Regional Compartmentalization in Multi-enzyme System

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Abstract: Multi-enzyme cascade reactions are widely utilized because they can function well to generate value-added products from simple raw materials. However, how to promote the catalytic efficiency and synergistic effect of the multi-enzyme system is proved to be a challengeable point. Recent discovery repeatedly emphasized the strategy of assembled multi-enzyme complexes or forming subcellular compartments for spacial optimization. This may contribute to various biochemical processes as a highly ordered and tunable organization. On the basis of the feasibility of regional compartments for natural or artificial biochemical reactions in vivo and vitro, this dissertation focuses mainly on analysis and progresses in this cascaded strategy.

Key words: compartments, spacial organization, enzyme assembly, bioreaction in vivo and in vitro

1. Introduction

Recently, enzymes are increasingly used in cascade reactions because of high selectivity to promote a biofriendly synthetic environment, like multi-enzyme cascade reactions. Compared with traditional multistep sequential biosynthesis methods, enzyme cascades can relieve the bulk diffusion from unstable or toxic intermediates and avoid the purification of intermediary state[1,2].

Additionally, cascade sequential reactions in vivo are more attractive in the stage of mutitienzyme assembled system. They can effectively eliminate product inhibition, improve cofactor regeneration and work without purification and immobilized enzymatic catalysts. Common methods of reaction pathways contain two following steps[1,3]. One is on the basis of optimization and modification of natural intermediate metabolism by gene knockout or overexpression in the early period. Lonza, et al.[4] found a biological process in natural metabolic pathways to synthesize L-carnitine from 4-butylamine. They got a strain of microorganisms that can degrade 4-butylamine by L-carnitine coenzyme A and Krebs cycle to knock out L-carnitine deaminase, functioning as the first step to catalyze the degradation. Next is to introduce heterologous enzymes to optimize the cascade metabolism and synthesize non-natural products. Ro et al.[5] founded a novel high-production process of cyanuric acid by sorting out the engineered Mevalonate pathway from Saccharomyces cerevisiae. The last mainly focuses on the ways of designing a non-natural metabolic routes to integrate enzymes from different origins into the same cellular space for expression. This can meet the demands in large production of the
enzymes with an extensive substrate spectrum, which may achieve coexpression in the same space like multienzyme biocoupling reactions[6].

Accordingly, many techniques in vitro sparks wide attention due to the improvements in the vitro multienzyme reactions with a vivid scaling effect in the sequential signal transfer. The vitro multienzyme process has a simple and easily controllable structure compared to the vivo traditional methods. Admittedly, with a mature stategy of reconstruction of multienzyme system or pseudo-natural synthesis by artificial design, it can freely choose initial substrates without effect from metabolic intermediates. Ox-red enzyme cascade system is one of the typical examples[7].

During the developing but tortuous period of research, the more cascade numbers it reached, the more uncertain parameters needed to be taken into account, like enzymatic stability, intermediate transfer and substrate inhibition in vitro, or rate-limiting enzymes, competitive inhibition and diffusion of toxic intermediates in vivo[8,9]. Tech in artificial multienzyme simulation is faced with a challengeable problem: How to establish optimized spacial organization for enzyme assemblies to handle the increasingly sophisticated requirements in metabolic engineering.

Natural pathway for cascade bioreactions is to make two or more active sites of enzymes close to each other to accelerate the enzymatic reactions, which indicates a regional optimization for spacial arrangement and energy barrier. Based on this, we may talk about a perfect structural assembly with the dynamic interaction in the multienzyme cascade reactions, as well as its progresses and applications.

2. Brief review on substrate channeling with multienzyme assemblies

As mentioned above, a common way of compartmentalization is called substrate channeling interaction, which means a metabolic transferring of intermediates from one enzyme directly to another without its bulk diffusion into solution. Based on its multi-function, it has been widespread in the process of enzymatic reactors and bioengineering. In nature, there are 3 kinds of pathways occuring the construction of spacial organization: One, which occurs in the pyruvate dehydrogenase complex, is by a substrate being attached to a flexible arm that moves between several active sites in a protein complex; Another possibility is by two active sites being connected by a tunnel where the enzymes and the substrate moving in the protein, such as tryptophan synthase; A third way is by a charged region on the surface of the enzyme acting as a "electrostatic highway" to guide a substrate that has the opposite charge from one active site to another, like bifunctional enzyme dihydrofolate reductase-thymidylate synthase.

Like Calvin cycle, biosynthesis of amino acids and microbodies, many primary metabolic pathways can be more effective with the existence of secondary metabolic factors, majorities known as multienzyme assembled complexes. Tryptophan synthase, existing in the form of (αβ)2 complexes as a domain of homologous proteins, catalyzes the last two steps of tryptophan
synthesis. Anderson et al.[10] discovered the evidence of the indole channel through steady-state experiments. Only a very small amount of indole was detected during the synthesis of L-tryptophan, which was about 1% of the concentration of D-3-glyceraldehyde, along with undetectable synthetic delay time of L-tryptophan. The results of kinetic experiments show that indole rapidly moves from the active site of the α subunit to β and rapidly converts to tryptophan on the β subunit. This demonstrates the existence of an ordered hydrophobic tunnel between 2 subunits, whose allosteric effect may further regulate the active space[11]. From this, roles in substrate channeling become clear: a) enhancingly sensitive to environmental parameters; b) protecting the unstable intermediates and cofactors; c) relieve the toxicity diffusion of metabolism; d) improving cellular dissolving capacity due to insoluble intermediates; e) promoting catalytic efficiency.

Thus, spatial organization plays a significant role in assembled strategy design, but more refined and pointed optimization to guarantee the highly ordered catalytic efficiency need further exploration.

3. Strategies of spatial organization by assembling enzymes

As mentioned above, more optimized artificial assemblies need introducing to build a efficient substrate highway ensuring the outstanding catalytic conversion. Presently, there are several successful multi-enzyme assembled methods, including co-immobilization, protein fusion assembly, protein cross-linked collocation assembly, mutienzymatic assembly of macromolecular scaffolds and so on.

3.1 Co-immobilization

Inspired by the natural multienzyme complexes, more attention is fixed on the multienzyme co-immobilized system. It can obviously improve the stability of enzymes, make bi-enzymes closer to each other and increase the concentration of substrates around the enzyme to promote the enzymatic efficiency[12]. In addition, the multi-enzyme system is suitable for in-situ coenzyme regeneration reaction process, greatly reducing the cost of large-scale biocatalytic reaction.

Based on the methods of single enzyme techniques, the common form of co-immobilized system contains immobilization on carriers and cross-linked aggregation of multienzymes, while the modern multi-enzyme ways are by multi-site covalent bonding, physical embedding, physical adsorption, site-specific affinity interaction, DNA directed self-assembly immobilization, and cross-linked enzyme aggregation with their combinations.

However, there are still many challenges in the field of multienzyme co-immobilization technology. First, the structure and function of a single enzyme need considering to optimize the optimal conditions to maintain the activity and stability of each enzyme; Secondly, the carrier should have a good compatibility so as not to affect the enzyme activity, also ensuring the
maximum loading of enzymes; Finally, the position of different enzymes in the carrier is effectively controlled, thereby reducing the limit of the intrinsic inherent diffusion[13].

3.2 Protein fusion assembly

Multienzyme fusion refers to the integration of multienzymes by gene fusion to generate a short-chain chimeric protein linked with a short peptide. As many results reveal, multifunctional enzymes shows greater activity than single enzymes, and catalytic efficiency grows with the increasing concentration of substrates[14]. Multi-fusion technology can not only maintain the independence of each single enzyme in function and steric structrue, but also make them closely associated with each other as proximity effect, from the result of small-angle scattering analysis[15].

With the booming of gene engineering, cascade multienzymes are more widely utilized and easily spatial-modified by means of multi-terminal fusion and so on. With the goal of constructing a fusion protein with expected characteristics, there is a high uncertainty because present synthesis counts primarily on subjective experience. It’s vital and urgent to understand more microinformation of fusion proteins, like spacial orientation, interactions and dynamics in folding and allosteric regulation. A systematc informatic tool need simultaneously keep pace for exact simulation and rational optimal design.

3.3 Protein cross-linked collocation assembly

The hybrid cross-linked enzyme is one of the most representative examples of chemical cross-linking methods for multi-enzyme assembly. The methods use the construction of single-enzyme cross-linked enzyme aggregates to effect the cross-linking aggregation of two or more enzymes. For example, cross-linked enzyme aggregates form by co-crosslinking of S-selective oxynitrilase and nitrilase catalyze, conversing benzaldehyde to S-mandelic acid. It can extraordinarily reduce the $K_m$ value during catalytic reaction and uniquely eliminate the enzyme-inhibition effects of nonspecific reactions.

Cross-linked aggregations are synthesized by disordered crosslinking of surface groups, unsure of spatial approach to each other, so reseachers have figured out some novel chemo-selective methods for protein aggregations to realise the heterologous assembly such as sulphydryl-sulfate exchanging reactions to form a new peptide bond[16].

3.4 Roles of protein scaffolds

In cellulosome, the existence of protein scaffolds, containing cellulose-binding proteins without catalytic reactivity and many fibronectin, makes contributions to combination with domain of anchoring protein to realize the assembly of hydrolase. The most representative scaffold is $(GBD)_x-(SH3)_y-(PDZ)_z$ by Dueber[17], which functioned well in the assembly of cascade enzymes
during metabolic pathways. By optimal regulating the ratio of x, y and z values, the output can reach the most splendid level.

On the basis of this, interactions among multi-proteins gradually play an important part in constructing scaffolds. For instance, SP1 protein originates from homomultimeric proteins in poplars, with a great high-temperature, pH and solvent resistance. Heyman et al. fused glucose oxidase with SP1 and expressed it in E. coli. Finally, the enzyme molecule was assembled into enzyme-labeled nanotubes by SP1 scaffolds. Each nanotube contained hundreds of enzyme molecules with an outstanding catalytic activity. Then, they accomplished the fusion between Coh and the SP1 gene and achieved the soluble expression of the fusion protein Coh-SP1 in E. coli so that there are 12 sites for co-binding proteins(Doc) connected to the Coh-SP1 per molecule. When the cellulase derived from Thermobifida fusca was fused with Doc and then mixed with Coh-SP1 in vitro, a scaffold cellulase was obtained, whose catalytic activity was remarkably improved[18].

3.5 Roles of DNA scaffolds

DNA stands for the expressing carrier of various molecular messages, storing and transmitting genetic instructions. With a unique hereditary property, DNA polymers can be extensively used in rational design by changing the base sequences.

Fu et al.[19] constructed a double-crosslinked scaffold-mediated complex containing swinging arm and multienzymes. The 6-phosphogluconate deaminase (G6pdH) and malate deaminase (MDH) were modified with a pre-encoded oligonucleotide that specifically binds the DNA scaffold so that G6pdH and MDH can self-assemble at a certain distance to the DNA. In order to enhance the substrate channeling between G6pdH and MDH, they used NAD+ modified by the oligonucleotide to bind a site on the scaffold between 2 enzymes to form a multienzyme complex that co-enzymes can shuttle back and forth. The results illustrate that the addition of swinging arm doesn’t only significantly increases the reactivity of the double-enzyme reaction, but also makes it highly specific in complicated reaction systems.

What’s more, the property that DNA and its conjugated protein specifically combine with each other can also involve in the DNA-scaffold-mediated methods to assemble multienzyme complexes. Usually, different zinc-finger-combined DNAs are inserted into the plasmids with a fusion between domains of zinc finger proteins and scaffold plasmids simultaneously[20]. After their co-expression in the E. coli, cascade enzymes can be anchored in the corresponding position under the driving interactions between domains of zinc finger proteins and their binding sites. By optimal changing the number and alignment of the zinc finger binding sites, more multienzyme catalytic efficiency may be achieved.

4. Comments on compartmentalization in metabolic pathways
It’s natural to accomplish the process of metabolism by regionally separated enzymes from vesicles, membranes, cellular organelles, or non-membrane bound cellular granules in the cytoplasm. Substrate channeling effect has long been regarded to promote metabolic efficiency by multienzyme complexes, but over four sequential cascade reactions in the tunnel may be structurally limit and more challengingly operated. Highly controlled spatial aggregations are in recent years proved to be well regulated with the media of cluster for substrate channeling. Under this circumstance, effective and ordered collocation of sequential enzymes may primarily contribute to the catalytic reactivity instead of associated active bindings.

Through gradually systematic researches, it’s also obvious that there isn’t a direct signal from the formation of assembled complexes to the metabolic flux enhancement, so cascade reactions in metabolism may contain more sophisticated mechanisms for multifunctional spacial assemblies[21]. In other words, spatial organization is only suitable for a certain bioenvironment with single enzymes of given pathways, which shows little potential functional diversities.

5. Outlook[22]

a. Multi-protein fusion lacks theoridical guidance because of lack of the understandings of interactions between intracellular structures with their relationship with functions. Like improving the soluble expression level of fusion protein and the impact on the folding protein, more inner information needs to be explored.

b. How to accomplish the rational design of enzymatic assemblies is still a big challenge, indicating that more bioinformatic methods or tools need to keep pace with the development of cascade reactions. By establishing a homologous structural model of intracellular multienzymes, a suitable binding site can be easily found to achieve the exact control of spacial orientation of enzymes on the surface for efficient immobilization.

c. For substrate channeling mechanism, contact via large numbers of sequential enzymatic reactions is hard to accomplish, which break the common sense that substrate channeling can promote the catalytic efficiency and relieve the bulk diffusion from toxic or unstable intermediates. Accordingly, the entire mechanism for assembled complexes needs further study.

d. For the precise control, more bioreaction mechanisms need exploring, like energy barrier change during catalysis and allosteric effect. To reveal the mechanisms of controlled assembly of protein complexes, more advanced strategies of dynamic simulation of conformational changes in assembling process concerning multi-level interactions should be developed.

e. Supramolecular interactions between active sites on different enzymes can be taken into key consideration, for the system is easily tunable under optimal regulation and form micro-partitions when multi-aggregating. In addition, it can be a effective methods for assemblies to eliminate the reducing enzymatic activity without purification, which can generate the ordered state.
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