

Scaffoldless engineered enzyme assembly for enhanced methanol utilization

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Edited by Arnold L. Demain, Drew University, Madison, NJ, and approved September 27, 2016 (received for review February 4, 2016)

Methanol is an important feedstock derived from natural gas and can be chemically converted into commodity and specialty chemicals at high pressure and temperature. Although biological conversion of methanol can proceed at ambient conditions, there is a dearth of engineered microorganisms that use methanol to produce metabolites. In nature, methanol dehydrogenase (Mdh), which converts methanol to formaldehyde, highly favors the reverse reaction. Thus, efficient coupling with the irreversible sequestration of formaldehyde by 3-hexulose-6-phosphate synthase (Hps) and 6-phospho-3-hexuloseisomerase (Phi) serves as the key driving force to pull the pathway equilibrium toward central metabolism. An emerging strategy to promote efficient substrate channeling is to spatially organize pathway enzymes in an engineered assembly to provide kinetic driving forces that promote carbon flux in a desirable direction. Here, we report a scaffoldless, self-assembly strategy to organize Mdh, Hps, and Phi into an engineered supramolecular enzyme complex using an SH3–ligand interaction pair, which enhances methanol conversion to fructose-6-phosphate (F6P). To increase methanol consumption, an “NADH Sink” was created using *Escherichia coli* lactate dehydrogenase as an NADH scavenger, thereby preventing reversible formaldehyde reduction. Combination of the two strategies improved *in vitro* F6P production by 97-fold compared with unassembled enzymes. The beneficial effect of supramolecular enzyme assembly was also realized *in vivo* as the engineered enzyme assembly improved whole-cell methanol consumption rate by ninefold. This approach will ultimately allow direct coupling of enhanced F6P synthesis with other metabolic engineering strategies for the production of many desired metabolites from methanol.

methane | methylotrophs | supramolecular | scaffold | substrate channeling

Fossil fuels are recognized as a limited energy source given the world's growing population and energy demands (1–3). Bio-fuels and bio-based chemicals have gained tremendous traction over the past decade as a means to produce both an alternative energy source as well as to replace bulk chemical production that is heavily reliant upon petrochemicals. Methanol, which can be produced inexpensively from natural gas or renewably through reduction of carbon dioxide by hydrogen (2, 4), can serve as a feedstock to produce biofuels, amino acids, and polymers (5, 6). Biological conversion of methanol to value-added chemicals may provide more favorable, economic operating conditions (i.e., lower temperature and pressure) than more traditional catalytic conversions.

Methylotrophic bacteria consist of a wide array of organisms that can use one-carbon (C1) compounds, and notably methane and methanol, as their sole source of carbon and energy (7). The ability to use methanol is initiated by its oxidation to formaldehyde by methanol dehydrogenase (Mdh) encoded by an *mdh* gene (8). There are two classes of Mdhs among methylotrophic bacteria: the well-studied pyrroloquinoline quinone (PQQ)-containing Mdhs, which are found in the most ubiquitous, Gram-negative, strictly aerobic methylotrophs, and the NAD(P)⁺-dependent, cytoplasmic Mdhs commonly found in thermophilic Gram-positive bacteria (9). Generation of most useful metabolites produced by industrial

organisms requires electrons in the form of NADH and culture conditions largely microaerobic or anaerobic (7, 10). Thus, organisms that can grow anaerobically or microaerobically and NAD-dependent Mdhs are essential for effective conversion of methanol to desirable metabolites (7). Although NAD(P)⁺-dependent Mdhs are the best candidates for engineering synthetic methylotrophs like *Escherichia coli*, these enzymes have poor predicted thermodynamic properties and are thus dependent on maintaining low intracellular formaldehyde and NAD(P)H concentrations to ensure the reaction continues to favor methanol oxidation (7). Formaldehyde, produced from the oxidation of methanol, is subsequently assimilated via distinct methylotrophic pathways, among which the ribulose monophosphate (RuMP) pathway is bio-energetically the most efficient (7). The best characterized NAD(P)⁺-dependent Mdhs were isolated from the thermotolerant *Bacillus methanolicus* MGA3 strain, which harbors two chromosomal *mdh* genes and one plasmid-borne *mdh* gene (6, 9). In particular, MGA3 Mdh3, a chromosomal *mdh*, is a decameric enzyme and has been shown to possess broad substrate specificity when tested with a range of primary alcohols (9, 11, 12).

In the widespread methylotrophic RuMP pathway for formaldehyde assimilation (Fig. 1A) (7, 13), D-arabino-3-hexulose 6-phosphate (6HP) is first produced by a condensation reaction between formaldehyde and ribulose 5-phosphate (Ru5P) by 3-hexulose-6-phosphate synthase (Hps) (6, 13, 14). This is followed by the isomerization of hexulose 6-phosphate (H6P) by 6-phospho-3-hexuloseisomerase (Phi) (6, 13, 14) into fructose 6-phosphate (F6P), which can either enter central metabolism

Significance

Methane, the major natural-gas component, can be converted industrially to syngas (a mixture of CO₂, CO, and H₂), which is used to produce methanol, an important industrial precursor for producing commodity and specialty chemicals. Unlike methanol's chemical conversion, which requires high temperature and pressure, its biological conversion, proceeding through its oxidation to formaldehyde, takes place at ambient conditions but suffers from relative low rates. As metabolite biosynthesis by industrial organisms utilizes NADH as an electron donor, methanol oxidation must be carried out by NAD-dependent methanol dehydrogenases, which have poor thermodynamic characteristics at mesophilic conditions. To solve this problem, we developed an engineering strategy for supramolecular enzyme assembly to dramatically enhance the carbon flux from methanol to the key intermediate fructose-6-phosphate.

Author contributions: J.V.P., E.P., and W.C. designed research; J.V.P., L.C., and W.B.W. performed research; J.V.P. contributed new reagents/analytic tools; J.V.P., W.B.W., E.P., and W.C. analyzed data; and J.V.P., W.B.W., E.P., and W.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1601797113/-DCSupplemental.

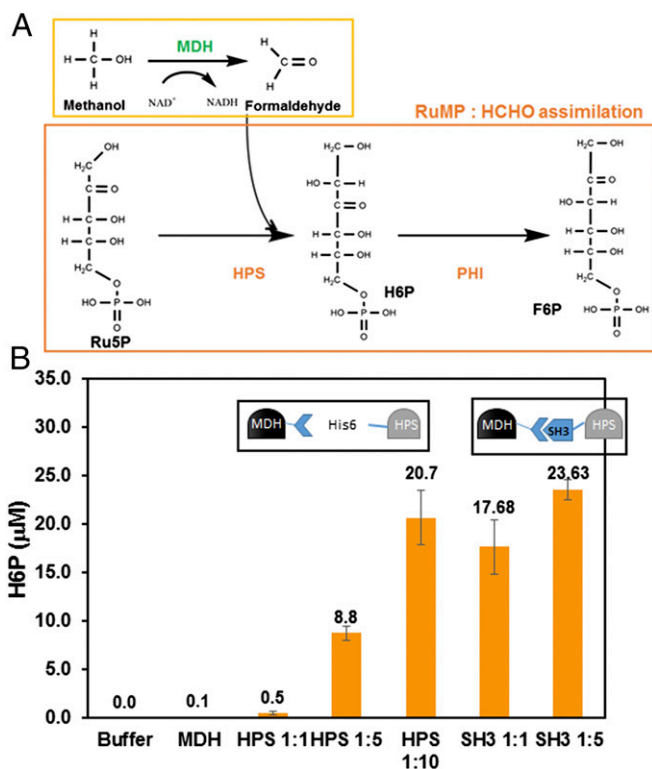


Fig. 1. (A) A schematic of the Mdh–RuMP pathway for methanol assimilation. (B) H6P formation with increasing ratios of Mdh3–sSH3lig:Hps or Mdh3–sSH3lig–SH3–Hps enzyme complexes. Purified Mdh–sSH3lig was mixed with equal molar ratios of purified Hps ranging from 1:1–1:10. H6P was assayed according to the previously established method by Arfman (39). Error bars represent the SD of at least three replicate experiments.

for biosynthesis (14) or be converted back to Ru5P to allow the pathway to cycle through (6, 14).

Recent work has demonstrated the ability to transfer methylotrophic genes to other organisms to enable nonnative methanol oxidation (15–17). Methanol utilization and ^{13}C -methanol-derived labeling of intracellular metabolites were first demonstrated in *Corynebacterium glutamicum* by expressing the *mdh* gene from *B. methanolicus* and the *hps* and *phi* genes from *Bacillus subtilis* (16, 17). A similar approach was also demonstrated in *E. coli*, where Mdh2 from *B. methanolicus* PB1 was coexpressed with Hps and Phi from *B. methanolicus* MGA3 to increase the production of ^{13}C -labeled intracellular metabolites compared with the control strain expressing Mdh3 alone (15). Although these reports provide evidence that the RuMP pathway can be transferred to nonnative methylotrophs, the rate of methanol consumption is significantly lower than those reported for native methylotrophs. Detailed kinetics analyses revealed that the reverse formaldehyde reductase activity for Mdh is at least 1,000-fold higher than methanol oxidation (9), and the irreversible sequestration of formaldehyde by Hps and Phi serves as a driving force to pull the pathway equilibrium toward the desired direction. In *B. methanolicus* MGA3, expression of both *hps* and *phi* is more highly up-regulated than *mdh* upon methanol addition, and carefully coordinated expression of *mdh* and the RuMP pathway genes is required for efficient methanol utilization (18). In the same organism, overexpression of *hps* and *phi* results in a higher growth rate on methanol, again indicating the importance of direct coupling between the reversible methanol oxidation and the irreversible formaldehyde assimilation (18).

Multienzyme supramolecular complexes, which self-assemble into spatially defined architectures, have been shown to be a

promising approach to improve the efficiency of cascade reactions (19–22). This spatial organization provides more stable enzyme structures, facilitates substrate channeling between active sites, and prevents the buildup of toxic intermediates (23–25). The use of driving forces such as ATP generation or NADH operation as a kinetic control element to enable directional operation of highly reversible enzyme reactions has also been reported (26, 27). We hypothesize that engineered supramolecular enzyme assemblies can be similarly created as a kinetic trap to enable fast and efficient methanol utilization. Unlike other reports based on the use of a scaffold (24, 28), which tend to produce large and disordered multienzyme complexes, we take advantage of the decameric nature of Mdh3 from *B. methanolicus* to construct a scaffoldless supramolecular enzyme complex composed of Mdh3 and a previously validated Hps–Phi fusion from *Mycobacterium gastri* to enhance methanol conversion to F6P. Our ability to enhance F6P synthesis is ideally suited for the recently proposed methanol condensation cycle (29), which combines the enzymes from the RuMP pathway and the nonoxidative glycolysis pathway for the in vitro synthesis of platform chemicals from methanol.

Results

High Ratio of Hps to Mdh Enhances in Vitro Methanol Utilization.

B. methanolicus MGA3 Mdh3 has a reported k_{cat}/K_m value roughly 1,000-fold lower for methanol oxidation than for formaldehyde reduction (9), indicating that a significant kinetic barrier must be overcome to achieve efficient methanol utilization. In methylotrophs, the irreversible conversion of formaldehyde by Hps and Phi is the driving force behind shifting the pathway equilibrium toward F6P formation. In *B. methanolicus*, it has been suggested that the level of Hps/Phi can impact methanol consumption and cell growth (18), and the stoichiometric effect of Mdh to Hps:Phi on the methanol consumption pathway remains unexplored either in native or synthetic methylotrophs. To investigate this issue, purified Mdh3 was mixed with different ratios of a highly active Hps from *M. gastri* (30) to create a kinetic trap to consume formaldehyde and increase H6P production. As expected, no H6P was detected in the absence of Hps. Even when Hps and the Mdh3 were mixed at a 1:1 molar ratio, only a small amount of H6P was detected over the background (Fig. 1B). This result is consistent with the roughly fourfold faster formaldehyde consumption rate by Mdh3 compared with Hps (Fig. S1). Increasing the molar ratio of Mdh3:Hps from 1:1 to 1:5 resulted in a significant increase in H6P production (Fig. 1B), supporting the notion that formaldehyde must be quickly and irreversibly removed to shift the pathway equilibrium. Although a higher level of H6P was achieved by further increasing the Mdh3:Hps ratio to 10, such a high ratio is difficult to maintain in vivo without resulting in a heavy cellular burden, especially if the same high ratio is needed for all subsequent enzymes in the pathway. One way to bypass this limitation is to directly couple these enzymes into a supramolecular enzyme complex to facilitate the rapid transfer of intermediates toward F6P formation.

Engineered Supramolecular Enzyme Complexes Enhance H6P and F6P Production.

Protein scaffolding is one of the most successful strategies used to create supramolecular enzyme complexes (24, 31). However, enzyme cascades involving multimeric enzymes are often difficult to assemble, as they tend to result in large and disordered structures because of the random cross-linking between enzymes and scaffolds (31). An alternative approach is to exploit the ability of multimeric proteins to self-assemble into defined nanostructures by engineering appropriate interaction domains (32). Here, we took advantage of the decameric nature of Mdh3 as a natural base for assembling Hps and Phi into a functional multienzyme cascade (Fig. 24). A well-known interaction pair, SH3 (Src homology 3 domain from the adaptor protein CRK), and its

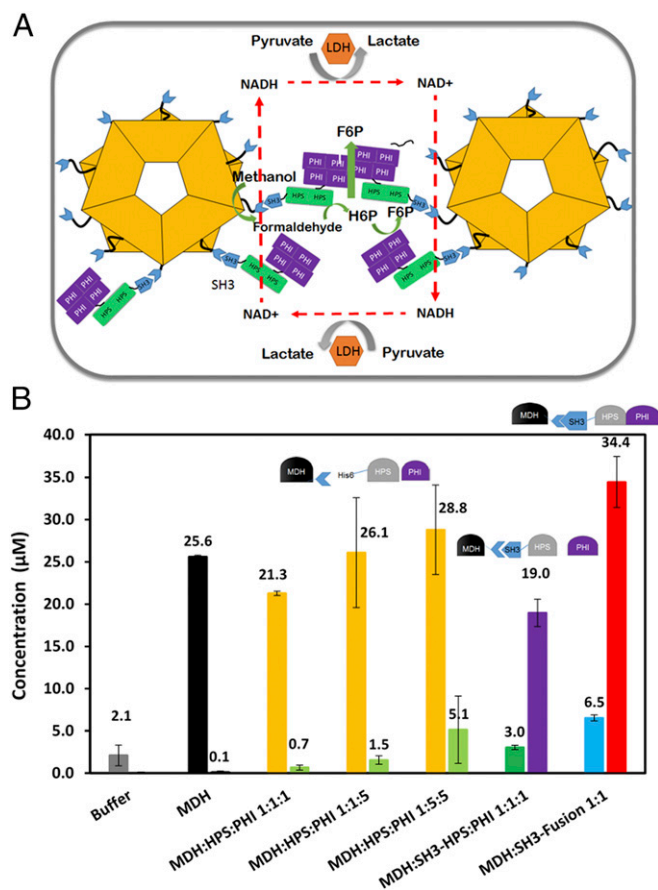


Fig. 2. (A) A schematic of the Mdh3-Hps-Phi supramolecular enzyme complex and the corresponding cascade reactions. Ldh was used as an NADH Sink to further minimize formaldehyde reduction. (B) Formaldehyde (first bar) and F6P (second bar) formation with and without SH3-tethered enzymes. Purified Mdh3-sSH3lig was mixed with either Hps and Phi, SH3-Hps and Phi, or the SH3-Hps-Phi fusion. Formaldehyde (HCHO) was assayed using the Nash reagent. Error bars represent the SD of at least three replicate experiments.

ligand (sSH3lig, PPPALPPKRRR), which have a reported K_d value of 0.1 μM (24), were used for protein assembly. The Mdh3-sSH3lig, SH3-Hps, and SH3-Hps-Phi fusions were solubly expressed in *E. coli* (Fig. S2). The functionality of both interaction domains was first confirmed by the coprecipitation of the fusion proteins with their respective elastin-like polypeptide (ELP) binding partners using the thermally induced phase transition property of ELP (33) (Fig. S2).

We next investigated the ability of the assembled Mdh3-Hps complex to drive H6P production. Even a 1:1 ratio of the two proteins resulted in higher levels of H6P production (Fig. 1B), which was roughly 35-fold higher than using nonassembled Mdh3 and Hps. More importantly, the level of H6P achieved was already twice the level achieved using a 1:5 ratio of Mdh3 and Hps. The use of a 1:5 ratio of Mdh3-sSH3lig to SH3-Hps improved H6P production further, although the impact was less significant than the 1:1 ratio (Fig. 1B). These results confirm the importance of enzyme assembly and substrate channeling in driving the conversion of methanol to H6P.

To investigate whether similar success could be achieved toward F6P production, Phi was added to the enzyme cascade. Consistent with the results with H6P, a barely detectable amount of F6P was produced using only a mixture of unassembled Mdh3, Hps, and Phi (Fig. 2B). Increasing the amount of Phi had virtually no impact on F6P production, whereas a higher amount of

Hps to the other enzymes elevated the F6P level by over sevenfold. This result again highlights the importance of Hps toward irreversible removal of formaldehyde to shift the pathway equilibrium. Direct coupling of Mdh3 with Hps again resulted in a more than 27-fold enhancement in F6P production even at a 1:1 ratio (Fig. 2B). More importantly, only the direct coupling of Hps resulted in a significant reduction in the formaldehyde level, indicating the efficient channeling of formaldehyde to H6P rather than back to methanol. Clustering Mdh3 with a bifunctional Hps-Phi fusion, which has been previously shown to be more active than an equimolar mixture of the individual enzymes (30), further improved F6P production by another twofold, resulting in an overall 50-fold improvement over the uncomplexed enzyme mixture (Fig. 2B). Compared with the unassembled enzyme system, a much lower level of formaldehyde was detected (Fig. 2B), and only a small amount of H6P was accumulated (Fig. S3), indicating the effective channeling of formaldehyde toward F6P by the supramolecular enzyme complex due to improved molecular proximity.

Dynamic Light Scattering Identification and TEM Visualization of the Supramolecular Enzyme Assemblies.

To confirm that the increased F6P production observed was the result of the formation of supramolecular enzyme complexes, we carried out biophysical characterization experiments to evaluate the enzyme assembly. Dynamic light scattering (DLS) was first used to identify the sizes of the individual enzymes and that of the enzyme complex. Mdh3-sSH3lig had a narrow size distribution with a peak at 11.6 ± 0.3 nm, consistent with the decameric nature of Mdh3 (Fig. 3A). The fusion enzyme, SH3-Hps-Phi, showed a much broader peak with an average diameter of 14.9 ± 1.2 nm (Fig. 3A) because of the formation of larger enzyme complexes between the dimeric Hps (30, 34) and the tetrameric Phi (30). Formation of a larger enzyme assembly with an average peak of 24.0 ± 1.9 nm was detected by mixing Mdh3-sSH3lig and SH3-Hps-Phi (Fig. 3A). This increase in size suggests that large, multienzyme complexes are not being formed, as the average size is roughly the same as the two individual components added together. To investigate this further, transmission electron microscopy (TEM) was used to visualize the complex formation. Individual Mdh3-sSH3lig enzymes have a decameric structure, composed of two planes with mirror symmetry, each composed of five monomers in a ring-like shape (12) (Fig. 3B). From the TEM images, it appears that the SH3-Hps-Phi enzyme fusion is globular and heterogeneous in size with little distinction between each of the components (Fig. 3B). When Mdh3-sSH3lig and SH3-Hps-Phi were mixed before TEM analysis, association between the two protein fusions is evident (Fig. 3B). In some instances, multiple Mdh3-sSH3lig proteins were found to decorate a single SH3-Hps-Phi, and large multimeric complexes were also visualized. Taken together, these data confirm that the beneficial effects on F6P production are due to successful enzyme assembly.

Lactate Dehydrogenase as An "NADH Sink" Further Enhances Methanol Consumption and F6P Production.

Although F6P production was significantly increased through complexation of Mdh3 with the bifunctional Hps-Phi fusion, a parallel approach to further improve MeOH utilization and F6P production is to prevent the reduction of formaldehyde back to methanol by consuming all of the NADH using an NADH Sink (Fig. 4A). Lactate dehydrogenase (Ldh) consumes NADH during the conversion pyruvate to lactate and can be used as an NADH Sink in synthetic pathways. *E. coli* Ldh was cloned from genomic DNA, expressed, and purified. Addition of Ldh to Mdh3 even at a 1:1 molar ratio improved methanol consumption and formaldehyde formation by 3.6-fold (Fig. 4B). This enhancement is the direct result of the efficient NADH recycling by Ldh as no net accumulation of NADH was

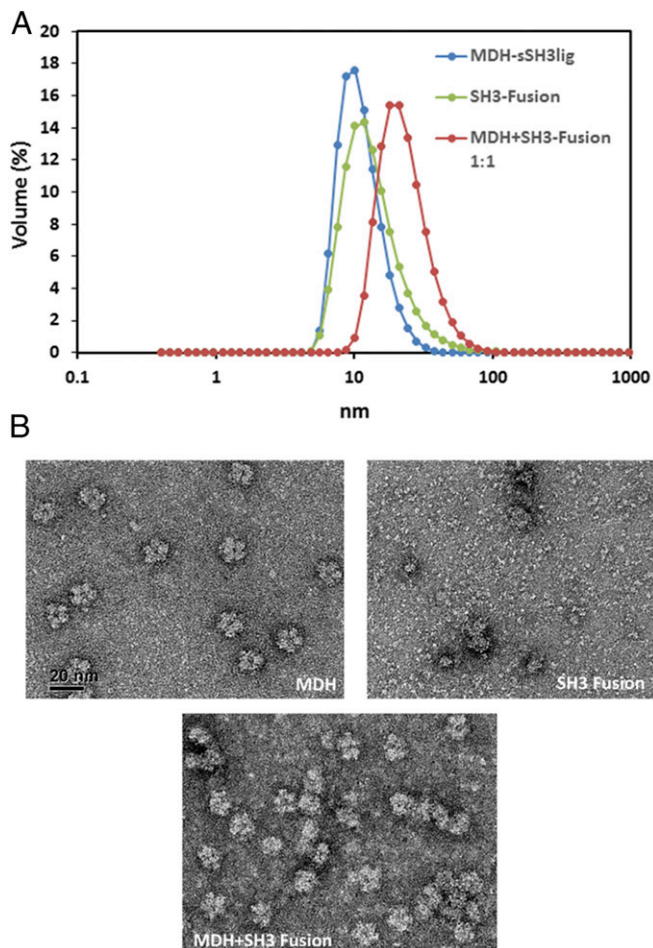


Fig. 3. Characterization of the Mdh3-Hps-Phi supramolecular enzyme complex. (A) DLS was used to characterize the size of the individual enzyme components and the 1:1 Mdh3-sSH3lig to SH3-Hps-Phi enzyme conjugates using the Zetasizer Nano ZS instrument. Three measurements were made at 25 °C, and the volume average is reported. (B) TEM of enzyme complexes. The TEM micrograph for Mdh3-sSH3lig showed the previously reported pentagonal shape of *B. methanolicus* MGA3 Mdh3, whereas the SH3-Hps-Phi fusion proteins were mostly larger and nonuniform enzyme clusters. Complex formation was clearly demonstrated by mixing the two proteins at a 1:1 molar ratio. Error bars represent the SD of at least three replicate experiments.

detected (Fig. S4). Increasing the Ldh-to-Mdh3 ratio further resulted in only a marginal improvement in methanol consumption (Fig. 4B), likely due to the very high specific activity of Ldh (Table S1).

We next investigated whether this substantial improvement in methanol consumption using the NADH Sink concept could be coupled with the enzyme clustering approach. Different enzyme combinations were evaluated as described earlier, except Ldh was also added at a ratio of 1:1 to Mdh3 (Fig. 5). In all cases, F6P production was increased in the presence of Ldh. Although the F6P level was elevated by more than 20-fold for the uncomplexed enzyme mixture, a significant amount of formaldehyde remained unprocessed. This result again suggests the inability of the uncomplexed Hps to effectively compete for formaldehyde to form H6P against the formaldehyde conversion back to methanol by Mdh3. A completely different phenomenon was observed when the engineered enzyme assembly was used. In both cases, virtually all formaldehyde formed was diverted to F6P, albeit at a lower efficiency without using the Hps-Phi fusion. This result

highlights that the synergistic action between the two parallel pathways, one in preventing reverse methanol formation and one in irreversibly removing formaldehyde toward F6P, is necessary not only to increase methanol consumption but also for the subsequent conversion to F6P. Overall, 67.7 μM F6P was produced using a combination of NADH Sink and enzyme assembly, which represents a 97-fold increase over the uncomplexed enzyme mixture.

In Vivo Engineered Enzyme Assembly Enhances Methanol Consumption by *E. coli*. For the in vitro experiments, all substrates were provided in excess in order to investigate only the influence of enzyme kinetics on F6P production, a condition very different from the in vivo setting. To investigate whether the supramolecular enzyme assembly can impact in vivo methanol consumption in a similar fashion, two recombinant strains were created where Mdh3 was either assembled with Hps-Phi or expressed as uncomplexed enzymes. A ΔfmA strain was used to eliminate the native formaldehyde oxidation system from competing with the RuMP pathway (35, 36). A high level of enzyme expression was first demonstrated by SDS/PAGE (Fig. S5A). To confirm the intracellular formation of enzyme complexes, cell lysates were fractionated by sucrose gradient centrifugation. Mdh3 was detected only in the later fractions (fractions 10–12), indicating the presence of intact larger protein particles (Fig. S5B). More importantly, TEM analyses confirmed the formation of protein complexes for cells expressing the assembled enzymes, whereas mainly individual Mdh3 proteins were detected for the uncomplexed enzymes (Fig. S5C).

After confirming complex formation, in vivo methanol consumption was compared using resting cell cultures in the presence of 500 mM methanol. Similar to the in vitro results, methanol consumption was detected only for cells expressing Mdh3, even after 24 h of cultivation (<0.5 mM evaporation within 24 h). Although the extent of improvement is lower for the in vivo system, the initial methanol consumption rate was roughly 9 times faster for the recombinant strain expressing both Mdh3-sSH3lig and SH3-Hps-Phi (1.7 mM/h) than the control strain without the SH3 interaction components (0.19 mM/h) (Fig. 6). This whole-cell methanol consumption rate of 1.7 mM/h is also 14 times higher

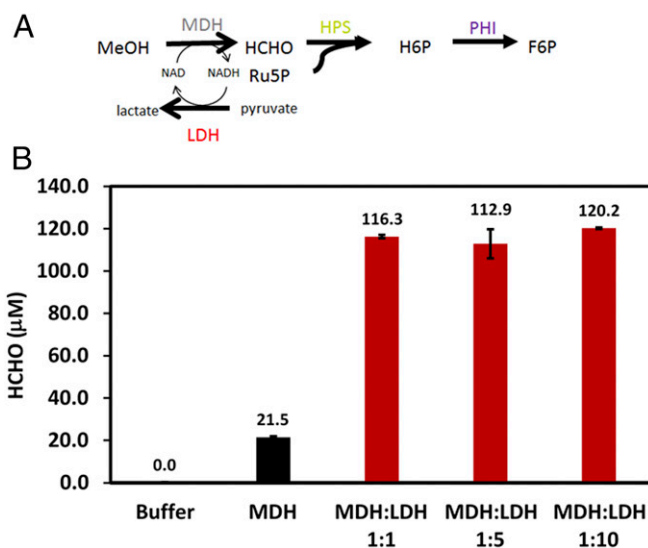


Fig. 4. (A) A schematic illustrating the concept of NADH Sink using *E. coli* Ldh. (B) Effects of NADH Sink on methanol (CH₃OH) conversion. Purified Mdh3-sSH3lig was mixed with increasing ratios of purified *E. coli* Ldh and assayed for formaldehyde (HCHO) according to the Nash method. Error bars represent the SD of at least three replicate experiments.

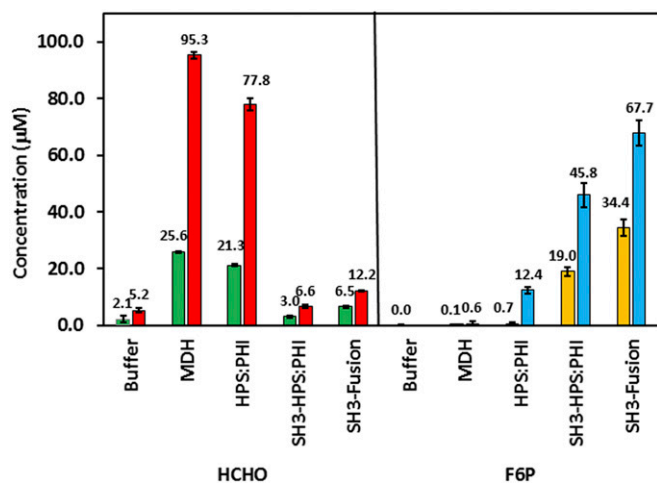


Fig. 5. Combinatorial effect of enzyme assembly and NADH Sink on methanol consumption and F6P production. Purified Mdh3-sSH3lig was mixed at a 1:1 molar ratio with either Hps and Phi, SH3-Hps and Phi, or SH3-Hps-Phi with or without an equal molar of Ldh. The HCHO and F6P concentration in the absence (first bar) and presence (second bar) of Ldh are shown. Error bars represent the SD of at least three replicate experiments.

than the 0.12 mM/h reported by Müller et al. in a similar resting cell assay (15). It should be noted that the overall enhancement in methanol consumption was only 2.3-fold after 24 h incubation, likely due to limitations in R5P in resting cell cultures. The consumed methanol was clearly diverted further down the RuMP pathway, as only micromolar levels of formaldehyde were detected in both cases. The majority of the carbon flux is likely channeled toward CO₂ formation via native glycolysis, although a small amount was diverted toward formate (Fig. 6). Collectively, these results indicate that the beneficial effects of enzyme assembly can be maintained in vivo and help improve methanol utilization by *E. coli*.

Discussion

Engineering *E. coli* as a synthetic methylotroph has been a challenging problem to date, as very little methanol utilization and no growth on methanol have been demonstrated (15). One of the key obstacles is the unfavorable kinetic properties of NAD-dependent Mdh3, such as Mdh3 (9), making the reversible reduction of formaldehyde far more favorable than methanol oxidation. Although the irreversible sequestration of formaldehyde by the RuMP pathway enzymes serves as a driving force to pull carbon flux to the synthesis of F6P, a 1:10 ratio of Mdh3 to Hps is necessary to drive a significant level of H6P production (Fig. 1B). This very high enzyme ratio is difficult to maintain, particularly if all of the downstream enzymes must be expressed at this high level.

The use of engineered enzyme assembly has been shown to substantially improve the efficiency of cascade reactions (19–22). We argue that this method is ideally suited for pulling formaldehyde toward F6P production because of the improved substrate channeling. Unlike other reports using a protein scaffold for enzyme assembly, we chose a scaffoldless self-assembly approach to cluster three multimeric enzymes. Our engineered supramolecular enzyme assembly yielded a 50-fold improvement in methanol conversion to F6P, a key metabolite that is capable of entering *E. coli* central metabolism. Although the improvement in F6P production was extremely promising, the overall improvement in methanol conversion remained modest. This observation suggested that an additional mechanism to slow down formaldehyde reduction was needed. This was accomplished by installing an NADH Sink to irreversibly consume NADH required for formaldehyde reduction using *E. coli* Ldh, which catalyzes the essentially unidirectional reduction of pyruvate (37). Addition of Ldh

successfully eliminated any NADH accumulation and led to over twofold more methanol oxidation and F6P production.

We next investigated whether the beneficial effects of supramolecular enzyme assembly can be similarly realized in vivo, as the intracellular substrate availability is significantly different.

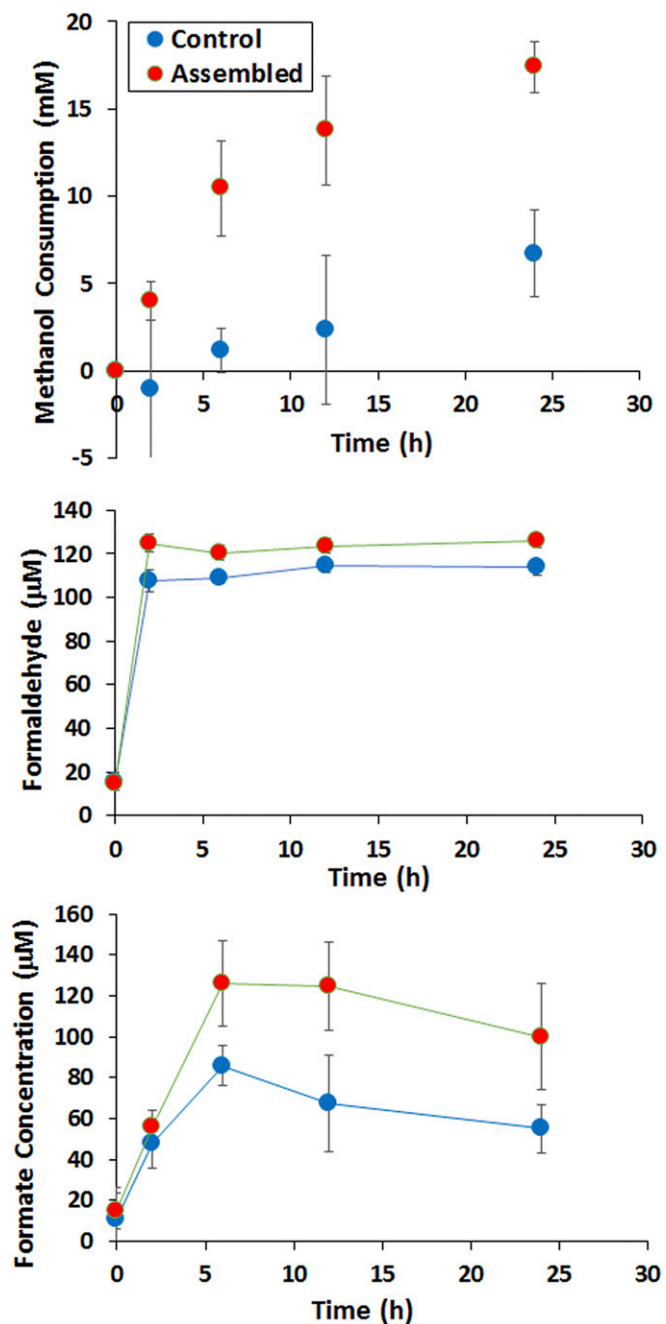


Fig. 6. In vivo methanol consumption and utilization. Two recombinant $\Delta firmA$ strains were tested for methanol consumption for 24 h. One strain harboring Mdh3 and Hps-Phi without the docking components (Control) and a second strain harboring Mdh3-sSH3lig and SH3-Hps-Phi fusion (Assembled). Cells were grown to an OD₆₀₀ of 1, washed, and resuspended in M9 medium and 500 mM methanol and incubated at 37 °C for 24 h. Methanol consumption was calculated as the difference between the starting and final methanol concentration. Formaldehyde accumulation was measured with the Nash reagent by harvesting cells and testing the clarified supernatant. Formate formation was determined by HPLC analysis of culture supernatants. Error bars represent SD; $n = 4$.

Although the overall enhancement is lower, we demonstrated that enzyme assembly improved methanol consumption and increased formate production by 4.5- and eightfold, respectively. Optimization of protein expression and fermentation conditions will likely lead to further enhancement in productivity, as reported by others (33).

In summary, we have demonstrated an engineered system that couples a supramolecular enzyme assembly with an NADH Sink to provide a highly effective strategy for overcoming essential obstacles associated with methanol utilization. The approach provides a platform for the direct coupling of enhanced F6P synthesis with many other metabolic engineering strategies such as the methanol condensation cycle (29) for biological conversion of methanol to higher value chemicals by *E. coli*.

Materials and Methods

Chemicals and Reagents. All reagents were purchased from Sigma-Aldrich, unless otherwise indicated. The following enzymes were also purchased from Sigma-Aldrich: glucose-6-phosphate dehydrogenase (G6dh) (*Saccharomyces cerevisiae*), phosphoglucose isomerase (Pgi) (*S. cerevisiae*), and phosphoribose isomerase (Rpi) (*Spinacia oleracea*). Phusion DNA polymerase, T4 DNA ligase, and all restriction endonucleases were purchased from NEB.

Cloning of Methanol Utilization Genes. All primer sequences are listed in Table S2. Detailed procedures for constructing the different expression vectors are described in *SI Materials and Methods*. Briefly, the gene encoding *B. methanolicus* MGA3 Mdh3 (9) was synthesized by Genscript. For the

pull-down experiments, ELP-sH3lig was created using SLIC (38) with 18 bp of homology on the ends of the linearized plasmid.

Methanol Conversion to F6P. A 1-mL reaction contained 50 mM Hepes (pH 7.4), 5 mM MgCl₂, 1 mM NAD⁺, 5 mM Ru5P, 500 mM MeOH, and purified enzymes at concentrations between 1 and 10 μM. When Ldh was used, 2 mM pyruvate was added to the reaction mixture. The reaction was initiated with the addition of MeOH and incubated at 37 °C for 14 h. The reaction was stopped by the addition of 1 M HClO₄ and neutralized with 1 M KOH. Following neutralization, reactions were analyzed for formaldehyde and F6P (39). Formaldehyde concentration was determined according to Nash (40).

In Vivo Testing of Enzyme Clustering. Characterization of the genes required for methanol assimilation in *E. coli* was performed in a BW25113 Δ*frmA* host strain. Methanol assimilation genes were expressed heterologously using the pETM6 vector that has been modified to contain the *tac* promoter using PCR and standard cloning procedures. Detailed culture conditions are described in *SI Materials and Methods*. Resting cell cultures were incubated at 37 °C with aeration for 24 h, and methanol, formaldehyde, and secreted metabolites were quantified. Methanol and formate concentrations were measured via high performance liquid chromatography (Agilent Technologies) using an Aminex HPX-87H column (Bio Rad), 0.005 M H₂SO₄ as the mobile phase, and a flow rate of 0.5 mL/min. Formaldehyde in the culture supernatant was analyzed as previously described (40).

ACKNOWLEDGMENTS. We thank Shannon Modla of the Bioimaging Center at the Delaware Biotechnology Institute for her assistance in acquiring TEM images and R. Kyle Bennett for help with in vivo experiments. Funding was provided by ARPA-E REMOTE Contract DE-AR0000432.

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