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Catalyzing NO to N_2O in the Nitrogen Cycle

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The great planetary nitrogen cycle, which includes the cyclic conversion of nitrogen gas (N_2) into "fixed" nitrogen that can be used by plants, is in large part mediated by metalloenzymes that catalyze the elementary chemical reactions. On page 1666 of this issue, Hino *et al*. (1) take an important step toward understanding the chemical function and evolution of one of these enzymes. They describe the structure of a nitric oxide reductase (NOR) from a common bacterium which plays an important role in the nitrogen cycle—and in human disease.

Today, human activities such as the widespread use of agricultural fertilizers and the burning of fossil fuels are influencing Earth's nitrogen cycle (2). Of considerable interest is the increasing release into the atmosphere of nitrous oxide (N_2O), a greenhouse gas (3) about 300 times as powerful, on an equimolar basis, as CO_2 (2). The vast majority of N_2O originates from microbes that break down nitrogen compounds in soil and water. Approximately equal quantities of N_2O come from two processes: nitrification (which converts ammonia into nitrite), in which N_2O is an unintended by-product of the oxidation of hydroxylamine (NH_2OH), and anaerobic denitrification, in which nitric oxide (NO) and N_2O are formed as diffusible intermediates (2, 4).

Hino *et al*. studied cytochrome c–dependent nitric oxide reductase (cNOR), in which the cytochrome acts as the electron donor in an enzyme that catalyzes the reduction of NO in *Pseudomonas aeruginosa*, a ubiquitous, denitrifying bacterium. It is also a nasty pathogen, particularly among burn victims and patients with chronic infections of their airways (5, 6). Similar infectious bacteria, such as *Neisseria meningitides* and *N. gonorrhoeae*, depend on NOR activity to withstand the defenses of host cells (7). Hino *et al*. describe the x-ray structure of cNOR at 2.7 Å resolution. In addition to offering insight into the detailed chemical mechanisms that affect the nitrogen cycle, the structure offers evidence for the existence of a common ancestor connecting the NORs to the heme-copper oxidase (HCO) superfamily of enzymes, and raises intriguing questions about how one diverged from the other. Finally, the structure provides the basis for an atomic-level understanding of the chemistry of both NOR and HCO enzymes.

Both HCOs and NORs are integral membrane proteins. The HCO superfamily contains at least three types that share a common core structure but vary in the number of peripheral subunits, heme types, physiological electron donors, and proton pathways that can generate the heme-copper cluster (8). The NOR family is also composed of several types (9). *P. aeruginosa* cNOR consists of a small (NorC) and a large (NorB) subunit. Although NorB

and the large subunits of HCOs are clearly homologs, both families show diversity in the smaller subunits (especially in the associated electron donor). It is hard to see how the predominantly β-sheet cupredoxin fold of subunit II containing Cu_A (see the figure, left panel) and the predominantly helical cytochrome c fold of NorC (right) could be linked evolutionarily via a series of mutations. Rather, it seems more likely that the smaller subunits evolve partly by "jumping" from one type of large subunit to another.

Hino *et al*. compare their structure to the simplest known HCO, the B-type cytochrome ba_3-oxidase from *Thermus thermophilus* (8, 10), and a comparison to the C-type cbb_3 oxidase from *P. stutzeri* (11) is also informative. Although the cbb_3 enzyme appears to be closer to cNOR than does ba_3, similarities in the amino acid sequences of NorB and subunit I of cbb_3 are low (<40%). Regardless, the two proteins exhibit highly similar three dimensional structures. Specifically, 12 central transmembrane helices are conserved that share a topology of tightly packed helices arranged around a low-spin heme and a binuclear active site (i.e., a heme-b_3/Fe_B or a heme-a_3/Cu_B). In ba_3, the active-site heme-a_3/Cu_B center is buried in the hydrophobic core of the enzyme, requiring that both polar (H^+, e^-, and H_2O) and lipophilic (O_2) reactants move along structurally specified pathways to their intended destinations. In cNOR, the active-site heme-b_3/Fe_B is found essentially at the same location in the hydrophobic core. The three histidine side chains that coordinate the Cu_B in HCO are conserved in cNOR and are supplemented with a glutamyl carboxylate group, providing a favorable coordination shell for an iron ion at the Fe_B site.

The structure of cNOR also shows that transmembrane pathways (D- and K-paths) for proton uptake are absent; this supports previous findings that cNOR does not pump protons (12) and sheds light on how heme-Cu oxidases acts as a proton pump. Hino *et al*. also propose pathways for electron transfer from the cytochrome c via the low-spin heme and proton entries from the periplasmic side to the binuclear center. As in cbb_3, a Ca^{2+} ion bridges and may stabilize an efficient electron transfer route between the two hemes in NorB. Another remarkable feature of the cNOR structure is the presence of a Y-shaped hydrophobic channel similar to that reported by Luna *et al*. (13) in ba_3 and by Buschmann *et al*. (11) in cbb_3. In cNOR, this channel must serve to carry NO from the lipid bilayer to the active site.

The authors do not assign the exit route of the N_2O product, but the factor of 10 increase in water solubility of N_2O relative to O_2 or NO may allow this small molecule to diffuse through both hydrophobic and hydrophilic channels. A question worthy of future investigation is whether the exit route of N_2O from cNOR influences its capture by the periplasmic, copper-containing N_2O reductase, and its escape into the atmosphere. The structure also leaves two other questions unanswered: (i) How do the two NO molecules bind at the diiron site? and (ii) How do the N-N bond formation and N-O bond cleavage occur? A distal glutamic acid within 5 Å of the iron cluster that appears conserved in NorB sequences may provide a proton to a putative hyponitrite anion ($N_2O_2^{2-}$) and facilitate N-O bond cleavage. Although most investigators might agree that the first NO binds to the ferrous heme b_3, the mode of addition of a second NO to this mononitrosyl complex remains to be defined, as do the respective roles of the two Fe ions (14). Bioengineering models of the heme b_3/Fe_B site in the simpler protein scaffold of myoglobin will be valuable tools for

conducting these mechanistic investigations (15). Structural characterization of other transmembrane NORs in parallel with HCOs will further clarify evolutionary variation in Fe_B/Cu_B coordination spheres, electron and proton transfer pathways, and hydrophobic channels to the catalytic dinuclear center.

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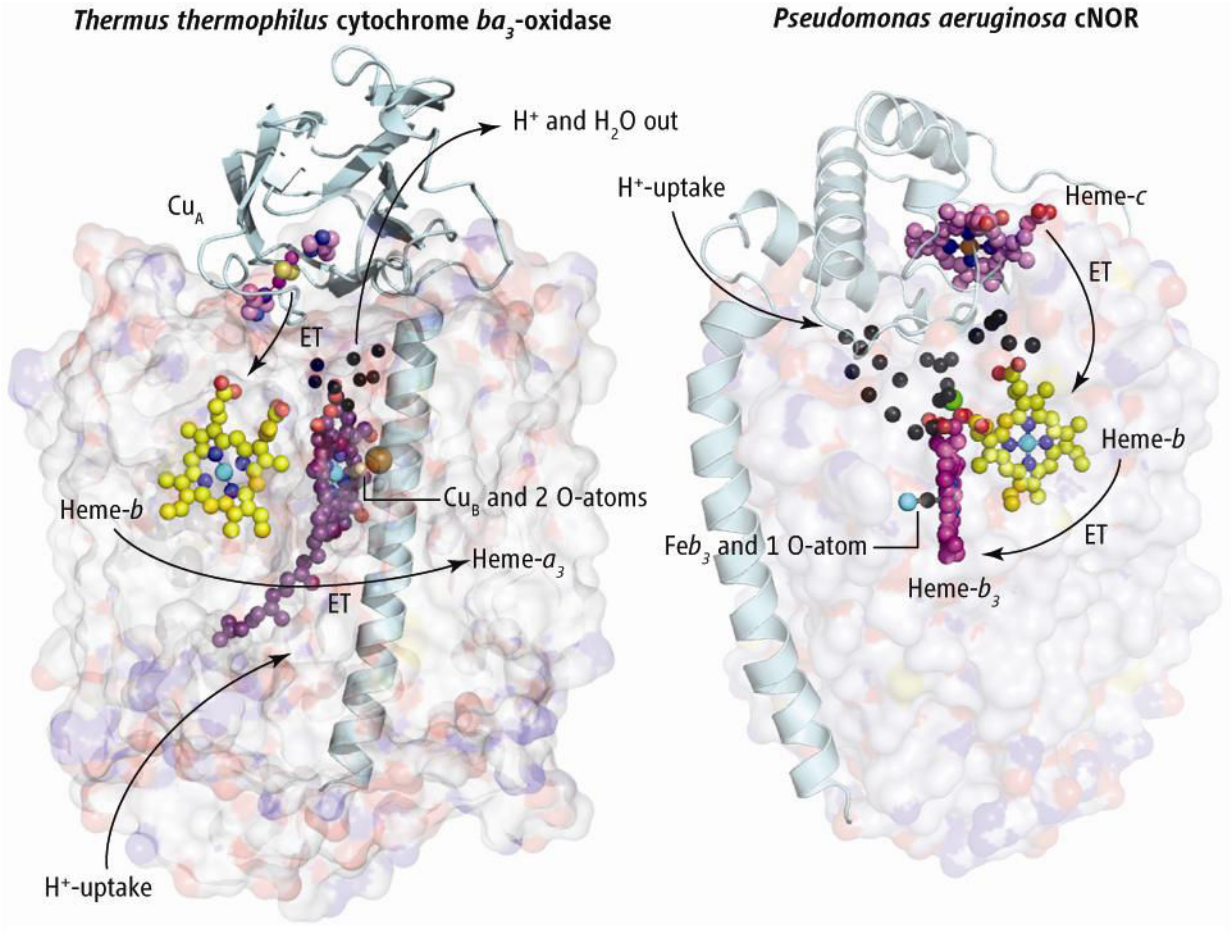

Figure. Compare and contrast

The structures of *Thermus thermophilus* (*Tt*) cytochrome ba_3 oxidase (left) and cNOR from *Pseudomonas aeruginosa* (*Pa*) (right), with large subunits shown as transparent surfaces. The two structures are aligned vertically at the level of the *b*-hemes and the cNOR structure is rotated approximately 180° about its vertical axis to bring the *b*-hemes into the same plane. Electrons flow from the subunit II of ba_3 that anchors the dinuclear copper cluster Cu_A, and from the cytochrome c in cNOR. These two redox centers and globular domains exemplify the variability in redox partners in the oxidases and NO reductases, as evolution may allow for occasional interchange of large and small subunits. Electrons flow into the *b*-hemes before passing to the binuclear active sites. Presumed proton flow in ba_3 oxidase and its water exit are represented by arrows. Black spheres represent crystallographically defined water molecules. The single Ca^{2+} ion is shown as a green sphere. In cNOR, protons appear to flow from the outside into the extensive water cluster, and from there into the binuclear heme-b_3/Fe_B active site.