研究説明資料

論文題目

# Study of enzyme reactions in the ordered assembly states

# (空間的に規制された配置にある酵素の反応解析)

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## Research background

Enzymes are natural biological catalysts that are responsible for cellular metabolic processes. In the multiple reaction steps of metabolic process, efficient transfer of intermediates from one enzyme to the other by spatial organization of enzyme molecules is believed to be the key feature for well-organized flow of metabolites. The enzymes arranged densely on the cell membrane or confined inside a micro-compartment exert an extraordinary efficiency in the metabolic sequential reactions. The specificity and the efficiency of enzyme reactions in such spatially organized conditions would be different from those in the test tube where enzymes are freely distributed in a dilute condition. The oligomeric assembly of enzymes is one of the typical examples in the cell, in which enzyme active sites for consecutive reactions are brought into close proximity to prevent the loss of intermediate by random diffusion thereby accelerating the substrate transfer. The hetero-multimeric enzyme found in carbamoyl phosphate synthetase, tryptophan synthase, and a few others are well studied. These studies revealed the mechanism of substrate channeling between active sites of enzyme subunits thereby preventing the metabolic crosstalk with other metabolic pathways. However, the advantage of enzyme being dimeric of identical subunits with each containing an active site remains to be answered.

Another strategy for the spatial organization is compartmentalization of enzymes. The organelles in eukaryotic cells, such as mitochondria, lysosomes, and transport vesicles, encapsulate enzymes in an enclosed single or double lipid layer membrane. The compartmentalization also occurs in prokaryotes. Bacterial microcompartments (BMCs)

commonly found in bacteria are protein-bound organelles with various functions including carbon fixation as anabolic carboxysomes or catabolic metabolosomes, such as the propanediol utilization (Pdu BMC) and the ethanol utilization (Etu BMC). A typical example is found in the carboxysomes of autotrophic bacteria, where ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) constitutes the final step of a $CO_2$ fixation in Calvin-Bensen-Bassham cycle. Around 2000 copies of RuBisCO and 80 copies of carbonic anhydrase are encapsulated inside a proteinous shell with diameter of 100 to 200 nm. CA catalyzes the cytosol $HCO_3^-$ thereby elevates the concentration of $CO_2$ in the vicinity of the active sites of RuBisCO. The co-encapsulation of CA provides a higher concentration $CO_2$ for this kinetically inefficient RuBisCO to ensure the high substrate selectivity for $CO_2$ against the competing substrate $O_2$. The proteinous shell would act not only as a diffusion barrier but also as a container to maintain the enzymes in high density to directly contact each other. These characteristics are considered to play important roles in the function of this microorganism, but a question of whether the reaction of enzymes in such a highly packed assembly is similar to that in the dilute condition as often chosen for biochemical studies remains opened. To address this issue, the protein assembly on DNA scaffold is an ideal system to study the reaction of enzymes in various arrangements. DNA nanostructures have been applied as the scaffolds for arranging functional molecules and nanomaterials and for constructing complex molecular devices. Especially, the DNA origami-based scaffolds enable the design of two- or three-dimensional nanostructures with a wide variety of shapes of defined size.

**Purpose of research**

To understand the effect of spatial organization of enzymes in nature system, especially when the enzyme molecules are confined in high density environment, I have constructed the enzyme assemblies using DNA nanostructure as a scaffold. A well-studied, monomeric enzyme human carbonic anhydrase II (CA) was chosen for this research as an enzyme of interest. Using the modular adaptor ZF-SNAP developed in Morii laboratory, four adaptor fused CA molecules (ZS-CA) were immobilized on DNA scaffold with the distance of less than 1 nm to mimic the packed state of a single type of enzyme. In the dispersed state, the four ZS-CA molecules were separated from each other with the minimum distance of 23 nm. The esterase reaction proceeded faster in the packed than in the dispersed state. The acceleration of reaction by packed assembly was more prominent for substrates with higher hydrophobicity. The entropic force by water was

proposed to increase the local substrate concentration within the domain confined between enzyme surfaces, thus accelerating the reaction. Another co-assembly of enzymes on DNA scaffold was constructed with the adaptor fused dimeric RuBisCO from *Rhodospirillum rubrum* and the above mentioned ZS-CA to mimic the stoichiometry in carboxysome. Although the natural carboxysome assembly is believed to enhance the RuBisCO activity, the co-assembly of RuBisCO and CA reduced the RuBisCO activity, possibly because the preferential $CO_2$ dehydration by CA reduced the RuBisCO reaction rate. The results suggested that the proximity in the interenzyme distance of RuBisCO and CA is not the crucial determinant for the enhanced RuBisCO activity in carboxysome. The assembly of RuBisCO and CA on DNA scaffold would provide a useful platform for further study on the spatial control of RuBisCO and associating enzymes.

**Contents of research**

Chapter 1 is a general introduction that describes the background of research carried out in this thesis. In chapter 2, I constructed the assembly of human carbonic anhydrase II (CA) on DNA scaffold using the modular adaptor ZF-SNAP consists of a DNA binding module zif268 and a covalent bond forming module ZNAP-tag. Adaptor fused CA (ZS-CA) was able to bind to DNA scaffold containing the target site irreversibly with fast reaction kinetics. A rectangular DNA scaffold with five cavities was prepared with the four binding sites for the adaptor in the center cavity or single binding site at each of the four outer cavities. Upon binding to DNA scaffold, ZS-CA will be filled up the center cavity to mimic the packed state or be separated with one molecule per cavity, representing a dispersed state. AFM images confirmed the binding of CA to the intended sites and the volume of CA assembly was measured to calculate the actual number of enzyme molecule per DNA scaffold in the packed state. The different orientations of enzyme molecules in packed state were also displayed by controlling the positions of binding sites on DNA scaffold and confirmed by AFM images.

In chapter 3, the activity of CA in packed state and dispersed state were evaluated for the esterase reaction of substrate *p*-nitrophenol acetate (*p*-NPA). In here I found that the reaction of enzyme in the packed assembly proceeded faster than the dispersed state and in free solution. The faster reaction profile was observed consistently at substrate concentrations from 0.1 to 1 mM with higher $K_m$ and $k_{cat}$ values for the packed state over those for dispersed assembly. I also investigated whether the specific interaction between two CA domains in certain orientations in the packed state could enhance the esterase

reaction. However, the results indicated that proximal alignment of CA molecules, regardless of orientation, was sufficient to increase the esterase reaction rate of ZS-CA.

To understand the enhancement in activity of enzyme in the packed state, several investigations were carried out in chapter 4. The macromolecular crowding reagents at high concentration decreased the enzyme activity, which was an opposite effect to that observed in the packed condition of ZS-CA on DNA scaffold. Beside that, the enzyme was remained stable in the packed state as well as in the dispersed state. The acceleration of reaction by the packed assembly was more prominent for the larger substrates with higher hydrophobicity. The packed state of enzyme was more tolerant to an inhibitor as compared to the enzyme in dispersed state. A contribution of water entropy effect was proposed for the enhanced activity of enzyme in the packed state, in which the entropic force by water increased the local substrate concentration within the domain confined between enzyme surface, thus accelerating the reaction.

In chapter 5, the enzyme RuBisCO from *R. rubrum* was chosen to assemble on a DNA scaffold by using a simple GCN4 binding protein adaptor. The enzyme assembly on DNA scaffold was characterized by means of AFM imaging and RuBisCO activity measurements. ZS-CA described in previous chapters was used to co-assemble with RuBisCO on the DNA scaffold to mimic the condition of cyanobacteria microcompartment, carboxysome. Carboxysome encapsulates two enzymes, RuBisCO and CA, to overcome the catalytic inefficiency of the main enzyme RuBisCO. However, the coassembly of RuBisCO and ZS-CA on DNA scaffold showed decrement in the reaction of RuBisCO which were explained by the $CO_2$ hydration activity of ZS-CA that reduced the local $CO_2$ concentration for RuBisCO. In line with the recent study by other group, the results suggest that the proximity of RuBisCO and CA is not the crucial determinant for the enhanced RuBisCO activity in carboxysome. Given the programmability in the scaffold design and the spatial and stoichiometric arrangements of enzymes, the RuBisCO assembly on DNA scaffold would provide a promising platform for further study on the chemistry of spatially organized RuBisCO systems.

**Significance of research**

In this study, the protein adaptor was applied to control the location and number of enzyme molecule of interest on the DNA nanoscaffold. The enzyme molecules in the packed state are located in the interenzyme distance of less than 1 nm, which is similar to the conditions often observed in the cell. Without increasing the concentration of enzyme

in solution, the enzymatic activity in packed state and dispersed state were compared by taking advantage of the DNA scaffold and adaptor system. The co-assembly of RuBisCO and CA on DNA scaffold mimicking the condition of carboxysome yet gave the unexpected results on the effect of proximal interenzyme distance on the RuBisCO activity, suggesting that the proximity of RuBisCO and CA is not the crucial determinant for the enhanced RuBisCO activity in carboxysome. The findings described in this thesis offer unprecedented insight on the efficiency of enzyme reaction in the highly packed state and how nature has harnessed the cellular crowded environment for metabolic enzyme reaction systems.

**Future perspectives**

The strategy to construct the packed state of enzyme was demonstrated by using carbonic anhydrase in this study, but it can potentially be applied to a wide range of enzyme systems, from single type of enzyme to multiple enzymes consisting of the cascade reaction. The packed state of enzyme provides higher reaction efficiency and higher resistance against inhibitors, which offers useful information for designing an artificial enzymatic reaction or for constructing a novel enzymatic compartment. The application can range from diagnostics to the production of high-value chemicals with highly efficient bioenergy utilization systems.

## List of publications

***Publications directly related to the contents of this thesis***

**1. DNA-binding proteins as the potential adaptors to locate functional protein on DNA nanoscaffold**

T. A. Ngo, H. Dinh, T. M. Nguyen, F. F. Liew, E. Nakata, and T. Morii

*Manuscript submitted to Chem. Commun. (in revision)*

*(Chapter 1)*

**2. DNA binding adaptors to assemble protein of interest on a DNA scaffold.**

E. Nakata, H. Dinh, T. M. Nguyen, and T. Morii.

*Methods Enzymol.* **2018**, *618*, 287

*(Chapter 2)*

**3. Enhanced enzymatic activity exerted by a packed assembly of single type of enzyme**

H. Dinh, E. Nakata, K. Mutsuda-Zapater, M. Saimura, M. Kinoshita, T. Morii

*Manuscript submitted to J. Am. Chem. Soc. (in revision)*

*(Chapter 3, 4)*

**4. Reaction of ribulose biphosphate carboxylase/oxygenase (RuBisCO) on DNA scaffold**

H. Dinh, E. Nakata, P. Lin, M. Saimura, H. Ashida and T. Morii

*Manuscript submitted to Bioorg. Med. Chem. (in revision)*

*(Chapter 5)*

***Publications not directly related to the contents of this thesis***

**1. A modular zinc finger adaptor accelerates the covalent linkage of proteins at specific locations on DNA nanoscaffolds**

E. Nakata, H. Dinh, T. A. Ngo, M. Saimura, T. Morii

*Chem. Commun.* **2015**, *51*, 1016

**2. Design of modular protein-tags for the orthogonal covalent bond formation at specific DNA sequences**

T. M. Nguyen, E. Nakata, M. Saimura, H. Dinh, and T. Morii

*J. Am. Chem. Soc.* **2017**, *139*, 8487

**3. DNA origami scaffolds as templates for functional tetrameric Kir3 K+ channels**

T. Kurokawa, S. Kiyonaka, E. Nakata, M. Endo, S. Koyama, E. Mori, N.H. Tran, H. Dinh, Y. Suzuki, K. Hidaka, M. Kawata, C. Sato, H. Sugiyama, T. Morii, Y. Mori

*Angew. Chem. Int. Ed.* **2018**, *130*, 2616

**4. Highly selective dual sensing of ATP and ADP using fluorescent ribonucleopeptide sensors**

S. Nakano, M. Shimizu, H. Dinh, T. Morii

*Chem. Commun.* **2019,** *55*, 1611

**5. Rational design of DNA sequence-specific modular protein tag by tuning the alkylation kinetics**

T. M. Nguyen, E. Nakata, Z. Zhang, M. Saimura, H. Dinh, and T. Morii

*Chemical Science,* **2019***, in press*

**6. Enzyme cascade reactions on DNA origami scaffold**

E. Nakata, H. Dinh, P. Lin and T. Morii

*Manuscript submitted to Methods Mol. Biol.*