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総説

Enzymes and Liquid-Liquid Phase Separation: A New Era for the Regulation of Enzymatic Activity

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Liquid-liquid phase separation (LLPS) is recognized as a mechanism for regulation of enzymatic activity. Biochemical mechanisms include concentrating reactants to enhance reaction rates or sequester enzymes and reactants from each other to reduce the reaction rate. On the other hand, LLPS might also regulate the diffusion of small molecules or important parameters for enzymatic activity (such as modulators, macromolecular crowding and changing the media physicochemical features) increasing or decreasing the reaction rate of the enzymes. Furthermore, the co-compartmentalization of specific enzymes can favour or speed up specific metabolic fluxes. Here, we discuss how LLPS contributed to generate a new era for enzyme regulation and the new possible subtle regulation mechanisms still unexplored.

enzyme / liquid-liquid phase separation / enzymatic activity / enzyme regulation / macromolecular crowding / cell metabolism

1. Enzymes and liquid-liquid phase separation

Enzymes are catalysts able to substantially increase the chemical reactions rate allowing ultimately to sustain life. Enzymes are also characterised by specificity for substrate/s and have an important role in regulating the metabolism of the cells. However, the dynamic motion of the enzymes' during catalysis is one of the most intriguing and still not completely understood events, along with other aspects of protein chemistry[1)-3)].

The cell is an organised and crowded environment, in which thousands of processes are active at the same time. Cellular organelles spatially organise the metabolism and the biological matter. These compartments can be physically separated by a membrane boundary from the surrounding cellular environment. Also, these organelles can be impermeable to most biological molecules and examples are given by Golgi apparatus, mitochondria, peroxisomes, and endoplasmic reticulum[4),5)].

However, the presence of micro-scale membrane-less liquid-like compartments have been recently reported in the cytoplasm, nucleus and along the membranes[6)-8)]. Evidence indicates that Liquid-Liquid Phase Separation (LLPS) is important in many biological processes, as the well-documented stress response (with the formation of stress granules), but also for transcription, signalling and metabolism[9)]. Further evidence associates aberrant forms of LLPS with several human diseases[10)].

These discoveries describe the LLPS as a new fundamental physicochemical mechanism for organising the biochemistry of the cells.

LLPS allows the formation of at least two different phases, one dense phase formed by concentrated biomolecules that usually interact by multivalent interactions (proteins and/or nucleic acids) and the surrounding dilute phase, depleted of biomolecules[11)]. The process is dynamic and reversible. To drive the formation of a phase-separated solution, the biomolecules must have specific features and reach a threshold concentration of participating biomolecules[12)].

Recently, it has been widely shown that LLPS plays important role in the regulation of enzymatic activity, providing spatiotemporal control over enzyme function. However, the physicochemical mechanisms that regulate the enzymatic activity and the possible structural changes at molecular level are largely unknown so far. May enzymes be structurally stabilised upon formation of LLPS? Or can it be only the more active conformation that is stabilised?

In this Review, we will discuss the mechanisms of regulation of enzymatic activity in the membrane-less compartments present in literature and the other possible mechanisms still unexplored.

2. Mechanisms for regulation of the enzymatic activity mediated by LLPS

A direct way for the cell to achieve control over space of

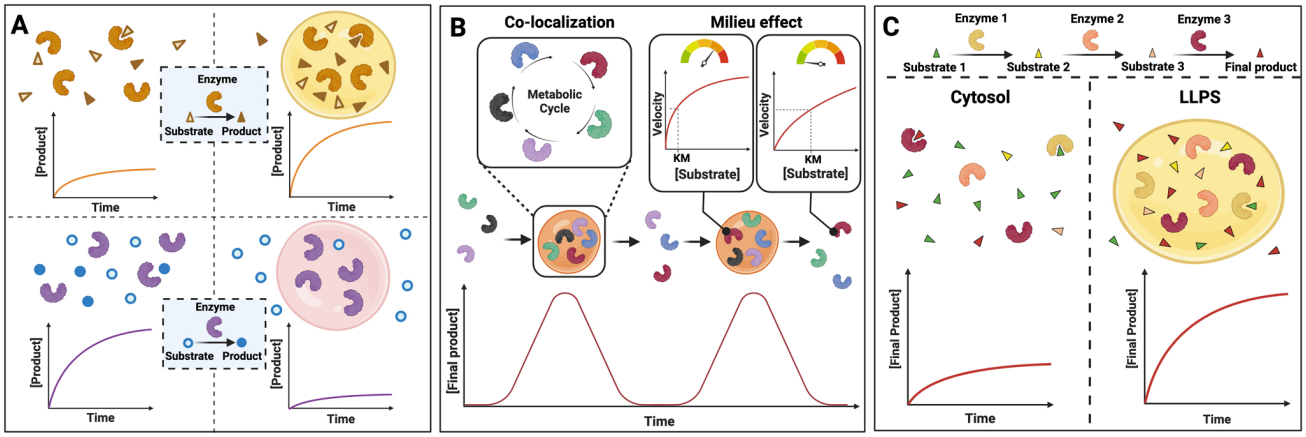

Fig.1
Mechanism of regulation of enzymatic activity mediated by LLPS. A: LLPS increase (upper part) or decrease (lower part) the rate of enzymatic reactions, concentrating or separating the reactants. B: Spatiotemporal activation and control of enzymatic reactions via LLPS. C: LLPS promotes the co-compartmentalization of multiple enzymes to boost the formation of key molecules involved in other metabolic cycles or in specific cellular functions.

an enzymatic reaction is to regulate the localization of the reactants[13]. Concentrating all the components of the reaction together can increase reaction rate. An interesting and recent example has been given by Peeples and Rosen[14] that demonstrates an increased enzymatic activity in their phase separated system due to mass action and scaffold induced organisation. Thus, not only the increased enzyme and substrate concentration in the dense phase led to an increased activity (as shown in the upper part of **Fig.1**, panel A) but also the organisation of the protein scaffolds that promotes the interaction and formation of the ES complex (enzyme-substrate complex, leading to a decrease of K_m value). Furthermore, sequestering substrates or enzymes separately can slow down the reaction (**Fig.1**, lower part of panel A). Conversely, concentrating all the reactants in a specific location over time allows the cell to have spatiotemporal control on the selected reaction, switching it on and off according to cell physiological demands (**Fig.1**, panel B). This concept is even more interesting if a certain reaction is a rate limiting step in a metabolic pathway or multistep reaction, or if the reaction in the crowded milieu is largely inefficient (low specificity or low concentration of reactants).

Co-compartmentalization of specific enzymes in biomolecular condensates involved in a multistep reaction can speed up the production of key molecules (substrates or specific signalling molecules) for the activation of a secondary metabolic pathway or to produce key molecules involved in specific cellular processes (**Fig.1**, panel C).

In multistep reactions or metabolic pathways, the way by which LLPS could regulate or spatially organise enzyme cascades enhancing the overall rate is still unclear. However, some studies report the increase in overall productivity of the multistep reactions[15].

Post-translational modifications can favour the phase separation of specific proteins and their regulation. As reported by O'Flynn and Mittag[16], post translational modifications may regulate alternate compartmentalization states of specific enzymes involved in a forked metabolic pathway. The metabolites can be used in two or more pathways, and according to the cell's necessity the kinetics of one of the two pathways can be temporarily increased.

Another possible level of regulation mediated by the combination of LLPS and enzyme catalysis is the modulation of the parameters important for catalysis, such as solvent condition and the production of catalytic modulators[17]. Interactions between macromolecules and reactants could also play an important role inside the crowded biomolecular condensates. The increased viscosity of the environment can affect substrate diffusion mediated by nonspecific interactions (**Fig.2** panel A) or alter structural features of the enzymes (plasticity or conformational changes), leading to slower overall kinetics (**Fig.2** panel B). On the other hand, macromolecular crowding could also play the opposite effect, shifting to a more catalytically active conformation the enzyme (for example stabilising the overall enzymatic structure), thus contributing to increased activity (**Fig.2** panel C)[18].

An interesting way to regulate the activity could be due to the presence of a background enzymatic activity in the

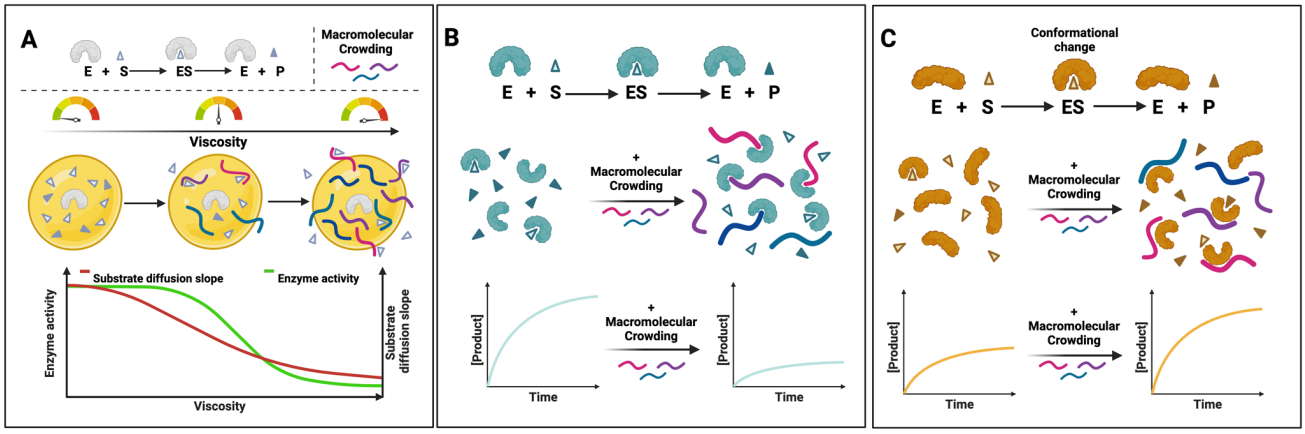

Fig.2

Possible effects of viscosity and macromolecular crowding on enzymatic activity inside LLPS. A: Viscosity may affect the diffusion of small molecules due to nonspecific interactions or sequestering them. B: Macromolecular crowding destabilizes the enzymes (nonspecific interactions) leading to a slower activity. C: Macromolecular crowding can stabilize a highly active conformation of the enzyme via LLPS, increasing its rate.

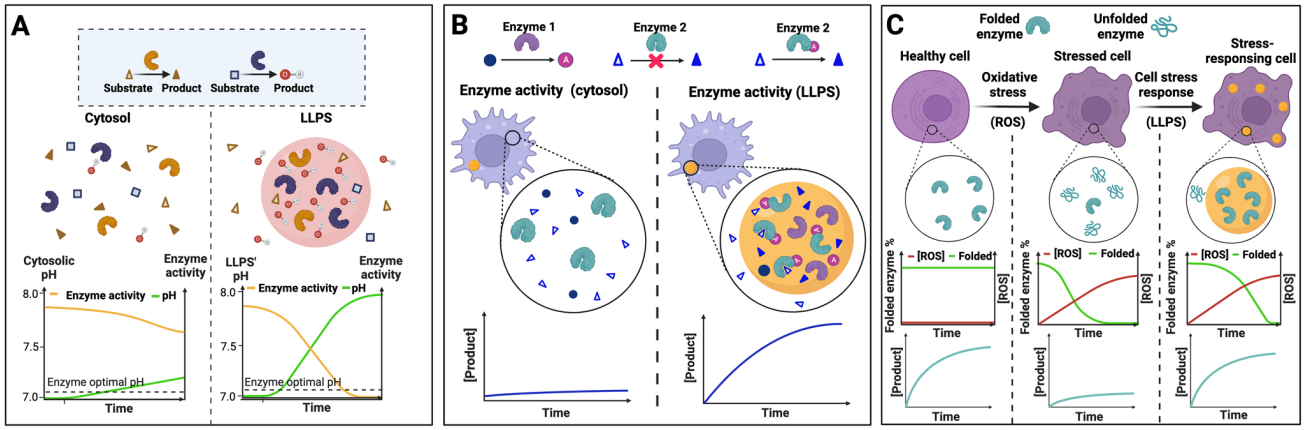

Fig.3

Regulation of the droplets physicochemical conditions mediated by enzymes. A: Specific concentrated enzyme generates optimal activity parameters in the LLPS for a second highly regulated enzyme only active under optimal conditions. B: Activation and control of specific enzymatic reactions mediated by modulators in the LLPS. C: LLPS protects enzymes from destabilizing conditions present in the cellular environment maintaining its enzymatic activity. ROS: Reactive Oxygen Species

biomolecular condensates that produce specific molecules (reaction intermediates) able to change locally the physicochemical features, as the pH, of the solvent affecting the catalysis of the target enzyme[19] (**Fig.3**, panel A). Also, the local production of molecules inside the biomolecular condensates, known as modulators, can affect the enzymatic activity (**Fig.3**, panel B). In this contest, LLPS could also exert other possible roles protecting specific/vital target enzymes from physicochemical changes in the cellular environment (**Fig.3**, panel C).

However, looking at the composition of the biomolecular condensates is a complicated task, for this main reason, researchers are focusing on the *de novo* design of artificial systems to investigate the effects of LLPS on enzymatic activity[19]. This is also necessary to further broaden our understanding on the possible mechanisms responsible for the regulation and activation of the biomolecular condensates.

Cellular biochemistry has seen a revolution in the last years due to the discovery of biomolecular condensates, generated via LLPS.

Their dynamic nature is important to tune chemical reactions, switching on and off enzymes and regulating their activity.

Although many processes and mechanisms related to enzymatic activity within LLPS are still unexplored, the intrinsic features of the enzymes are now seen from a new point of view. Researchers are looking at new and subtle mechanisms for enzymatic regulation via LLPS.

However, many open and intriguing questions still need

to be answered. Correlation between the size of LLPS and the enzyme concentration for the regulation of the reaction rates is one example. In this contest, modulating the enzyme concentration in the LLPS allows us to investigate the effects of the high metabolic activity in the system[20].

In fact, we have recently shown that our protein-based droplets are able to exceed the metabolic densities of even the most voracious unicellular microorganism, using just one, highly concentrated enzyme. Also, the high metabolic density can be sustained for hours, increasing the lifetime of experiments using concentrated enzymes.

The increased concentration of the active enzyme could also lead to a change in the physicochemical features of the LLPS that directly then affect the enzymatic activity or the surrounding environment. Also, what is the main mechanism responsible for the increased enzymatic activity or better which step of the catalysis is mainly involved? Formation of enzyme-substrate (ES) complex, enzyme-coenzyme interaction, product release or other not yet mentioned effects?

A priority to better understand the mechanism underlying the regulation of enzymatic reaction rates would be given by detailed studies of several enzymes modulating LLPS conditions. In this contest, it is still missing a comprehensive study about enzyme inhibition in a LLPS system.

The development of a specific cell-like LLPS model system will allow us to understand the effect of the macromolecular crowding on the enzymatic catalysis steps, under conditions that resemble the cellular features.

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