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Enzymatic reactions inside biological condensates

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Abstract

Biological enzymes significantly speed up chemical reactions in living organisms. The complex environment within cells has long been appreciated as a major regulator of enzymatic activities. Recent advances in the rapidly evolving field of biological condensates, which are spontaneously formed by macromolecules through phase separation, suggest new possibilities for how enzymatic reactions may be modulated within cells. Here, we review the latest studies of enzymatic reactions in biological condensates focusing on basic concepts in enzymology and discussing some context-dependent roles of phase separation in regulating biochemical reactions.

Introduction

Enzymes play critical roles in biology by accelerating the rates of diverse types of biological reactions [1]. Most fundamentally, catalysis is defined as stabilization of the transition state of a reaction relative to its ground state [2]. Such transition-state specific stabilization effectively reduces the activation barrier for the reaction. Within this framework, biological enzymes are particularly good at specifically stabilizing the transition state. Biological enzymes consistently out-perform the best human-made catalysts by several orders of magnitude, achieving rate accelerations of up to 10^{13}-fold [2]. How catalysts function and how enzymes achieve such large rate enhancements has been a topic of active study for almost a century [1]. These studies have identified some key strategies used by enzymes that we summarize next.

For bi-and multi-molecular reactions, a large component of the activation barrier is the entropic cost of bringing together the reactants in the correct orientation for chemistry to

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occur [3]. Enzymes pay for this entropic cost by using binding energy to stabilize the substrates within an active site. Once bound to the enzyme, active site residues then enable specific stabilization of the chemical transition state by exploiting small electrostatic and geometric differences between the ground state and the transition state. These active site residues can serve as general acids, general bases, or act as ligands for metal ions that serve to stabilize developing negative charges on a transition state [1]. Layered on top of these two strategies of using binding energy and active site residues, is the ability of an enzyme active site to regulate the environment of the chemical reaction. For example, the local environment of the active site can perturb the pK_as of residues involved in transition state stabilization [1]. Analogously, the specific composition and arrangement of residues within an enzyme can tune the hydrophobicity and electrostatic nature of the environment experienced by the substrates. Thus, for example, a hydrophobic active site may enhance electrostatic interactions that are preferentially made in the transition state.

To date, the effectiveness of the above strategies has been largely assessed relative to reactions in dilute aqueous solutions [2]. However, several key biological processes are now proposed to occur within liquid-like phase-separated compartments [4]. The solvent conditions are likely to be substantially different in such phase-separated compartments than the aqueous buffer conditions that are commonly used for biochemistry. Here we revisit the catalytic strategies used by enzymes in the context of the potentially diverse environments created within phases. We imagine that the mechanisms described above can be regulated (up or down) in the following ways. Firstly, when the solvent changes, how well the enzyme interacts with the substrate relative to solvent also changes. Such changes would affect the observed free energy change associated with substrate binding (also measured in terms of its dissociation constant, K_d), and the specific transition state stabilization that the enzyme can achieve (k_{cat} or k_{max}). Secondly, a phase-separated compartment may alter the kinetics of substrate binding by affecting substrate diffusion and concentration. Thirdly, given that many biological enzymes can be partitioned into condensates through multi-valent interactions, phase-separation can also be coupled to allosteric regulation of enzymatic activity in cases where formation of multivalent interactions drives a conformational change in the enzyme.

1. Phase-separated droplets have unique solvent properties

The formation of phase-separated droplets provides opportunities to regulate the electrostatic and hydrophobic environment experienced by the enzyme and substrate, thereby altering enzymatic parameters. The intracellular environment, which contains high concentrations of macromolecules (300~400 g/L), has long been known to differ drastically from dilute aqueous solutions used to measure enzymatic activity in a test tube [5]. Phase-separated droplets may mimic similar levels of crowdedness while also mimicking the diverse and unique local conditions found within cells —such as crowding, viscosity, and polarity— leading to alterations of biochemical reactions inside. Using water-soluble neutral cosolutes as mimetics to induce crowding conditions and shift solvent properties, the Sugimoto group found a 2–7 fold increase of RNA hydrolysis catalyzed by a hammerhead ribozyme [6]. In addition, the Sugimoto group also show that the dependence of the ribozyme reaction rate on the relative dielectric constant of solvent correlates with the dependence

on monovalent salt-concentration [7], supporting the idea that solvent properties alone can modulate catalysis (buffer vs. additives). Another example of phase-separated droplets altering solvent properties is provided by experiments with Ddx4. The disordered tails of Ddx4, a DEAD-box RNA helicase capable of unwinding short RNA duplexes, were shown to form condensates both *in vitro* and in cells [8]. The dielectric constant of the droplet was measured to be ~ 45, resembling the polar organic solvent such as DMSO (~47) rather than the hydrophobic interior of a protein core (4). Interestingly, despite the lack of the energy input, short double-stranded DNA which spontaneously partitioned into these droplets was destabilized and melted. This observation suggests that the unique interior of condensates can modulate the physical properties of macromolecules [9].

Collectively, these findings raise the question of the diversity of internal environments present within the biologically relevant condensates. Recent studies have identified a wide variety of membraneless compartments inside the cell, including stress granules and P-bodies in the cytoplasm, nucleoli and Cajal bodies in the nucleus, and even on membranes (reviewed in [4]). Given the large compositional variance of biological condensates, it is difficult to imagine uniform physical properties inside these droplets. A recent study by the Forman-Kay group reconstituted minimal RNA-containing condensates formed by intrinsically disordered regions (IDRs) from two RNA-binding proteins (FMRP and CAPRIN1), both of which are involved in mRNA stabilization and translational repression and colocalize in cytoplasmic condensates [10]. The authors show that FMRP, CAPRIN1 and RNA can form a range of different condensates, depending on phosphorylation of Ser/Thr in FMRP and Tyr in CAPRIN1. Notably, Kim *et al.* demonstrate that these different condensates, although all formed by FMRP, CAPRIN1 and RNA, exhibit distinctive mRNA processing preferences, in which deadenylation inversely correlates with translation. This study underscores the great potential of biological condensates in creating unique micro-environments for chemical reactions. Not only do condensates separate the reactants from the surrounding aqueous environment, but they also fine-tune the droplets' interior characteristics through altering composition and/or post-translational modifications of proteins.

In parallel with showing that phase-separation by biological macromolecules *in vitro* can recapitulate relevant cellular processes, researchers have also developed artificial compartments in order to examine principles of biochemical reactions inside condensates. For example, Küffner *et al.* fused low complexity regions (derived from DEAD-box proteins) to both the N- and C-terminus of the model enzyme adenylate kinase and created chimeric proteins capable of phase separation [11]. Inside the droplets, the authors measured a 50–150 fold increase of protein concentration and observed a more non-polar environment closer to methanol than water. This less polar environment preferentially partitioned hydrophobic molecules and green fluorescent protein (GFP). This strategy can be valuable for future studies to quantitatively examine the physical properties of condensates formed through different mechanisms.

2. Sequestering reaction components in phases and its effect on rates

In principle, the crowded environment within phases can lead to a two opposing effects on biochemical reactions: increased intermolecular collisions can cause a significant reduction of diffusion rates while the increased concentrations of the reacting macromolecules can lead to an increase in the rate of association. Thus, apart from the unique solvent environments inside the condensates, the local concentrations of enzymes, cofactors, substrates, and products which may be different from surrounding areas can also affect association and catalytic rates [12–14]. Further, each component of a reaction can be enriched individually or simultaneously into the same compartment, and therefore may have different net effects on catalytic reactions. Some of the most straight-forward scenarios are listed in Figure 1 and are discussed in detail below.

2.1 Enrichment of enzyme and/or substrates in phases accelerate reactions

The concentration effect inside phase-separated condensates is very straight forward and has been widely observed *in vitro* and in cells. Typically, as the local concentrations of enzymes and substrates increase, biochemical reactions are accelerated, as has been demonstrated in many recent studies [4] [15] [16]. It has also been found that a reaction mixture containing microdroplets spontaneously formed by cationic peptides and mononucleotides (thus containing a high local concentration of ATP, the substrate), shows enhanced activity of hexokinase by two-fold [15]. In a PEG-salt mixture resembling a cell-like environment, Sokolova *et al.* detected a remarkable 50-fold increase of mRNA production in dense and viscous droplets [16]. The authors also note that the effect of substrate and enzyme concentration alone could not account for the significant increase of the rate and propose that other factors such as altered association constants and transcription rate constants collectively contribute to the enhancement of the transcription rates.

How much benefit could the concentrating effect of enzymes provide on accelerating biochemical reactions? Strulson *et al.* report a 3,000-fold enrichment of the hammerhead ribozyme in phases, leading to a ~70-fold increase in the rate of cleavage [17]. Similarly, the aforementioned chimeric adenylate kinase inside the droplets displays only a 3-fold catalytic enhancement compared to the exterior continuous phase, despite a 50–150 fold enrichment of the enzyme [11]. Another hammerhead ribozyme derived from tobacco ringspot virus shows a ~50-fold enrichment in the microdroplets compared to the bulk coacervate phase (a polymer-rich state produced by spin-down of aqueous dispersion of microdroplets and removing supernatant) though it enhances the reaction rate constant only by 4-fold [18]. Intriguingly, the authors note that the reaction rate in the bulk coacervate phase appears to be 60-fold slower than in buffer conditions (10 mM Tris), which indicates an overall reduced activity within the coacervate phase despite the concentrating effect. These studies collectively show that although the concentration of enzymes is significantly higher inside the phase-separated condensates, reaction rates are only slightly increased or even reduced, pointing to other mechanisms that negatively contribute to the catalytic reaction.

2.2 Diffusion rates are altered in phases

Slowed diffusion may be one of such mechanisms that reduce catalytic reactions in condensates [19, 20]. Phase-separated droplets are usually found to be highly viscous compared to the diluted solution, therefore restricting free diffusion of enzyme and substrate. The Kay group has measured the motion of IDP from Ddx4 (residues 1–236) inside phase-separated condensates [8, 21]. The authors found that the IDP remains highly dynamic in condensates but diffuses ~ 100-fold slower than the nonphase-separating control. A similar reduction in the diffusion rate and an increase in viscosity was also reported for other condensates [11] [22].

However, as discussed in prior sections, the composition and physical properties of cellular condensates can vary drastically. Additionally, the intracellular environment is typically crowded and differs from dilute solutions commonly used to measure enzymatic properties. Therefore, whether the diffusion rate inside a particular type of condensates is faster or slower than the surrounding areas depends on the exact local environment. The Brangwynne group had shown that frog nucleolus is organized into distinct liquid-phase sub-compartments, including the granular component, dense fibrillar component (DFC) and the fibrillar center, with each sub-compartment displaying different biophysical properties, such as surface tension and viscosity [22]. *In vivo* studies of human nucleoli further show that these compartments exhibit very low surface tension and are surrounded by highly viscous nucleoplasm [23], implying a faster diffusion rate inside the condensates. Moreover, the diffusion rates inside the condensates are also subjected to change, depending on ongoing biochemical reactions. For example, Feric *et al.* showed that in the frog nucleolus, ATP-depletion results in the significantly slower FIB1 (enriched in DFC) dynamics, which suggests that enzymatic activity may maintain the fluidity of DFC [22]. It will be necessary to characterize extent to which the diffusion rate is altered in the condensates and to determine its impact on biochemical reactions in future studies.

2.3 Exclusion of matured products or competing enzymes from condensate may accelerate the reaction rate

Another factor that may alter the reaction rate is the release of products. Riback *et al.* has recently shown that endogenous phase separation is dominated by heterotypic multicomponent interactions among protein and RNA components, which suggests that thermodynamics of a biomolecular interaction network controls the composition of condensates [24]. The authors quantified the transfer free energy of a biomolecule *in vivo* and *in vitro* under single- or multi-component phase separation conditions, and found that multi-component droplets recapitulate the free energy curve measured in cells. More specifically, in the case of the nucleolus, the products of RNA-processing — fully assembled ribonucleoprotein complexes — are available for a smaller number of interactions with other nucleolus components, thereby becoming less prone to phase separate. Such a change allows coupling of product formation to its thermodynamically driven exit from nucleoli. This selective removal of the reaction product can also serve to drive the chemical equilibrium leading to a higher reaction rate.

Living cells are incredibly complex systems with thousands of reactions happening concurrently, some of which even compete for the same substrate. Thus, besides the direct contribution to the catalytic rate through the concentration effect, it is possible that phase-separation allows selective enrichment of one enzyme, while simultaneously excluding other competing enzymes to drive a specific reaction pathway. A case study reported by the Rosen and Vale groups on T cell receptor (TCR) signal transduction provide a compelling example [25]. Following TCR activation, the downstream signaling proteins, including the transmembrane protein LAT (linker for activation of T cells) and its binding partners, spontaneously form phase-separated condensates. These micro-compartments concentrated kinases but excluded phosphatases (which oppose TCR signaling), promoting LAT phosphorylation and amplifying TCR signaling.

3. Condensates accelerate multi-step reactions and increase specificity

As the definition of phase-separated condensates continues to expand, some multienzyme metabolic complexes have also recently been recognized as phase-separated condensates [26]. Analogous to the single-step reactions discussed above, the formation of phase-separated condensates also enhances the reaction rates for multi-step reactions. One such example is the puriosome, the *de novo* purine biosynthetic enzymes co-cluster that forms cellular bodies in the cytosol under high cellular purine demand [27]. In stressed puriosome-rich cells, the Benkovic group observed a 50% enhancement of metabolic flux and an overall 3-fold increase in the purine nucleotide precursor (inosinic acid, IMP) formation compared with non-stressed cells [28].

Several advantages may be expected for compartmentalizing multi-step reactions, including the stabilization of reaction intermediates and enhancement of processivity. The benefit of co-clustering multiple enzymes has been quantitatively modeled by Castellana *et al.*, who predicted a maximum 6-fold improvement in the efficiency of a two-step pathway [29]. The Avalos and Toettcher groups engineered the two-step deoxyviolacein biosynthesis pathway allowing the formation of inducible phase-separated droplets using optogenetic tools [30]. Coincidently, the authors observed a 6-fold enhancement in product formation and an 18-fold improvement of product specificity. An increase of the reaction rate was also observed in the menaquinone biosynthesis pathway by assembling enzymes in condensates [31].

Benefits of multi-step reactions are not limited to metabolic pathways and have also been reported for RNA processing condensates [22] and the ubiquitination pathway. Gallego *et al.* show that phase-separated droplets formed by a scaffolding protein (Lge1) recruit the histone-modifying enzymes Rad6 and Bre1, ubiquitin-conjugating (E2) enzyme and ubiquitin-ligating (E3) enzyme to a shell on the surface of condensates [32]. The authors propose a model in which these layered condensates form spatially organized reaction chambers that stimulate histone H2B ubiquitination.

4. Inhibition of biochemical reactions through phase separation

In contrast to promoting specific biological reactions, phase separation can also play an inhibitory role. Sequestration of substrates alone without enzymes in phases may deplete the available substrates and therefore impede reactions. Several recent studies have shown that the phase-separated stress granules and processing-bodies accumulate non-translating mRNAs leading to translation inhibition and protection from decay [33] [34] [35]. Moreover, Banerjee *et al.* report that increasing the RNA concentration in stress granules triggers disassembly of the droplets by charge inversion following initial phase separation [36]. The authors proposed that the reentrant phase transition in ribonucleoprotein droplets allows spatiotemporal control of the organization and dynamics of granules. It is also possible that the inter-molecular interactions that partition an enzyme into phase may block its active site, suppressing the catalytic reaction. Consistent with such a possibility, Prouteau *et al.* found that yeast TORC1 oligomerizes into a cylindrical assembly and consequently inactivates itself in response to glucose withdrawal [37].

5. Coupling of phase separation and enzymatic reactions

The recent study of human HP1 protein proposes a model in which in the absence of phosphorylation and ligand (DNA), HP1α protein exists in an auto-inhibited and compact conformation incapable of phase separation [38]. A similar compact and auto-inhibited state was also proposed for G3BP under non-stressed conditions, which is alleviated upon encountering stress-induced unfolded mRNA [39]. If the enzyme itself participates in phase-separation, there could be implications for the coupling release (or reinforcement) of auto-inhibition to phase-separation. Coupling of enzyme activation and phase-separation was reported for the MORC3 protein, which phase separates in the presence of the ATP cofactor and nucleosomal ligands [40]. RNA-dependent ATPases (DDXs) were also shown to promote phase separation only in the ATP-bound form, whereas active ATP turnover was found to trigger the release of its RNA client and subsequent disassembly of condensates [41].

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Highlights

- Internal environments of biological condensates may alter enzymatic reactions
- Concentration of reactants in condensates has diverse effects on catalytic reactions
- Regulation of catalytic reactions by phase separation is context-dependent

Perspectives

Accumulating evidence suggests that cellular condensates can play major roles in regulating the activities of biological enzymes. Many enzymes, particularly those inside the nucleus, contain IDRs and are frequently shown to colocalize in condensates. Such phase-separated droplets have diverse yet unique interior properties, which are likely fine-tuned by the composition and post-translational modifications of the proteins. Regulation of the interactions, conformations and concentrations of enzymes and substrates as well as products inside the condensates could serve to modulate catalytic reactions across a large dynamic range. In this rapidly advancing area of research, we anticipate that as more enzymatic reactions are characterized within phases, the diversity of bio-regulatory effects enabled by phase-separation will be more fully uncovered.

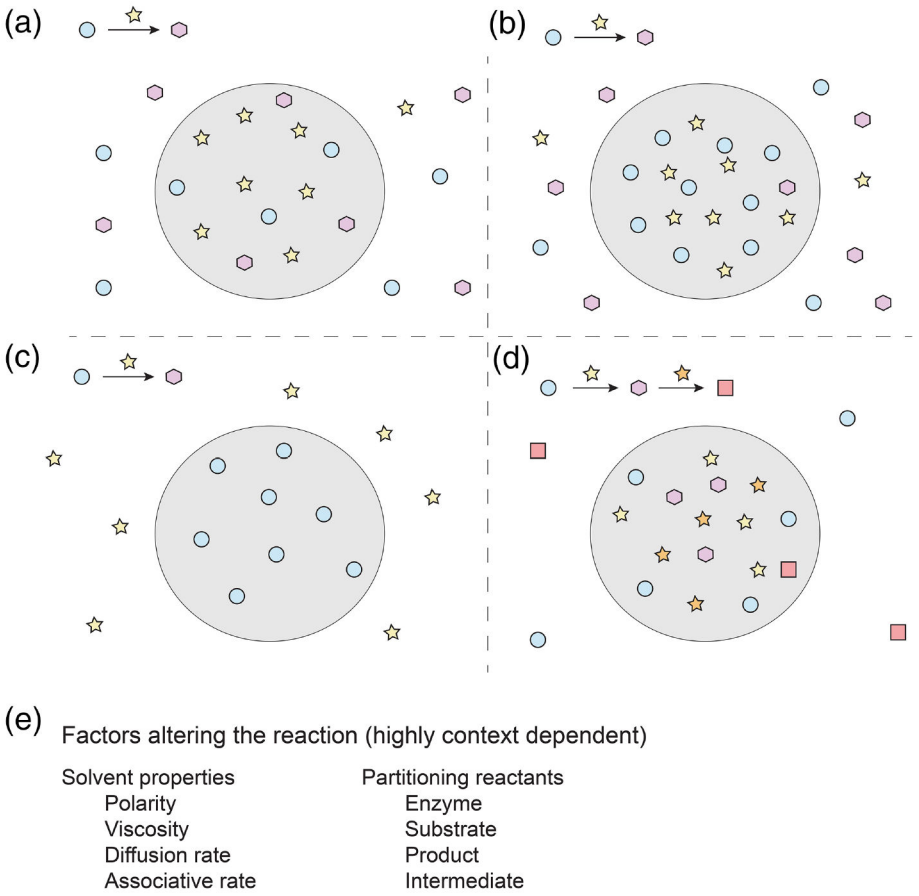

Figure 1.

Schematic diagrams of biochemical reactions in condensates. (a, b) Enrichment of enzyme (a) and substrate (b) in phases accelerate reactions. Further rate enhancement may be achieved by the exclusion of product. (c) Sequestration of reaction components, such as the substrate, may inhibit biochemical reaction. (d) Phase-separation accelerates reaction rate and specificity by compartmentalizing multi-step reactions. (e) A list of factors alters reaction rates.