Modulation of Enzyme Reactions on DNA Scaffold

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Cells have developed intelligent systems to implement the complex and efficient enzyme cascade reactions via the strategies of organelles, bacterial microcompartments and enzyme complexes. The scaffolds such as the membrane or protein in the cell are believed to assist the co-localization of enzymes and enhance the enzymatic reactions. Inspired by nature, enzymes have been located on a wide variety of carriers, among which DNA scaffolds attract great interest for their programmability and addressability. Integrating these properties with the versatile DNA–protein conjugation methods enables the spatial arrangement of enzymes on the DNA scaffold with precise control over the interenzyme distance and enzyme stoichiometry.

DNA scaffold enzyme reaction catalytic enhancement

1. Introduction

Living organisms have evolved over millions of years to build a complex metabolic network containing thousands of enzymatic reactions for their survival [1]. Enzymes are spatially organized in the cell to implement specific sequential reactions via the strategies of compartmentalization [2]. The spatial organization of enzymes often relies on the specific scaffolds of proteins or the membrane to achieve the high efficiency and the specificity of enzymatic reactions [3]. Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and carbonic anhydrase packed in the protein shell of carboxysome [4] and cytochrome P450 enzymes anchored on the membrane of endoplasmic reticulum [5] are the typical examples. In these compartments, the reactants in low concentration are believed to be effectively transferred through spatially arranged enzymes, thereby channeling metabolites to drive favorable reactions and preventing the toxic side reactions by intermediates [6]. It is the challenge to understand the efficient bioenergetic processes of nature and to construct human-engineered energy utilization systems [7].

To mimic and understand the natural systems, a wide variety of carriers has been built for the attachment of enzymes, as has been reviewed previously [8][9][10][11]. The further applications of the carriers such as protein, liposome or polymersome are challenged by the structural programmability of carriers and the spatial arrangement of enzymes [12]. These obstacles are tackled by means of DNA nanotechnology [13]. A typical example of DNA nanostructures, DNA origami, folds a long, single-stranded DNA into the predesigned, two-dimensional (2D) and three-dimensional (3D) DNA scaffolds with accurate addressability, providing the ideal templates for the assembly of enzymes [14][15]. Individual or multiple enzymes have been spatially arranged on the DNA scaffolds with precise control over the enzyme orientations, interenzyme distance and the stoichiometry of enzymes [16].

Interestingly, the catalytic enhancement of single type of enzyme assembled on the DNA scaffolds has been widely observed [17]. This phenomenon has been attributed to the substrate affinity to the negatively charged DNA scaffold surface by electrostatic interactions, lower local pH on the DNA scaffold surface, reduced adsorption of scaffolded enzymes on the reaction vessels or the ordered hydration layer attracted by the DNA surface [18]. However, the notion of whether these proposed mechanisms can apply in general for the catalytic enhancement of DNA-scaffolded enzymes remains controversial. Besides the single type of enzyme, the cascade reactions of multi-enzyme assembled on the DNA scaffold were also extensively studied [19][20]. While the close interenzyme distance, optimal spatial organization of enzymes and a confined DNA environment have been proposed to be the main factors enhancing the efficiency of enzyme cascade reactions on the DNA scaffold, the actual mechanisms remain to be elucidated [21].

2. Construction of the Artificial Enzymatic Reaction Systems Inspired by Nature

To implement the efficient enzyme cascade reactions for the biochemical transformation, cells utilize the strategy of compartmentalization by forming the membrane-bound organelles (e.g., mitochondria, chloroplasts and peroxisome), bacterial microcompartments and multi-enzyme complexes [22]. The spatial organization of enzymes on the specific scaffolds (e.g., proteins or membrane) exerts the high efficiency and specificity of cascade reactions by increasing the concentration of reactants, reducing the toxic intermediates and competing reactions and overcoming the unfavorable enzyme kinetics [23]. To mimic the natural systems, a wide ranges of materials have been applied to construct the artificial scaffolds for enzyme reactions in vitro [18].

2.1. The Spatial Organization of Enzymes in Nature

Substrate channeling is the transportation of the intermediates from one enzyme active site to the next without the release into solution [23]. The typical example of substrate channeling is tryptophan synthase. This $\alpha_2\beta_2$ tetrameric enzyme complex catalyzes a two-step cascade reaction that converts indole-3-glycerol-phosphate and serine to tryptophan. As the intermediate, indole is directly transferred through the 25 Å long hydrophobic tunnel bridging the two active sites [24]. Such hydrophobic tunnel is also found in carbamoyl–phosphate synthetase that channels carbamates to the neighboring active site. The substrate channeling couples the first hydrolysis reaction of glutamine and the successive reactions to maintain the proper stoichiometry despite of the three-order-of magnitude difference in K_m for NH_3 in the carbamate synthesis and that for glutamine in the first reaction [25]. Instead of using hydrophobic tunnel, the malate dehydrogenase–citrate synthase (MDH–CS) complex utilizes the electrostatic guidance to channel the metabolite oxaloacetate to achieve a high flux though the MDH–CS pair by overcoming the unfavorable kinetics of MDH forward reaction in the citric acid cycle [26].

Metabolons feature the dynamic assembly and disassembly of the enzyme complexes [27]. These highly transient protein assemblies are believed to allow the direct channeling of the intermediates from one enzyme to the next consecutive enzyme in the metabolic pathway. Purinosome [28], dhurrin metabolon [29] and glucosome [30] are the typical examples. While the spatiotemporal enzyme assemblies are proposed to enhance the metabolic flux and

biochemical transformation, the study of the spatial organization of metabolon remains challenging due to the transient and complex interactions of proteins involved [27]. In 2008, the human purinosome was first identified, in which the ten chemical steps of de novo purine biosynthesis (DNPB) were catalyzed by six enzymes. Two human enzymes involved in DNPB were fused to either a green fluorescent protein (GFP) or an orange fluorescent protein (OFP) and transiently expressed in the purine-rich or purine-depleted cells. The cytoplasmic clusters formed by these two enzymes were observed by fluorescence microscopy in purine-depleted cells. This provided the evidence for the formation of the enzyme complex "purinosome" [31]. Afterward, the protein–protein interactions of purinosome and the regulation of its formation were extensively studied [32]. In 2020, Pareek et al. [33] used metabolomics and in situ three-dimensional sub-micrometer chemical imaging of single cells by gas cluster ion beam secondary ion mass spectrometry (GCIB-SIMS) to directly visualize DNPB by purinosome. It was proposed that purinosome consisted of at least nine enzymes that functioned synergistically to increase the pathway flux. Moreover, these DNPB metabolons were hypothesized to locate proximal to the mitochondria. In a recent study, Pedley et al. [34] found the heat shock protein 90 kDa (Hsp90) would help to regulate the physical properties of the purinosome and maintain the liquid-like state inside HeLa cells. This finding provides novel insights into how the liquid–liquid phase separation drives the formation of metabolon in the human cell.

As the best-studied bacterial microcompartments, carboxysomes play a central role in the carbon concentrating mechanism [4]. Two enzymes, RuBisCO and carbonic anhydrase (CA), are packed in the polyhedral protein shells of carboxysome. CA converts bicarbonate (HCO_3^-) to CO_2, which is subsequently consumed by RuBisCO in the carbon fixing reaction. The diffusion of negatively charged HCO_3^- into the protein shell is promoted by the positively charged pores of carboxysome, while the concentration of uncharged CO_2 is increased in the compartment, resulting in the enhanced reactivity of RuBisCO [4]. Moreover, RuBisCO molecules have been hypothesized to form the condensate by the liquid–liquid phase separation, which provides the understanding of carboxysome biogenesis. The function of the protein shell of carboxysome in RuBisCO condensation remains to be elucidated [35].

Membrane plays important roles in many biological processes in cells. Cyanobacterial thylakoid membrane possesses a unique structure carrying both photosynthetic and respiratory electrons transfer chains, allowing the performance of both oxygenic photosynthesis and aerobic respiration in the same cellular compartment [36]. The membrane of endoplasmic reticulum or mitochondria provides the scaffold for anchoring the cytochromes P450, an important class of enzymes involved in the biotransformation of many endo- and exogenous compounds. The structure and dynamics of cytochrome P450 on biomembranes have been reviewed [5]. While the scaffolds in nature are proposed to assist the co-localization of enzymes, maintain the enzyme functions and enhance the enzymatic reactions, the actual working mechanisms of scaffolds in the natural compartments remain unclear.

2.2. Enzymatic Reactions on Various Carriers

The reactions of individual or multienzymes have been conducted on a wide range of carriers, such as metal–organic frameworks (MOFs) [37], hydrogel [38], graphene oxide [39], liposome [40], polymersome [41], proteins [42] and DNA nanostructures [43]. These artificial scaffolds are suggested to enhance the enzyme stability, reusability or

catalytic ability, expanding the applications of enzyme in different fields such as the biosynthesis of value-added chemicals. These scaffolds are also applied to mimic and understand the natural systems such as complex metabolic pathways or molecular transport.

Yoshimoto et al. [44] entrapped glucose oxidase (GOx) and catalase into the liposome membrane. The hydrogen peroxide (H_2O_2) produced by GOx in the glucose oxidation reaction inside the liposome was decomposed by catalase. A remarkable protection effect of the liposome membrane on catalase activity inside the liposome was observed. The presence of outer membrane protein F (OmpF) enhanced the transport of glucose molecules from the exterior of the liposomes to the interior and increased the enzyme activity of GOx by 17 times compared with that of GOx encapsulated in the liposome in the absence of OmpF. This system demonstrates the typical example of the application of liposome as an enzyme carrier. Klermund et al. [41] compartmentalized the three-step reaction in a polymersome to synthesize CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac). *N*-Acetylneuraminate lyase (NAL) and CMP-sialic acid synthetase (CSS) were inserted into polymersome, while *N*-Acyl-D-glucosamine 2-epimerase (AGE) with allosteric activator ATP was encapsulated inside the polymersome. Channel proteins OmpF enabled a selective mass transport across the polymer membrane. The incorporation of OmpF into the membrane restrained the cross-inhibitions in enzyme cascade reactions. The overall throughput was enhanced 2.2-fold compared to the reaction by free enzymes.

Protein scaffolds are also widely applied as the templates for enzymatic reactions. Zhang et al. [42] utilized the shell proteins from the ethanolamine utilization bacterial microcompartment (EutM) to attach enzymes by SpyTag–SpyCatcher conjugation strategy. Alcohol dehydrogenase (ADH) and amine-dehydrogenase (AmDH) were co-immobilized on the protein scaffold to convert alcohols to chiral amines in a highly enantioselective manner. To construct an artificial carboxysome, Raphael et al. [45] used an electrostatic tagging system to co-encapsulate RuBisCO and carbonic anhydrase (CA) in an engineered protein cage based on lumazine synthase from *Aquifex aeolicus*, AaLS-13. RuBisCO and CA were genetically fused to the positively supercharged variants of green and yellow fluoresce proteins, GFP(+36) and TOP(+36), to obtain G-RuBisCO and T-CA, respectively. These positively charged constructs were encapsulated by the AaLS-13 capsids containing a negatively charged lumenal surface. While a significant kinetic effect of co-entrapped CA on the enzyme activity of RuBisCO was not observed under ambient or oxygen saturated conditions, this system nonetheless provided the new strategy of constructing the artificial organelles.

3. Catalytic Enhancement of Single Type of Enzyme Assembled on the DNA Scaffold

While the soft materials show their own advantages and the potential as the enzyme scaffolds, the further applications of carriers such as liposome, polymersome or protein face the difficulties in controlling the enzyme loading positions and stoichiometry. Therefore, the scaffolds that can overcome these challenges are required for the enzyme assembly in vitro. Given the predominant advantages of structural programmability and accurate addressability, DNA nanostructures are considered as the ideal platforms for the assembly of enzymes [46]. The reactions of a single type of enzyme assembled on the DNA scaffolds and the previously proposed mechanisms for

the catalytic enhancement of enzyme by the DNA microenvironment have been reviewed. Understanding the origins of enhanced activity of DNA-scaffolded enzymes will expand their practical applications.

3.1. DNA Origami Scaffold

The past two decades have witnessed the rapid development of DNA origami and its applications [47]. In 2006, Rothemund [14] created DNA origami that folds a long, circular, single-stranded DNA template (7-kilobase) into desired two-dimensional (2D) shapes with the aid of over 200 short oligonucleotides (staple strands). Nonperiodic structures, such as square, rectangle, star and smiley face, were obtained. Featuring preparation simplicity, structural programmability and high folding yield, DNA origami plays an important role in the development of structural DNA nanotechnology. In 2009, Douglas et al. [15] extended the DNA origami method to build custom three-dimensional structures formed as pleated layers of DNA helices in the honeycomb lattices, providing a general route to the construction of complex 3D DNA nanostructures. By using the computer-aided design software for DNA origami nanostructures such as caDNAno, the staple sequences for folding newly designed DNA nanostructures are easily generated [48][49].

With the development of the DNA origami technique, a number of DNA nanostructures with complexity were constructed, such as DNA box, DNA nanoflask and hollow DNA sphere [50][51][52]. Featuring tremendous self-assembly properties and addressability, DNA scaffolds provide the ideal platforms for the assembly of functional macromolecules such as proteins. To locate proteins of interest on DNA scaffolds, various strategies of DNA–protein conjugation have been developed with noncovalent and covalent conjugations [53]. Besides the static DNA origami structures, the dynamic DNA nanotechnology enables the construction of various reconfigurable DNA origami structures induced by the hybridization of short DNA, aptamer–ligand interactions, temperature, pH, ion and electric field with controlled translational, rotational or more sophisticated movement [54][55][56][57][58][59][60]. These dynamic DNA structures have been widely applied in the fields of drug delivery, diagnosis, biosensing and biocatalysis, which has been reviewed previously [61][62][63][64][65].

3.2. “Favorable Microenvironment” Provided by DNA

The enzyme scaffolded by DNA structures often displays enhanced activity and stability over its free form; however, the actual mechanisms for the higher catalytic ability are still under debate [66]. As studied previously, Glettenberg et al. [67] covalently conjugated peroxidase to different DNA oligonucleotides (ODN). The ODN markedly influenced the catalytic of tethered-enzyme in a DNA sequence-dependent manner. This phenomenon was attributed to the interactions such as hydrogen bonding or electrostatic contacts between ODN and the heme-containing catalyst. Rudiuk et al. [68] conjugated β-lactamase with a branched DNA complex constructed by four λ DNA (48.5 kbp), which underwent a dramatic and reversible higher-order structural transition regulated by spermine (SPM^{4+}) and NaCl. The enhanced catalytic activity of enzyme was attributed to the “favorable microenvironment” composed of the giant and ordered DNA molecules. Then an interesting and important question arises: What is “favorable DNA microenvironment”, and what is the chemistry behind it?

3.3. Protection Effect Derived from DNA Scaffold

Featuring the characteristics of a highly negatively charged surface, DNA scaffolds have been reported to protect enzymes from deactivation. Timm et al. [69] tethered the *S*-selective $NADP^+$/NADPH-dependent oxidoreductase Gre2 from *S. Cerevisiae* on rectangular DNA origami structures through chemo- and site-selective protein–DNA coupling methods. Gre2 was first fused with the Halo-tag or SNAP-tag to obtain Halo-Gre2 or SNAP-Gre2. Chlorohexane (CH) or benzylguanine (BG) was then incorporated as suicide ligand into the DNA origami scaffold to facilitate the crosslinking reaction of the Halo-Gre2 or SNAP-Gre2. To assess the enzyme stability, the activity of the free enzyme or DNA-scaffolded enzyme was measured immediately after preparation (incubation for 0 h) or after incubation for 2.5 h. A significantly increased activity of the DNA origami-tethered enzymes as compared to free enzymes in solution was observed. Considering the phenomenon that the free enzymes lost significant activity upon incubation for 2.5 h, it was suggested that the large, highly charged DNA nanostructures would protect the enzyme against denaturation. In particular, the adsorption of scaffolded enzymes on the surface of the reaction vessels would be reduced. Moreover, DNA structures have been reported to protect the encapsulated protein from the hydrolysis catalyzed protease [70].

3.4. Substrate Affinity to the DNA Scaffold

DNA scaffolds with the high-density negative charge have been proposed to attract molecules through electrostatic interactions. Lin et al. [71] assembled horseradish peroxidase (HRP) on a triangular DNA scaffold to study the substrate–scaffold interactions by using various substrates. *p*-Aminophenol (AP) with a positive charge was first used as a substrate for the oxidation reaction catalyzed by HRP. An over 250% increase in the activity of scaffolded HRP over the free enzyme was observed with AP. This enhancement was decreased to 153 ± 21, 131 ± 11 and 158 ± 24% in the presence of 50, 150 and 300 mM NaCl, respectively, suggesting that the electrostatic interaction between the positively charged AP and the negatively charged DNA scaffold partly contributed to the catalytic enhancement. In addition, the reactions of scaffolded HRP with phenol (P), *p*-hydroxybenzoic acid (HBA), *o*-phenylenediamine (OPD) and 3,3′,5,5′-tetramethylbenzidine (TMB) showed the kinetic enhancement of 244 ± 6%, 197 ± 6%, 130 ± 16% and 138 ± 12%, respectively. The reduced K_m values of scaffolded HRP for these substrates supported the increased affinity to the substrates. The activity of the scaffolded HRP with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was lower than the free HRP due to the negative charge of ABTS. Interestingly, the plot of the kinetic enhancement of scaffolded HRP against the binding energy of substrate to HRP suggested that the DNA scaffold enhanced HRP activity following the Sabatier Principle, a “just and right” manner. In a recent study, Kosinski et al. [72] encaged thrombin, a model of allosterically regulated serine proteases, into the cavities of DNA scaffolds with distinct structural and electrostatic characteristics. The hydrolysis reactions with peptide substrates carrying different charge indicated that the reaction rates were affected by DNA/substrate electrostatic interactions. These examples indicate the effect of DNA–substrate interactions on the enzymatic reactions.

3.5. Ordered Hydration Layer on the DNA Scaffold Surface

Zhao et al. [73] have observed 3- to 10-fold activity enhancements of five different enzymes, GOx, HRP, glucose-6-phosphate dehydrogenase (G6pDH), malic dehydrogenase (MDH) and lactic dehydrogenase (LDH), individually encapsulated in DNA cages. It was hypothesized that enzymes were stabilized by the highly ordered, hydrogen-bonded water environment formed by the negatively charged DNA cage surface, in which the stabilization of the hydrophobic interactions of a folded protein was induced by the solvent entropy penalty upon protein unfolding. The activity of encapsulated G6pDH reduced to approx. 25% activity in the presence of 1 M NaCl. This was suggested that Na^+ would shield the negative charge on the DNA surface and disrupt the surface-bound hydration layer. However, the high concentration of salts containing the cations such as Na^+, K^+ and NH_4^+ also strongly inhibited the activity of free G6pDH. The DNA hydration layer may play an important role in the modulation of enzyme reactions on the DNA scaffold, but the stabilization of proteins may not be the general mechanism for enhancing the activity of the DNA-scaffolded enzymes.

3.6. Local pH Environment

Besides the above factors, local pH environment near the enzyme was proposed as a critical factor to improve the enzyme activity on DNA scaffold. Zhang et al. [74] suggested that activity enhancement of enzymes (GOx or HRP) located on DNA scaffolds derived from the lower pH on the negatively charged DNA scaffold surface compared with the bulk solution. Such lower local pH would provide more optimal pH for GOx or HRP. In another study, Xiong et al. [75] positioned GOx at different locations of a 3D octahedral DNA scaffold, providing different polyanionic environments for enzymes. By using the electrical sensing based on a bipolar junction transistor, the proton generations by enzyme at different locations were measured. The activity enhancement of DNA scaffold-tethered GOx was observed over the free GOx in the solution. In particular, GOx positioned at one vertex of the DNA octahedral exhibited a faster oxidation rate than when embedded between the bundles of the scaffold edge. This was explained by the different local pH environments induced by the DNA scaffold due to the exposure of GOx to different amounts of negatively charged DNA scaffold. The local pH change may be one of the reasons resulting in the catalytic enhancement, but the enhancement effect should be limited to particular enzymes, such as GOx or HRP that exhibits similar pH dependence with an increased maximal turnover rate under more acidic conditions.

3.7. General Factors for the Catalytic Enhancement of DNA-Scaffolded Enzymes

As reported previously [76], two enzymes with different pH preferences, xylose reductase (XR) and xylitol dehydrogenase (XDH), were individually assembled on the fully open state of a 3D DNA scaffold through the modular adaptor method [77][78][79][80][81][82] in high loading yields. XR was genetically fused to the modular adaptor ZF-SNAP to obtain ZS-XR. The zif268 bound with the specific DNA sequence, while SNAP-tag would react with benzylguanine incorporated in the DNA sequence to form the covalent linkage [78]. Similarly, XDH was fused to the C-terminal of modular adaptor Halo-GCN4 to construct enzyme HG-XDH. The Halo-tag substrate 5-chlorohexane (CH) was incorporated in the GCN4-binding DNA sequence. The catalytic enhancements were observed for both the DNA-scaffolded ZS-XR (sXR) and scaffolded HG-XDH (sXDH) over the respective free enzyme. In the enzyme reactions, XR converted xylose with the cofactor NADH to xylitol, while XDH produced xylulose from xylitol using NAD^+. Such neutral or net negative charge of their substrates and cofactors indicated that the surface–substrate or

–cofactor electrostatic attractive interaction could not account for the increase in activities of assembled enzymes. Instead, the large scaffold with high packing density of DNA helices improved the enzyme stability and reduced the adsorption of scaffolded enzymes to the reaction vessels, which could partly contribute to the catalytic enhancement of DNA-scaffolded XR or XDH. To assess the local pH environment of DNA scaffold, a dual-emission ratiometric pH indicator SNARF derivative was loaded on the DNA scaffold either facing near the surface or locating 6.7 nm away from the surface, which corresponded to the distance between the enzyme and the surface of the DNA scaffold. The local pH near the surface of the DNA scaffold or near the enzyme loaded position in the reaction buffer (pH 7.0) was deduced to be 6.2 or 6.5. Such local pH shifts would result in 25% enhancement of the catalytic activity for sXR and 30% reduction for sXDH since XR and XDH displayed the optimal pH at 6.0 and 8.0, respectively. Therefore, the postulated modulation of enzyme activity by the lower pH shift near the DNA scaffold surface unlikely explained the catalytic enhancements of both scaffolded enzymes [76].

This evaluated the DNA scaffolding effect on enzyme reactions from various aspects. The protection of the enzyme against adsorption to the reaction vessels by a DNA scaffold to benefit the enzyme reactions would be one of the general factors for the catalytic enhancement. However, this factor alone could not account for the large enhancement. The question that remains unanswered is, what is the critical character of the DNA scaffold for accelerating the enzyme reaction? The high-density charge of the DNA scaffold surface exerts the formation of an ordered hydration layer. Such a local microenvironment has been suggested to affect the catalytic reactions [73]; however, the chemical mechanisms remain to be elucidated.

3.8. Packed State of Enzymes on the DNA Scaffold

In cells, enzyme reactions were performed in highly packed conditions. To mimic this “cellular crowed environment” in vitro still remains challenging. Huyen et al. [83] applied the modular adaptor method to assemble the monomeric carbonic anhydrase (CA) on a DNA scaffold in the packed state or dispersed states. CA was genetically fused to the modular adaptor ZF-SNAP to obtain ZS-CA. In the packed state, the interenzyme distance between CA regions was less than 1 nm. The reactions of ZS-CA assembled on the DNA scaffold were performed with the substrate *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl butyrate (*p*-NPB) or *p*-nitrophenyl valerate (*p*-NPV). Interestingly, the enzymatic reactions proceeded faster in the packed than in the dispersed state under same enzyme and substrate concentrations. Acceleration of the reactions in the packed assembly was more predominant for substrates with higher water-excluded volumes (higher hydrophobicity), in which the reactions were accelerated by 1.3-fold, 1.5-fold or 1.6-fold in the packed state over the dispersed state with *p*-NPA, *p*-NPB or *p*-NPV as the substrate. The entropic force of water increasing the local substrate concentration within the domain confined between enzyme surfaces was attributed to the acceleration of enzyme reactions in the packed assembly. The acceleration of the enzyme reaction in the packed state over the dispersed state was also observed for xylose reductase assembled on the same type of DNA scaffold. This system provides a reasonable molecular model of enzymes in a packed state inside the cell, such as the condensate in the liquid–liquid phase separation.

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