One Site Fits All: Design of a Functional Nitric Oxide Reductase within a Myoglobin Scaffold

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In recent years, significant progress has been achieved in the computational design of functional artificial enzymes. When combined with directed evolution protocols, their efficiencies are comparable to those obtained with catalytic antibodies.[1]

In comparison, the *in silico* creation of artificial metalloenzymes remains challenging. This may be due to the difficulty in computing both the transition states for metal-catalyzed reactions and the corresponding entatic state for a metalloenzyme.[2]

To circumvent these challenges, two complementary approaches have been successfully pursued:

i) Small molecule "enzyme models" can be designed to mimick the active site of a metalloenzyme of interest. Although these rarely rival natural enzymes in terms of catalytic efficiency, they often yield precious structural and mechanistic information.[3]

ii) Catalytically competent moieties can be introduced in a protein scaffold to yield artificial metalloenzymes. In this context, sperm whale myoglobin – a globular protein consisting of eight alpha helices and a heme prostetic group surrounded by a hydrophobic pocket, Figure 1a – has been exploited as a stable scaffold for the creation of artificial metalloenzymes.[4]

In a recent study, Yi Lu and coworkers have rationally designed and structurally characterized a functional bacterial nitric oxide reductase (NOR) within a myoglobin scaffold.[4b] This achievement is particularly noteworthy as neither the X-ray structure nor an unambiguous reaction mechanism of NOR have been elucidated.[5]

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Nitric oxide reductase (NOR) is a key enzyme in the nitrogen cycle and thus is critical to all life forms on Earth. It catalyzes the two electron reduction of NO to N_2O according to Equation 1.

Sequence alignment and structural threading suggested that NORs are structurally homologous to subunit I of heme copper oxidases (HCO, Figure 1c).[5a] Most interestingly, some heme copper oxidases (HCOs) and NORs show reciprocal promiscuity towards their substrates albeit with significantly reduced efficiencies: NOR from *Paracoccus denitrificans* reduces dioxygen to water (according to Equation 2) and cytochrome *cbb₃* from *Pseudomonas stutzeri* catalyzes the reduction of NO to N_2O.[6]

Major structural differences between both enzymes include:

i) the substitution of Cu_B in HCO by Fe_B in NOR

ii) the presence of conserved glutamate(s) near the catalytic site in NOR

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O \qquad (Eq. 1)$$

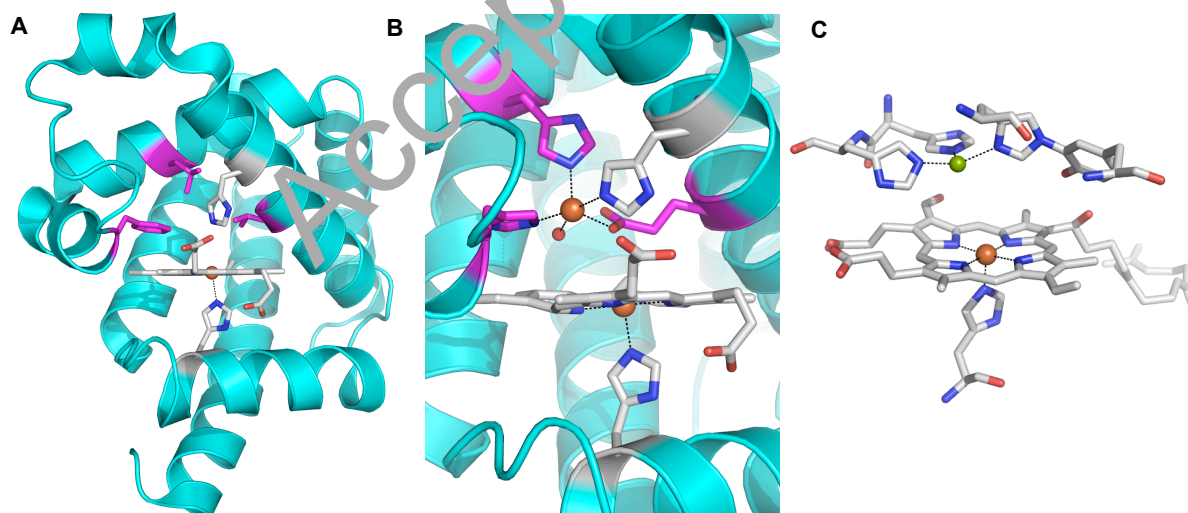$$O_2 + 4H^+ + 4e^- \rightarrow 2 H_2O \qquad (Eq.2)$$

Figure 1: A) Cartoon representation of wildtype holo-myoglobin with histidines H64 (Fe_B), H93 (heme) involved in metal complexation in NOR-model Fe_BMb. Side chains of residues (L29, F43, V68) targeted for mutagenesis are highlighted in magenta (pdb code 1JP6). B) Active site of NOR-model Fe_BMb with mutations L29H, F43H, V68E (pdb code 3K9Z); the water molecule coordinated to Fe_B is depicted as a red sphere. C) Active site of

cytochrome c oxidase from bovine heart (pdb code 2OCC); copper is depicted as a green sphere; the peroxo-bridge between iron and copper has been omitted. Pictures from pdb files have been generated with Pymol from DeLano Scientific Inc.

In 2000 Yi Lu and coworkers engineered an HCO-model within myoglobin (termed Cu_BMb). For this purpose, they created a metal binding site by introducing two histidine residues which, combined with the distal His64, provide a tetrahedral coordination environment for copper similar to cytochrome c oxidase from bovine heart (Figure 1c).[4f] As reported for natural HCOs, this artificial metalloenzyme Cu_BMb was later shown to catalyze the reduction of NO to N_2O.[4g]

Building upon this and relying on force field modelling, the copper binding site was modified to accommodate an iron ion by introduction of an additional critical glutamate residue, thus providing a $(His)_3Glu$ binding site for the non-heme Fe_B (Figure 1b).

The resulting artificial Fe_BMb NOR was characterized by X-ray crystallography, confirming the predictive power of the semi-quantitative computational design (*ie.* Zn parameters were used to model the Fe_B site). Additional striking features of the Fe_BMb are:

i) In addition to the $(His)_3Glu$ coordination, a water molecule is bound to Fe_B thereby expanding the coordination sphere to 5+1 (with a weak contact with the second oxygen of glutamate).[5,7]

ii) The Fe-centers (heme-Fe and Fe_B) seem to be spin coupled in analogy to NOR as demonstrated by EPR-spectroscopy and redox potential determination.

Next, the behaviour of the fully reduced artificial NOR Fe_BMb was studied under single turnover conditions. Subsequently a second turnover was approximated by re-reduction of the artificial NOR by addition of dithionite. A control experiment with wildtype Mb in the presence of added Fe(II) did not lead to the production of N_2O even after the addition of

dithionite. This is surprising as Fe(II) combined with dithionite in the absence of protein led to N_2O formation: approximately half of what was produced in the presence of NOR Fe_BMb. The mode of action of NOR remains a matter of debate while different mechanisms have been presented in the literature.[5] A critical issue in this context is the role of both metals in the binding and activation of the substrates, (Figure 2a):

i) Do both NO-molecules bind to Fe_B (the *cis:Fe_B*-mechanism)?[8]

ii) Does one NO bind to the heme b_3 center and one to Fe_B (the *trans*-mechanism)?[9]

iii) A third, intermolecular mechanism (the *cis:b_3*-mechanism) has been suggested for NO reduction in cytochrome *cbb_3* oxidase from *Pseudomonas stutzeri*: a free NO attacks a heme-bound nitrosyl.[10]

Recently, Collman, Solomon and coworkers presented the first functional small molecule model for the active site of NOR (Figure 2 b).[11] Spectroscopic characterisation of the NO complexes in different oxidation states point towards a *trans*-mechanism.
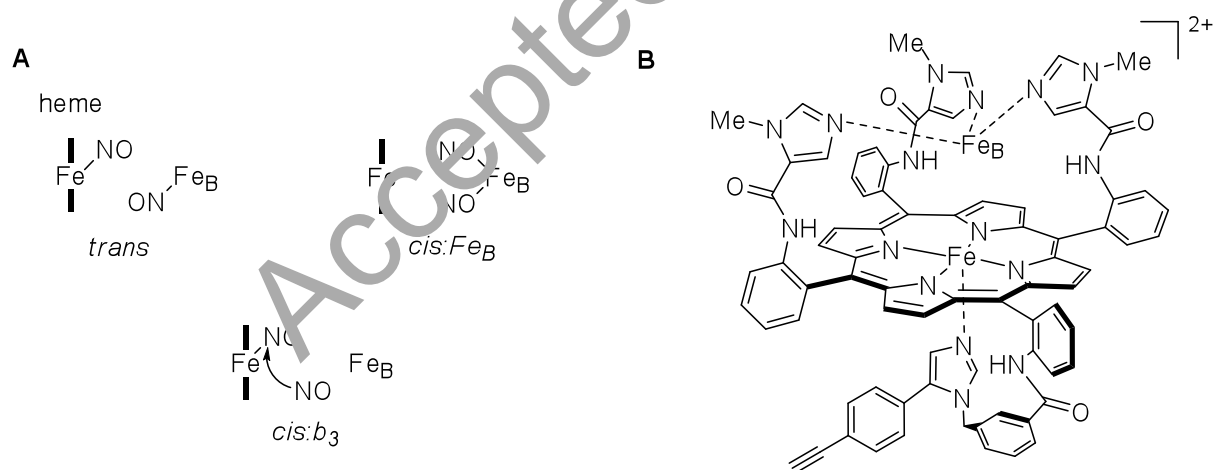

Figure 2: A: Proposed intermediates in the formation of N_2O from NO by NOR; B: functional small molecule model reported by Collman, Solomon and coworkers.[11]

Overall, the groundbreaking study by Lu and coworkers has opened the way towards the rational design of artificial metalloenzymes for more challenging reactions. For the artificial

NOR, several issues deserve further scrutiny: catalytic efficiency (k_{cat}, K_M and total turnover number) as well as detailed reaction mechanism (mode of NO binding, proton shuttling, product release etc.).

In a broader perspective, the use of more elaborate computational algorithms, combined with efficient directed evolution protocols should enable the creation and optimization of highly versatile artificial metalloenzymes in a variety of protein folds.[12]

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$$2\,NO + 2e^- + 2H^+ \longrightarrow N_2O + H_2O$$
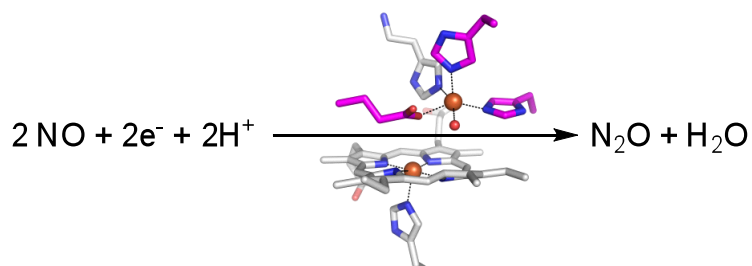
One Site Fits All! Yi Lu and coworkers have reported the conversion of sperm-whale Myoglobin into a functional nitric oxide reductase. For this purpose, they designed a second metal binding site into the wildtype holo-protein and demonstrated NO reduction with the structurally characterized model, making thereby a significant contribution to the rapidly developing field of artificial metalloenzymes.