DOI: 10.1002/anie.201202441

Biocatalysis

Facilitated Substrate Channeling in a Self-Assembled Trifunctional Enzyme Complex**

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Most cascade enzymes in metabolic pathways are spatially held together by noncovalent protein–protein interactions.[1] The formation of a cascade enzyme complex often allows the product of one enzyme to be transferred to an adjacent enzyme where it acts as the substrate, thereby resulting in an enhanced reaction rate, because reaching equilibrium in the cytoplasm is not required; this mechanism is called substrate channeling.[1,2] In nature, most intracellular enzyme complexes are dynamic so that they may be dissociated or associated, thereby resulting in forestallment of substrate competition among different pathways, regulation of metabolic fluxes, mitigation of metabolite inhibition, and circumvention of unfavorable equilibrium and kinetics.[3] The simplest way to facilitate substrate channeling between cascade enzymes is the construction of fusion proteins,[4] but substrate channeling in fusion proteins might not take place.[5]

The assembly of numerous enzymes and/or co-enzymes in vitro is called cascade enzyme biocatalysis and has been proposed for the implementation of complicated bioconversion that microbes and chemical catalysts cannot do,[6] such as hydrogen production from cellulosic materials and water with high yield.[7] Inspired by natural enzyme complexes (e.g., metabolons, which are complexes of sequential enzymes of a metabolic pathway), the construction of static rather than dynamic enzyme complexes could be an important approach to accelerating reaction rates among cascade enzymes and to avoiding the regulation of enzyme–enzyme interactions. For example, Wilner et al.[8] linked glucose oxidase and horseradish peroxidase by DNA scaffolds of different lengths, resulting in reaction rates that were enhanced by 20–30-fold. However, DNA scaffolds may be too costly for scale-up as compared to protein scaffolds. Minteer and co-workers[9] demonstrated that chemical cross-linking of proteins within the mitochondria of *Saccharomyces cerevisiae* resulted in significant increases of the power output in enzymatic fuel cells. But chemical covalent linking often impairs enzyme activity so that it may not be applied to most intracellular enzymes.

Herein we demonstrate a general approach for constructing a static self-assembled enzyme complex by using the high-affinity interaction between cohesin and dockerin modules, which occur in natural extracellular complexed cellulase systems, called cellulosomes. Cohesin domains are part of the natural scaffoldin protein of the cellulosome, which is crucial to the construction of the cellulase complex by binding to enzymes carrying dockerin domains. Bayer et al.[10] proposed to construct designed enzyme complexes by utilizing species-specific dockerins and cohesins, which can bind tightly in these complexes at a molar ratio of 1:1. Later, several synthetic mini-cellulosomes containing various extracellular glycoside hydrolases were constructed.[11] However, no one attempted to construct an enzyme complex containing cascade enzymes from a metabolic pathway by using dockerins and cohesins and investigated its potential applications in cascade enzyme biocatalysis.

Triosephosphate isomerase (TIM, EC 5.3.1.1), aldolase (ALD, EC 4.1.2.13), and fructose 1,6-bisphosphatase (FBP, EC3.1.3.11) are cascade enzymes in the glycolysis and gluconeogenesis pathways. TIM catalyzes the reversible conversion of glycer-aldehyde-3-phosphate (G3P) to dihydroxy-acetone phosphate (DHAP). ALD catalyzes the reversible aldol condensation of G3P and DHAP to fructose-1,6-bisphosphate (F16P). FBP catalyzes the irreversible conversion of F16P to fructose-6-phosphate (F6P; Scheme 1). Previous studies reported that substrate channeling existed in dynamic metabolons of enzymes such as TIM, ALD, or FBP.[12] Three dockerin-free proteins: *Thermus thermophilus* HB27 TIM (TTC0581) as well as the *Thermotoga maritima* ALD (TM0273) and FBP (TM1415) were expressed in *E. coli* and purified to homogeneity by using nickel–nitrilotriacetate (Ni–NTA) resin[13] or a self-cleaving intein.[14] However, a mixture of these three enzymes did not form a putative enzyme complex, as examined by affinity electrophoresis (data not shown).

The synthetic static three-enzyme complex was assembled in vitro through a synthetic trifunctional scaffoldin containing a family 3 cellulose-binding module (CBM3) at the N terminus followed by three different types of cohesins from the *Clostridium thermocellum* ATCC 27405 CipA,[15] *Clostridium cellulovorans* ATCC 35296 CbpA,[16] and *Ruminococcus flavefaciens* ScaB[17] (cohesins CTCoh, CCCoh, and RFCoh,

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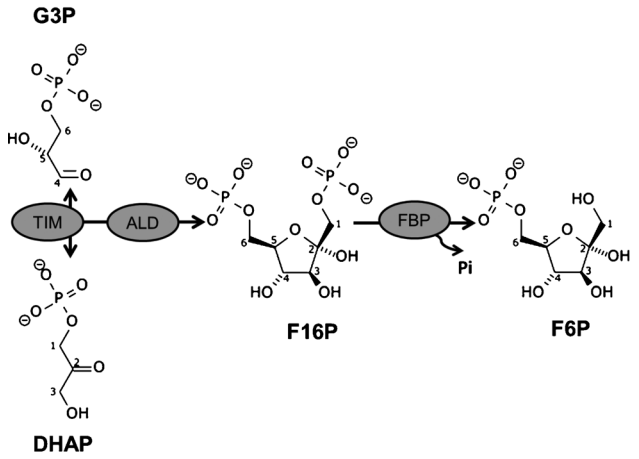
[**] This work was supported partially by the DOE BioEnergy Science Center (BESC) and DOE ARPA-E Petro project. This work was also partially supported by the College of Agriculture and Life Sciences Bioprocessing and Biodesign Research Center at Virginia Tech.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201202441.



Scheme 1. The cascade reactions catalyzed by the enzymes TIM, ALD, and FBP.

respectively; Figure 1). The DNA sequence encoding this mini-scaffoldin was synthesized by Genescript (Piscataway, NJ). The dockerin-containing enzymes TIM, ALD, and FBP were constructed by the addition of one dockerin from the *C.*

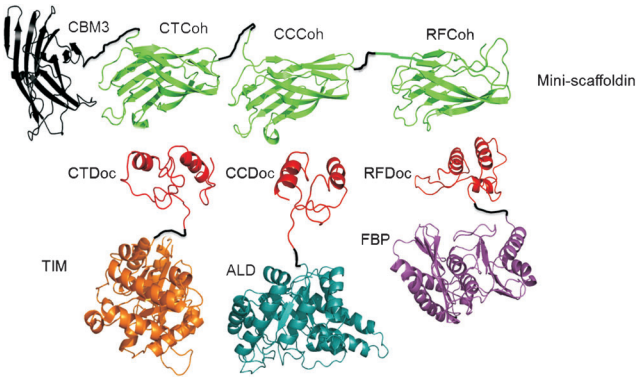


Figure 1. Schematic representation of the self-assembled three-enzyme complex containing TIM-CTDoc, ALD-CCDoc, FBP-RFDoc, and a mini-scaffoldin containing three different types of cohesins (i.e., CTCoh, CCCoh, and RFCoh) and one family 3 carbohydrate-binding module.

thermocellum CelS (GenBank Accession number: L06942), *C. cellulovorans* EngE (GenBank Accession number: AAD39739.1), and *R. flavefaciens* ScaA (GenBank Accession number: CAC34384.3) at their C termini, respectively. All the genes were subcloned into plasmid pET20b (Novagen, Madison, WI) by the simple-cloning method,[18] yielding plasmids: pET20b-mini-scaf, pET20b-tim-ctdoc, pET20b-ald-ccdoc, and pET20b-fbp-rfdoc. The details in cloning, primers, and amino acid sequences of four recombinant proteins are listed in the Supporting Information.

Four soluble recombinant proteins (mini-scaffoldin, TIM-CTDoc, ALD-CCDoc, and FBP-RFDoc) were produced by four *E. coli* BL21(DE3) strains harboring the respective expression plasmids. After induction with isopropyl-β-D-thiogalactopyranoside followed by ultrasonication and centrifugation, the SDS-PAGE gel of each cell extract contained a major band representing the desired soluble recombinant protein (Figure S1 a in the Supporting Information, Lanes 1–4). When the cell extract containing mini-scaffoldin was mixed with one cell extract containing dockerin-carrying enzymes TIM-CTDoc, ALD-CCDoc, or FBP-RFDoc,[19] the high-affinity interaction between the cohesin and dockerin resulted in the formation of the enzyme complexes consisting of one mini-sacffoldin and one dockerin-containing enzyme. Because mini-scaffoldin contained a CBM3 domain, the scaffoldin-containing enzyme complexes were adsorbed on the surface of regenerated amorphous cellulose (RAC). The adsorbed enzyme complexes that were eluted by using ethylene glycol exhibited two bands in the SDS-PAGE gel (Figure S1 a in the Supporting Information, Lanes 6–8), thus suggesting that enzyme complexes contained one mini-scaffoldin and one dockerin-containing enzyme. The activities of dockerin-containing enzymes TIM-CTDoc, ALD-CCDoc, or FBP-RFDoc in the presence of mini-scaffoldin were similar with those of dockerin-free enzymes TIM, ALD, and FBP (data not shown), thus suggesting that the dockerin addition did not influence the activity of each enzyme.[20]

When the cell extracts of four cell cultures were mixed together in the presence of RAC, the three dockerin-containing enzymes bound to the mini-scaffoldin simultaneously, thereby forming the trifunctional enzyme complex. This enzyme complex eluted from RAC by using ethylene glycol exhibited four bands in the SDS-PAGE gel (Figure S1 a in the Supporting Information, Lane 9). The binding ratio between cohesin and dockerin together with the band intensity in the SDS-PAGE gel (Figure S1 a, Lane 9) suggested that one mini-scaffoldin can bind one TIM, one ALD, and one FBP (Figure 1). The formation of the static three-enzyme complex was further validated in native gel (Figure S1 b in the Supporting Information).

The formation profiles of F6P from G3P mediated by the enzyme complex (2 μM) and a noncomplexed dockerin-free three-enzyme mixture (2 μM each enzyme) were examined at 60 °C (Figure 2). The enzymatic activity was measured in a HEPES buffer (200 mM, pH 7.5; HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid) containing $MgCl_2$ (10 mM), $MnCl_2$ (0.5 mM), $CaCl_2$ (1 mM) and G3P concentrations of 0.5, 1.0, 2.5, or 5.0 mM. An aliquot (65 μL) of the reaction sample was withdrawn and then mixed with perchloric acid (35 μL of 1.88 M) for stopping the reaction. The pH value of the reaction solution was adjusted to neutral with KOH (13 μL of 5 M). After centrifugation, the F6P concentration in the supernatant was measured by using a glucose hexokinase/glucose-6-phosphate dehydrogenase assay kit (Pointe Scientific, Canton, MI, USA) supplemented with phosphoglucose isomerase.[14]

At a concentration of 2.5 mM of G3P, the initial F6P generation rate of the reaction mediated by the synthetic three-enzyme complex was approximately 1 $\mu M s^{-1}$, which is approximately 13.4-fold higher than that of the noncomplexed mixture (Figure 2 a). The acceleration factor was defined as the ratio of the initial reaction rate of the synthetic enzyme complex to that of the noncomplexed enzyme mixture at the same enzyme concentration. When the G3P concentration decreased from 5.0 to 0.5 mM, the acceleration

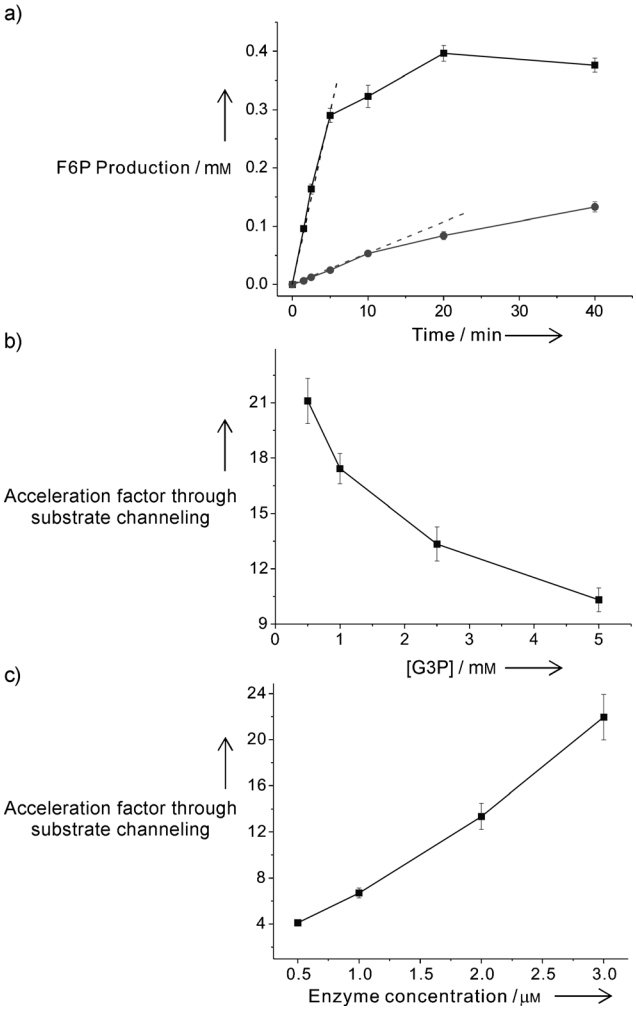


Figure 2. a) Profiles of F6P production catalyzed by 2 μM synthetic enzyme complex (■) and 2 μM noncomplexed enzyme mixture (●) in 2.5 mM glycerealdehyde-3-phosphate at 60 °C, where the slope of the dashed lines was defined as the initial reaction rate. b, c) Acceleration factors through substrate channeling in terms of substrate concentration (b), and enzyme concentration (c). Values shown are means of triplicate determinations.

factor increased from 10.3 to 21.1 (Figure 2b), thus suggesting the substrate channeling was more significant when substrate levels were lower. The kinetic parameters (Table 1) for the three-enzyme complex and the noncomplexed three-enzyme mixture can be determined based on the Michaelis–Menten equation (Figure S2a in the Supporting Information). The k_{cat} value of the enzyme complex was 36.3 min^{-1}, 9.3 times of that of the enzyme mixture. Also, the K_m value of the enzyme complex was approximately a quarter of that of the enzyme mixture. As a result, the catalytic efficiency (k_{cat}/K_m) of the three-enzyme complex was about 33-fold of that of the three enzyme mixture. It was noted that the apparent k_{cat} and K_m values of the free enzyme mixture (Table 1) were valid only for 2 μM of the enzyme mixture because of the nonlinear dependence of the initial rate on the enzyme concentration (Figure S3 in the Supporting Information).

Table 1: Apparent kinetic parameters for the three-enzyme complex and three-enzyme mixture at 60 °C based on the G3P concentration.

Name	K_m [mM]	k_{cat} [min^{-1}]	k_{cat}/K_m [$mM^{-1}min^{-1}$]
Enzyme complex	0.46 ± 0.12	36.3 ± 2.9	79.7
Enzyme mixture	1.63 ± 0.28	3.90 ± 0.29	2.39

When the enzyme concentration increased from 0.5 to 3 μM, the acceleration factor increased from 4.1 to 22.0 (Figure 2c). This trend was completely different from a previous report pertaining to substrate channeling,[15] which was due to a different mechanism. The enzyme complex can be regarded as a whole so that the overall apparent activity of the enzyme complex was linearly related to its concentration (Figure S2b in the Supporting Information). On the contrary, the overall apparent reaction rate of the noncomplexed enzyme mixture increased slowly and even decreased when the enzyme concentration increased (Figure S2b in the Supporting Information); this increase was attributed to two facts: 1) the specific activity of TIM was approximately 3000-fold of that of ALD so that ALD was the rate-limiting enzyme, and 2) the equilibrium ratio of DHAP to G3P was approximately 20.[21] At very high TIM loading, G3P was quickly converted to DHAP by TIM so that the DHAP concentration was much higher than the G3P concentration, thus resulting in low apparent reaction rates of ALD, which cannot access enough G3P in the enzyme mixture (Figure S3 in the Supporting Information). As a result, the acceleration factor for the enzyme complex to the enzyme mixture increased when the total enzyme concentration increased.

The F6P generation rates by the enzyme complex decreased significantly after five minutes and then leveled off after 20 min (Figure 2a). At that time, the G3P conversion efficiency was 32%. The slow reaction rate after five minutes was not attributed to the denaturation of the enzyme complex, because the enzyme complex and free enzyme mixture were stable at 60 °C (Figure S4a in the Supporting Information). The decrease in reaction rate after five minutes was due to product inhibition, as supported by the fed-batch experiment. When another aliquot of 2.5 mM G3P was added after 20 min (Figure S4b in the Supporting Information), the reaction restarted and its initial reaction rate was approximately a third of the initial rate when no product was present at the beginning.

The enzymes TIM, ALD, and FBP could exist in multiple forms from monomer, dimer, even to tetramer. It is known that only dimeric TIM is active;[22] all monomeric, dimeric, and tetrameric ALDs are active;[23] and both dimeric and tetrameric FBPs are active.[24] The fact that the addition of dockerins to the C terminus of these three enzymes did not influence their activities implies that the three-enzyme complex possibly forms a dimer with TIM, ALD, and FBP facing each other, as hypothesized previously.[25] The formation of this dimeric three-enzyme complex was partially supported by the result that the particle size of the synthetic enzyme complex was increased by approximately five-fold relative to the single mini-scaffoldin estimated by their zeta potentials with the Malvern Zetasizer Nano ZS system (Worcestershire, UK; data not shown).

The construction of synthetic static enzyme complexes by using small-size polypeptide tags, cohesins and dockerins from cellulosomes, would be a powerful tool for cascade enzyme biocatalysis, because it not only can result in increased reaction rates among cascade enzyme complexes through substrate channeling without requiring the use of large amounts of enzymes,[26] but also may prevent the degradation of labile metabolites as occurring in nature.[3,14] The effects of numerous factors, such as the number of cohesin proteins and their order in a scaffoldin, linker length, number of enzymes, and enzyme orientation, on the kinetic performance of synthetic enzyme complexes and substrate channeling are interesting subjects of further investigations pertaining to the structural orientation among cascade enzymes and metabolite transfer among them.

Received: March 29, 2012
Revised: June 27, 2012
Published online: July 23, 2012

Keywords: biocatalysis · enzymes · enzyme cascades · metabolon · self-assembly

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