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Design of a Functional Nitric Oxide Reductase within a Myoglobin Scaffold

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In recent years, significant progress has been made 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 could 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:

1) Small-molecule "enzyme models" can be designed to mimic the active site of a metalloenzyme of interest. Although these rarely rival natural enzymes in terms of catalytic efficiency, they often yield invaluable structural and mechanistic information.[3]
2) Catalytically competent moieties can be introduced into a protein scaffold to yield artificial metalloenzymes. In this context, sperm whale myoglobin—a globular protein consisting of eight α-helices and a heme prosthetic group surrounded by a hydrophobic pocket, Figure 1 A—has been exploited as a stable scaffold for the creation of artificial metalloenzymes.[4]

In a recent study, Yi Lu and co-workers 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 has been elucidated.[5]

Nitric oxide reductase 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).

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

Sequence alignment and structural threading suggested that NORs are structurally homologous to subunit I of heme copper oxidases (HCO, Figure 1 C).[5a] Most interestingly, some HCOs and NORs show reciprocal promiscuity towards their substrates albeit with significantly reduced efficiencies: NOR from *Paracoccus denitrificans* reduces dioxygen to water [according to Eq. (2)] and cytochrome cbb_3 from *Pseudomonas stutzeri* catalyzes the reduction of NO to N_2O.[6]

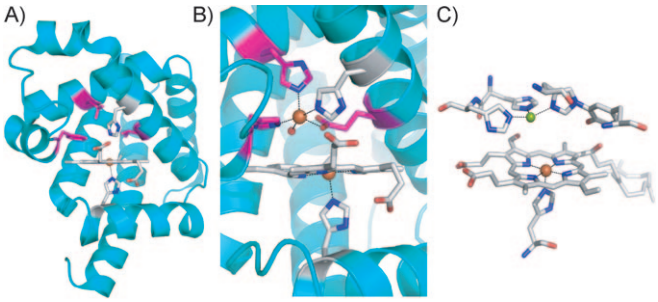

Figure 1. A) Cartoon representation of wild-type holo-myoglobin with histidines H64 (FeB) and H93 (heme) involved in metal complexation in NOR model FeBMb. The side chains of residues targeted for mutagenesis (L29, F43, V68) are highlighted in magenta (PDB ID: 1JP6). B) The active site of NOR model FeBMb with mutations L29H, F43H, and V68E (PDB ID: 3K9Z); the water molecule coordinated to FeB is depicted as a red sphere. C) The active site of cytochrome c oxidase from bovine heart (PDB ID: 2OCC); copper is depicted as a green sphere; the peroxo bridge between iron and copper has been omitted. Pictures from PDB files were generated with Pymol (DeLano Scientific Inc.).

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

The major structural differences between the enzymes include:

1) the substitution of Cu_B in HCO by Fe_B in NOR
2) the presence of conserved glutamate(s) near the catalytic site in NOR

In 2000 Yi Lu and co-workers engineered an HCO model within myoglobin (termed Cu_BMb). For this purpose, they created a metal binding site by introducing two histidine residues that, combined with the distal His64, provide a tetrahedral coordination environment for copper similar to that in cytochrome c oxidase from bovine heart (Figure 1 C).[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 modeling, the copper binding site was modified to accommodate an iron ion by introduction of an additional critical glutamate residue,

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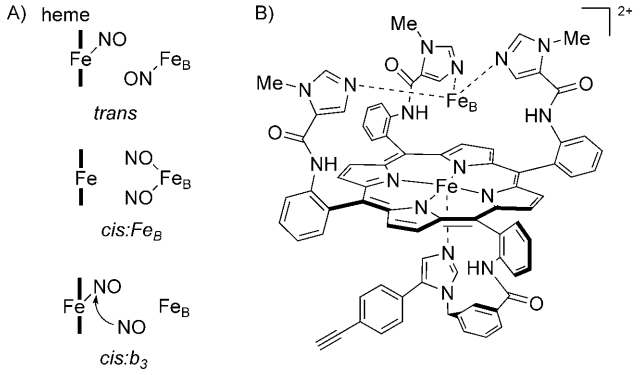
thus providing a $(His)_3Glu$ binding site for the nonheme Fe_B (Figure 1 B).

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

1) 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 carboxylate oxygen of glutamate).[5,7]
2) 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 behavior 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 the addition of dithionite. A control experiment with wild-type 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, with different mechanisms having 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 (Scheme 1 A):



Scheme 1. A) Proposed intermediates in the formation of N_2O from NO by NOR. B) functional small-molecule model reported by Collman, Solomon and co-workers.[11]

1) Does one NO bind to the heme b_3 center and one to Fe_B (the *trans*-mechanism)?[8]
2) Do both NO molecules bind to Fe_B (the *cis:Fe*$_B$-mechanism)?[9]
3) 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 co-workers presented the first functional small-molecule model for the active site of NOR (Scheme 1 B).[11] Spectroscopic characterization of different NO complexes points towards a *trans* mechanism.

Overall, the ground-breaking study by Lu and co-workers 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]

Acknowledgements

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Keywords: bioinorganic chemistry · enzyme models · heme proteins · nitrogen oxides · protein design

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HIGHLIGHTS

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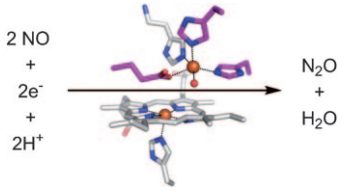

One site fits all: Yi Lu and co-workers have reported the conversion of sperm-whale myoglobin into a functional nitric oxide reductase. For this purpose, they designed a second metal binding site in the wild-type holo-protein and demonstrated NO reduction with the structurally characterized model, making thereby a significant contribution to the rapidly developing field of artificial metalloenzymes.

