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研究課題名（和文）Study of enzymes inside liquid-liquid phase separated crowded droplets

研究課題名（英文）Study of enzymes inside liquid-liquid phase separated crowded droplets

研究代表者
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交付決定額（研究期間全体）：（直接経費）　　3,100,000円

研究成果の概要（和文）：これまでに我々は、生体内でみられるliquid dropletsにおける酵素の機能を解析してきた。具体的には、LLPS現象によって発生するliquid protein crowded dropletsに着目し、そのliquid protein crowded droplets内で機能する生体内酵素の動力学的なパラメーターに関して研究した。

研究成果の学術的意義や社会的意義
The current project gives a better understanding about how enzymes works in liquid droplets. The liquid droplets are a new biochemical organization of the metabolism recently reported across all the living forms.

研究成果の概要（英文）：The current research project explored the function fo the enzymes within liquid droplets. In detail, we have studied the kinetic parameters of enzymes partitioned within liquid protein crowded droplets generated through liquid-liquid phase separation.

研究分野：Biochemistry

キーワード：LLPS　enzymes　catalytic efficiency　liquid droplets　pH gradient

１．研究開始当初の背景

Enzymes play fundamental roles in almost all life processes. They are biological catalysts able to substantially increase the rate constants of a great variety of chemical reactions, thus controlling energy transduction and signalling, as well as the transcription and translation of genetic information. Therefore, they have an important role in regulating the metabolism of cells and there is a broad interest in understanding the origin and the mechanism of this catalytic power on a molecular level. The compartmentalization of enzymes within membrane-enclosed organelles has represented the natural framework for the spatiotemporal regulation of metabolic reactions in the cell. However, the recent discovery of membrane-less liquid-like compartments, called biomolecular condensates (BMCs) or liquid dropleys has forced the scientific communities to look at the metabolism regulation under a new perspective. BMCs often form through a process called liquid-liquid phase separation (LLPS). LLPS allows the formation of at least two different phases, one dense phase formed by concentrated biomolecules (proteins and/or nucleic acids) that usually interact by multivalent interactions and the surrounding diluted phase, depleted of biomolecules. The process of BMC formation is dynamic and reversible. The potential of BMCs to selectively sequester enzymes (and other molecules) from the cellular environment in a more flexible and dynamic manner has introduced a novel regulatory mechanism in biology and biochemistry, whose full potential is under investigation from several points of view. Indeed, from the enzyme perspective, it is still largely unknown how BMCs affect enzyme kinetics or the mutual effects of the material properties of BMCs on the structural and kinetic features of the enzymes and vice versa. Regarding the regulation of biochemical reactions, it has been proven that biomolecular condensates can concentrate reactants enhancing the rate of the biochemical reactions or sequestering enzymes or reactants from each other to reduce the efficiency of the targeted reactions. However, this is probably just a part of the story, and more complex phenomena likely participate in the overall regulation of enzymatic reactions within BMCs. On this regard, it is known that proteins in solution can adopt different conformations, active, partially active or inactive, based on the thermodynamic distributions in their environment. These conformational changes result in energy fluctuations that impact the protein structure and their dynamics.

Protein dynamics refers to protein structural fluctuations or conformational exchanges, which are essential for protein functions. In most cases, enzymes do have one static and stable structural state but undergo interconversion between multiple conformational states, driven by thermodynamic conditions. Importantly, many function-related excited states are sparsely populated and thus “invisible”, especially in the crowded cellular environment. The importance of protein conformation equilibria has an enormous importance in biology, biochemistry, protein design, drug design, medicine, and

biotechnology. It has been shown that even local structural changes on the sub-Angstrom scale in the active site of an enzyme alter its reaction kinetics enormously. Therefore, instead of a single static structure, protein structures must be viewed as conformational ensembles that fluctuate around energy minima. However, this vision of the enzymes within BMCs is still unexplored and was one of the main aims of this project (Fig.1). Another important aim of the current research project was understanding the effects of the enzymatic activity on the behavior or properties of the liquid droplets as well as the influence of the protein-protein interactions withing the droplets, which can modulate the kinetic parameters of the enzymes and the metabolism.

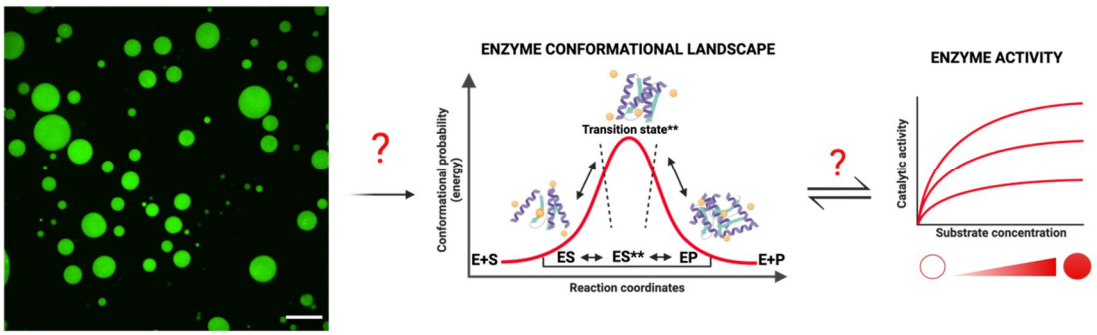

Fig.1. Representation of the research aims of the following project. Enzyme (in green) partitioned inside the liquid-liquid phase separated protein droplets could have different activities based on the stabilization of the reaction intermediates (transition states and rate limiting steps during catalysis).

2．研究の目的

The purpose of the current research project was divided in two big questions:

1-How is enzyme's function modified/altered within liquid liquid phase separated droplets and how the enzymatic activity influences the behavior of liquid droplets?

We have studied urease enzymatic activity within the liquid droplets by analyzing the kinetic features and the effects of the catalysis on the droplets behavior https://pubs.acs.org/doi/full/10.1021/jacs.4c02823).

2-How is protein-protein interaction happening within the droplets?

Second, we have studied the enzyme-enzyme interaction (metabolon) of two non-consecutive enzymes (isocitrate dehydrogenase and malate dehydrogenase) involved in the TCA cycle in *Bacillus subtilis* within the protein droplets (https://www.biorxiv.org/content/10.1101/2023.11.01.565101v1).

3．研究の方法

We have used several techniques in OIST to study the enzymes and their activity within the phase separated protein droplets.

In detail:

-synthetic biology tools to prepare the liquid-liquid phase separated droplets;
-enzymatic assays using commercially available kits to characterize the kinetic

parameters (kcat and KM) of the enzymes within the protein droplets;
-imaging techniques using confocal microscopes, to visualize the enzymes within the droplets and to analyze and comprehend the behavior of the droplets when the enzyme was active.

In the manuscripts accepted for publication there are also other techniques employed for the characterization of the phenomenon under study. However, these parts have been carried out by collaborators in different labs and not mentioned here.

4. 研究成果

1 How is enzyme's function modified/altered within liquid liquid phase separated droplets and how the enzymatic activity influences the behavior of liquid droplets?

This study published in JACS represents a significant stride in increasing the degree of complexity of synthetic droplets to emulate cellular features and effectively integrate recent advances in this field.
In this study, we discovered that membraneless enzymatically active droplets respond to a pH gradient in a chemotactic manner and that we can tune their migration by varying substrate and enzyme concentrations. These synthetic droplets mimic cytosolic protein crowding and resemble rudimental cellular features such as sensing and migration toward neighboring droplets by following the chemotactic gradient. Each droplet enzymatically produces ammonia, generating a pH gradient that forms a pH halo extending outside the droplets.
Our study does not merely demonstrate enzyme-generated random motion, but it demonstrates droplet migration exclusively toward other droplets, resembling sensing and selective migration phenomena similar to cellular systems. This work represents an essential step for assessing biologically relevant questions related to chemotactical sensing and long-range interactions. Furthermore, our study shows the relevance of fluid mechanics in these ubiquitous biological processes, and it opens up new perspectives for the PEG-BSA droplet system as a synthetic biological tool, drug delivery method, and bioinspired smart materials.

2 How is protein-protein interaction happening within the droplets?

Enzymes of the core energy metabolism pathways tend to assemble into transient supramolecular complexes, yet the functional significance of the interactions within these complexes, particularly between enzymes catalyzing non-consecutive reactions, remains unclear. In this study, by co-localizing two non-consecutive enzymes of the TCA cycle from *B. subtilis*, malate dehydrogenase (MDH) and isocitrate dehydrogenase (ICD), in highly crowded liquid-liquid phase separated droplets we discovered that MDH-ICD interaction causes an enhancement of ICD catalytic rate and an apparent sequestration of its reaction product, 2-oxoglutarate. Theory suggests that the observed phenomena are explained by the MDH-mediating clustering of ICD molecules. In vivo validation with targeted GC-MS and ^{13}C tracer analyses revealed that when *B. subtilis* is grown on glucose and ammonia, overexpression of MDH leads to accumulation of 2-oxoglutarate with a concomitant reduction of fluxes flowing through both the catabolic and anabolic branches of the carbon-nitrogen intersection occupied by 2-oxoglutarate, resulting in impeded ammonium assimilation and reduced biomass production. Our findings thus suggest that in *B. subtilis* the MDH-ICD interaction is an important coordinator of carbon-nitrogen metabolism, thereby expanding the list of types of functionally understood unconventional enzyme-enzyme interactions.

５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　2件／うちオープンアクセス　2件）

１．著者名 Dindo Mirco、Bevilacqua Alessandro、Soligo Giovanni、Calabrese Vincenzo、Monti Alessandro、Shen Amy Q.、Rosti Marco Edoardo、Laurino Paola	４．巻 Not available yet
２．論文標題 Chemotactic Interactions Drive Migration of Membraneless Active Droplets	５．発行年 2024年
３．雑誌名 Journal of the American Chemical Society	６．最初と最後の頁 0
掲載論文のDOI（デジタルオブジェクト識別子） 10.1021/jacs.4c02823	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Jasinska Weronika、Dindo Mirco、Correa Sandra M.、Serohijos Adrian W.R.、Laurino Paola、Brotman Yariv、Bershtein Shimon	４．巻 Not available yet
２．論文標題 Non-consecutive enzyme interactions within TCA cycle supramolecular assembly regulate carbon-nitrogen metabolism	５．発行年 2023年
３．雑誌名 Nature Communications (accepted)	６．最初と最後の頁 0
掲載論文のDOI（デジタルオブジェクト識別子） 10.1101/2023.11.01.565101	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関