Development of Designer Enzymes for Biomolecular Systems Engineering

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Life processes are sustained by a complex network of interconnected biochemical reactions. There has been a growing interest in re-engineering these biochemical reaction networks, which has implications for synthesis of chemicals and medical applications. We believe that the integration of unnatural chemical reactions, not found in nature but developed by humans, into this biochemical reaction network will pave the way for new ventures, leading to the production of various high-value-added compounds and the development of novel drugs with unique modes of action. With this ultimate objective in mind, our group is focusing on designer enzymes that catalyze unnatural chemical transformations. We are conducting a comprehensive study on the development of designer enzymes, drawing on coordination chemistry, catalytic chemistry, and protein engineering, as well as the development of technologies for their delivery into cells and organisms.

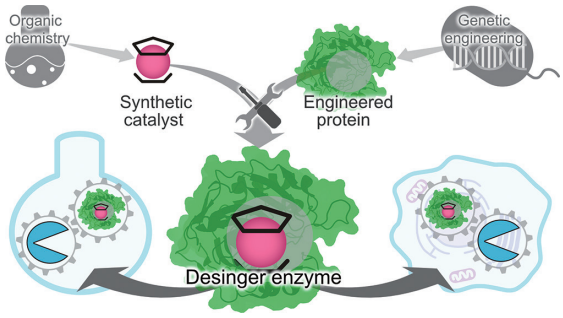

Figure 1. By combining synthetic catalysts developed through organic synthetic chemistry and proteins engineered through genetic optimization, we construct designer enzymes that possess non-natural functions. Using these designer enzymes, we aim to design chemical reaction networks within flasks and cells.

Selected Publications

- A. Ueno, F. Takida, T. Kita, T. Ishii, T. Himiyama, T. Mabuchi and Y. Okamoto, "A Cytokine-Based Designer Enzyme with an Abiological Multinuclear Metal Center Exhibits Intrinsic and Extrinsic Catalysis," *Nat. Commun.* **16**, 6781 (2025).
- Y. Okamoto, T. Mabuchi, K. Nakane, A. Ueno and S. Sato, "Switching Type I/Type II Reactions by Turning a Photoredox Catalyst into a Photo-Driven Artificial Metalloenzyme," *ACS Catal.* **13**, 4134–4141 (2023).
- H. J. Davis, D. Häussinger, T. R. Ward and Y. Okamoto, "A Visible-Light Promoted Amine Oxidation Catalyzed by a Cp* Ir Complex," *ChemCatChem* **12**, 4512–4516 (2020).
- Y. Okamoto, R. Kojima, F. Schwizer, E. Bartolami, T. Heinisch, S. Matile, M. Fussenegger and T. R. Ward, "A Cell-Penetrating Artificial Metalloenzyme Regulates a Gene Switch in a Designer Mammalian Cell," *Nat. Commun.* **9**, 1943 (2018).

1. Rational Design of a Synthetic Trinuclear Metal Complex Structures in a Protein Scaffold

Enzymes facilitate diverse chemical transformations in nature, with metal ions significantly expanding reaction capabilities. Examples include soluble methane monooxygenase (diiron enzyme for methane hydroxylation), nitrogenase (iron-molybdenum cofactor for nitrogen fixation), and photosystem II's oxygen-evolving complex (manganese-calcium cluster for water oxidation). In such metalloenzymes, protein scaffolds serve dual functions: Amino acid side chains act as ligands controlling metal ion reactivity, while defined internal spaces create reaction compartments enhancing rates and selectivity.

Designer metalloenzymes, created by incorporating synthetic molecules into proteins or constructing metal centers by using amino acid residues in protein, has proved the importance of reaction compartments by demonstrating enhanced reactivity and selectivity. However, using proteins as ligands lags behind their compartment applications due to difficulties in designing coordination chemistry at atomic levels. Designer mononuclear metalloenzyme development relies mainly on metal-substitution approaches and designing metal-binding site from scratch is further challenging. While some studies report construction of multinuclear metal centers using proteins and peptides as ligands, catalytically active examples remain limited with restricted scaffold variety.

Here, we successfully developed a designer enzyme containing a synthetic multinuclear metal complex structure by using proteins as the only coordination ligands.

As a model for grafting a multinuclear metal center into a protein scaffold, we have selected a synthetic trinuclear zinc complex (Figure 2a, b). This specific trinuclear zinc complex is a unique structural motif not typically found in natural enzymes. The choice of zinc ion was made due to its prevalence as one of the most abundant metal ions in biological systems.

For our study, we selected human macrophage migration inhibitory factor (MIF) as the scaffold protein because of its trimeric structure, which contains an internal pore suitable for hosting a synthetic trinuclear zinc complex structure (Figure 2c).

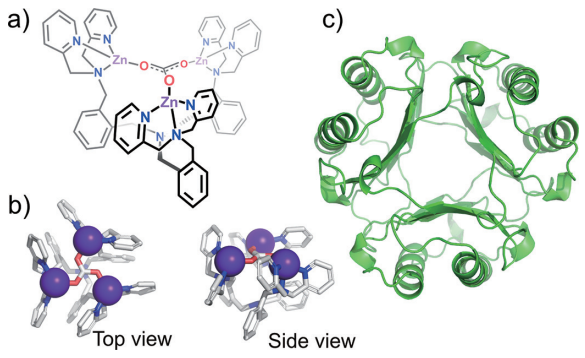

Figure 2. Building blocks used in this study. (a) Schematic and (b) crystal structures of the synthetic trinuclear zinc complex (CCDC 931956), and (c) crystal structure of human cytokine MIF (PDB code: 1MIF).

We have conducted a computational geometry search to identify suitable locations for placing histidine residues as ligands for the trinuclear zinc center. Subsequently, DFT calculations were performed to further refine the selection of candidate sites. As a result, we have prepared the identified variant, MIF(S61H/Y100H), along with an additional derivative, MIF(Y100H).

X-ray crystallography has verified the successful formation of the trinuclear zinc center in both MIF(S61H/Y100H) and MIF(Y100H) variants (Figure 3). The experimental structures align closely with the predictions from DFT calculations, illustrating the accuracy of our design strategy. Results from ITC analysis and DLS measurements confirm that the trimeric structures of the MIF variants are maintained in solution, with three zinc ions binding to the trimeric MIF variants.

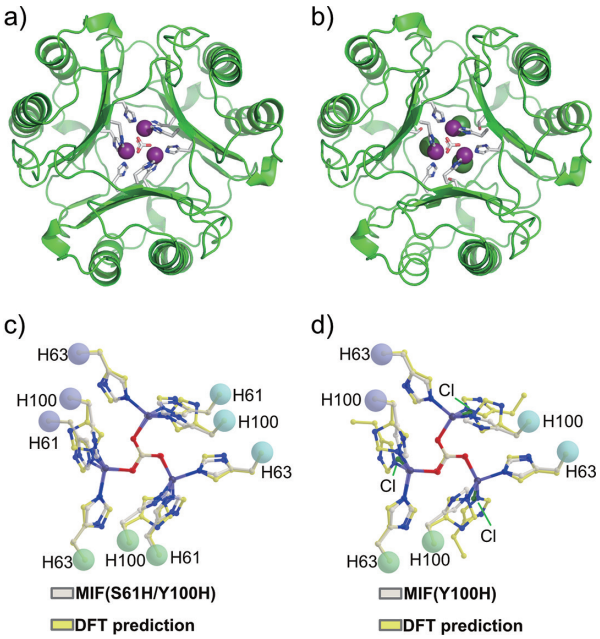

Figure 3. Crystal structures (a and b) of MIF(S61H/Y100H) (PDB code: 9JIZ; carbon: cyan) and MIF(Y100H) (PDB code: 9JJ0; carbon: cyan) in the presence of zinc ion. Superimposed images of the DFT-optimized trinuclear zinc center with (c) MIF(S61H/Y100H), and (d) MIF(Y100H) in the presence of zinc ion.

In the case of both MIF(S61H/Y100H) and MIF(Y100H) variants, hydrolytic activity was enhanced in the presence of zinc ions. This acceleration was not observed in the wild type MIF, indicating that the trinuclear zinc center plays a crucial role in catalysis. Interestingly, the trinuclear zinc center in the MIF(Y100H) variant exhibited higher activity compared to that in MIF(S61H/Y100H), achieving k_{cat}/K_M = 46.0 ± 0.4 M^{-1} s^{-1} at pH 7.9.

Reference

1) Ueno, F. Takida, T. Kita, T. Ishii, T. Himiyama, T. Mabuchi and Y. Okamoto, *Nat. Commun.* **16**, 6781 (2025).