., and Haidu, J. (1995) Cell 81, 369–377
11. Averill, B. A. (1996) Chem. Res. 96, 2951–2964
12. Halfen, J. A., and Toman, W. B. (1994) J. Am. Chem. Soc. 116, 5475–5476
13. Halfen, J. A., Mahapatra, S., Wilkinson, E. C., Gengenbach, A. J., Young, Y. G., Que, L., and Toman, W. B. (1996) J. Am. Chem. Soc. 118, 763–776
14. Tolman, W. B. (1993) Inorg. Chem. 30, 4877–4880
15. Jackson, M. A., Tiedie, J. M., and Beppu, T. (1991) FEBS Lett. 295, 111–116
16. Howes, B. D., Abraham, Z. H. L., Lowe, D. J., Bruser, T., Eady, R. R., and Smith, B. E. (1994) Biochemistry 33, 3171–3177
17. Kim, C. H., and Hollocher, T. C. (1984) J. Biol. Chem. 259, 2092–2099
18. Adman, E. T., Godden, J. W., and Turley, S. (1995) J. Biol. Chem. 270, 27458–27474
19. Strange, R. W., Dodd, F. E., Abraham, Z. H. L., Grossman, J. G., Bruser, T., Eady, R. Y., Smith, B. E., and Hasnain, S. S. (1995) Nat. Struct. Biol. 2, 287–292
20. Nishiyama, M., Suzuki, J., Kimimoto, M., Ohnuki, T., Horinouchi, S., and Beppu, T. (1993) J. Gen. Microbiol. 139, 725–733
21. Turley, S., Adman, E. T., Sicker, L. C., Liu, M.-Y., Payne, W. J., and LeGall, J. (1988) J. Biol. Chem. 263, 417–419
22. Orwinowski, Z. (1993) In Proceedings of the CCP4 Study Weekend (Sawyer, L., Evans, P. R., and Leslie, A. G. W., eds), pp. 87–91, United Kingdom Science and Engineering Research Council Daresbury Laboratory, Daresbury, UK
23. Higashi, T. (1990) J. Appl. Cryst. 23, 253–257
24. Murphy, M. E. P., Turley, S., Kimimoto, M., Nishiyama, M., Horinouchi, S., Sasaki, H., Tanokura, M., and Adman, E. T. (1995) Biochemistry 34, 12107–12117
25. Brunger, A. T. (1992) Nature 355, 472–475
26. Brunger, A. T. (1990) X-PLORE, Version 3.1, Yale University, New Haven, CT
27. Engh, R. A., and Huber, R. (1991) Acta Crystallogr. Sec. A 47, 392–400
28. Jones, T. A., Zou, J.-Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sec. A 47, 110–119
29. Collaborative Computational Project, Number 4 (1994) Acta Crystallogr. Sec. D 50, 760–763
30. Laskowski, R. A., Moss, D. S., and Thornton, J. M. (1993) J. Mol. Biol. 231, 1049–1067
31. Kraulis, P. (1991) J. Appl. Cryst. 24, 946–950
32. Arnez, J. G. (1994) J. Appl. Cryst. 27, 649–653
33. Murphy, M. E. P., Lindley, P. F., and Adman, E. T. (1997) Prot. Sci. 6, 761–770
34. Peters-Libeu, C. A., and Adman, E. T. (1997) Biochemistry, in press
35. Merritt, R. A., and Murphy, M. E. P. (1994) Acta Crystallogr. Sec. D 50, 869–873
36. Talman, W. B., Carrier, S. M., Ruggiero, C. E., Anthrione, W. E., and Whitterk, J. W. (1993) In Bioinorganic Chemistry of Copper (Karlin, D. K., and Tykela, Z., eds) p. 406, Chapman and Hall, Inc., New York
37. Sibiryan, R. T., Potenza, J. A., and Schugar, H. J. (1996) Inorgano-Chimica Acta 243, 33–37
38. Argos, P., Garavito, R. M., Eventoff, W., Rossman, M. G., and Branden, C. I. (1978) J. Mol. Biol. 126, 141–158
39. Gemius-Ruth, F. X., Grant, T., Vialleur, L., Nar, H., Kusthardt, U., Zwilling, R., Bode, W., and Stocker, W. (1994) J. Biol. Chem. 269, 17111–17117
40. Dodd, F. E., Hasnain, S. S., Abraham, Z. H. L., Eady, R. R., and Smith, B. E. (1997) Acta Crystallogr. Sec. D 53, 469–476

42860