Insights into Cell-Free Conversion of CO₂ to Chemicals by a Multienzyme Cascade Reaction

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Supporting Information

ABSTRACT: Multienzymatic cascade reactions have garnered the attention of many researchers as an approach for converting CO₂ into methanol. The cascade reaction used in this study includes the following enzymes: a formate dehydrogenase (ClFDH), a formaldehyde dehydrogenase (BmFaldDH), and an alcohol dehydrogenase (YADH) from Clostridium ljungdahlii, Burkholderia multivorans, and Saccharomyces cerevisiae, respectively. Because this cascade reaction requires NADH as a cofactor, phosphite dehydrogenase (PTDH) was employed to regenerate the cofactor. The multienzymatic cascade reaction, along with PTDH, yielded 3.28 mM methanol. The key to the success of this cascade reaction was a novel formaldehyde dehydrogenase, BmFaldDH, the enzyme catalyzing the reduction of formate to formaldehyde. The methanol yield was further improved by incorporation of 1-ethyl-3-methylimidazolium acetate (EMIM·Ac), resulting in 7.86 mM of methanol. The key to the success of this cascade reaction was a novel formaldehyde dehydrogenase, BmFaldDH, the enzyme catalyzing the reduction of formate to formaldehyde. The methanol yield was further improved by incorporation of 1-ethyl-3-methylimidazolium acetate (EMIM·Ac), resulting in 7.86 mM of methanol. A 500-fold increase in total turnover number was observed for the ClFDH-BmFaldDH-YADH cascade system compared to the Candida boidinii FDH-Pseudomonas putida FaldDH-YADH system. We provided detailed insights into the enzymatic reduction of CO₂ by determining the thermodynamic parameters (K₆ and ΔG) using isothermal titration calorimetry. Furthermore, we demonstrated a novel time-dependent formaldehyde production from CO₂. Our results will aid in the understanding and development of a robust multienzyme catalyzed cascade reaction for the reduction of CO₂ to value-added chemicals.

KEYWORDS: cascade reaction, formaldehyde, methanol, multienzyme, CO₂, FDH, FaldDH

INTRODUCTION

Carbon dioxide (CO₂) is a major greenhouse gas which has accumulated steadily over the past few decades.¹,² It is generated mainly by fossil fuel combustion, volcanic eruptions, industrial byproducts, and products of respiration.³ Increasing atmospheric concentration of CO₂ and a constant need for alternative sources of energy have inspired many researchers to investigate different methods to produce methanol from CO₂.⁴ Recently, reduction of CO₂ to yield methanol and other renewable fuels has received considerable attention from the scientific community.⁵⁶ There are several routes to achieve CO₂ conversion, such as chemical, photochemical, electrochemical, and enzymatic reduction. Of particular interest is enzymatic reduction of CO₂ owing to its product selectivity, mild experimental conditions, and environmental friendliness.⁷ However, electrochemical CO₂ reduction presents various
fundamental and practical challenges, mainly because of its inefficient conversion performance and high cost.8

Enzymes are natural catalysts. In living cells, enzymes often work together or in a specific order to catalyze multistep biochemical reactions, which play a crucial role in the synthesis of natural products.9 To mimic microbial multistep reactions, multienzyme in vitro systems have been explored for various catalytic reactions where single enzyme catalysis is not effective.10 Enzymatic reduction of CO2 is a multienzymatic, multistep process which employs three dehydrogenases, namely formate dehydrogenase (FDH), formaldehyde dehydrogenase (FaldDH), and alcohol dehydrogenase (ADH), acting in a cascade where the product of the first reaction serves as a substrate for the subsequent, downstream enzyme.7,11 In vitro biocatalytic reduction of CO2 to methanol is considered to be a “green” chemical process and occurs at ambient temperature and atmospheric pressure.12 Cazelles and colleagues have reported the reduction of CO2 to methanol by a polyenzymatic system using FDH from Candida boidinii, FaldDH from Pseudomonas putida, and ADH from Saccharomyces cerevisiae.7 Similarly, the enzymatic reduction of CO2 to methanol using three dehydrogenases was achieved by reversing the biological metabolic pathways.13–17 However, this multienzymatic conversion of CO2 to methanol was found to be quite inefficient, taking several hours to produce a very low concentration of methanol (less than 1 mM). Additionally, the need for high NADH concentrations (up to 100 mM) makes the multienzymatic CO2 conversion to methanol more expensive. Here, the low reduction activity of FDH and FaldDH is a major challenge for the reduction of CO2. In order to improve the catalysis efficiency of the multienzyme cascade reaction, more active FDH and FaldDH are required. Additionally, each of the three dehydrogenases require reduced nicotinamide adenine dinucleotide (NADH) as a cofactor for their activity. Therefore, in situ regeneration of the cofactor in the multienzymatic conversion of CO2 will further improve the productivity.

The low solubility of CO2 in aqueous media is one of the possible challenges encountered during design of the enzymatic reduction of CO2.18 Suitable cosolvents may be introduced to increase the substrate solubility. Ionic liquids (ILs), as an alternative to conventional organic cosolvents, can be used owing to their green properties.19 ILs have been used in various chemical and enzymatic reactions, involving enzymes such as lipases, cellulases, and alcohol dehydrogenases.20–22 ILs have also been reported to enhance the stability of various enzymes. Furthermore, Rosen and coworkers reported that the IL 1-ethyl-3-methylimidazolium tetrafluoroborate (EMIM-BF4) lowers the reduction overpotential for CO2 conversion in aqueous media.22 They proposed that the effectiveness of this newly observed catalytic reduction relies on the ability of the IL to lower the energy barrier. Wipple et al. also proposed that the overpotential of CO2 reduction could be lowered by stabilizing intermediate using a suitable catalyst.6

In this study, we constructed a multienzymatic, artificial cascade reaction using dehydrogenases from three different organisms, which may not share similar reaction conditions for their respective catalytic activity (Figure 1). We used purified recombinant FDH from Clostridium ljungdahlii (CIFDH), FaldDH from Burkholderia multivorans (BmFaldDH), and ADH from S. cerevisiae (YADH) in the overall enzymatic cascade reaction for the conversion of CO2 to chemicals. To overcome the limitation of reducing cofactor levels during catalytic cycles, an in situ NADH regeneration system was incorporated using phosphate dehydrogenase (PTDH). Cazelles et al. have reported that PTDH shows highest activity in NADH regeneration.7 This work also demonstrates the role of EMIM-Ac in the overall process, by comparing the thermodynamic parameters of the multienzymatic artificial cascade reaction in the absence and presence of EMIM-Ac. Transient time analysis for the CIFDH-BmFaldDH-YADH and BmFald-DHP-PTDH-YADH cascade systems was performed to demonstrate the effect of novel enzymes (CIFDH and BmFaldDH) on the entire cascade reaction.

## MATERIALS AND METHODS

**Materials.** The genes encoding CIFDH, BmFaldDH, and PTDH used in this study were synthesized by GenScript (Piscataway, NJ, USA). Alcohol dehydrogenase (YADH) enzyme (homotetramer, 141 kDa) from S. cerevisiae and oxidized and reduced nicotinamide adenine dinucleotide (NAD+, NADH), were purchased from Sigma-Aldrich (St. Louis, MO, USA). For PCR, Ex-Taq DNA polymerase was acquired from Promega (Madison, WI, USA). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA, USA). pET 28(a) and pQE-80L expression vector and Ni-NTA Superflow column were purchased from Novagen (Madison, WI, USA) and Qiagen (Hilden, Germany), respectively. Oligonucleotide primers and a plasmid isolation kit were obtained from Bioneer (Daejeon, Korea). Electrophoresis reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA), and all assay chemicals were purchased from Sigma-Aldrich. All reagents were of analytical or biotechnological grade.

**Screening FDHs and FaldDHs.** To identify a suitable FDH and FaldDH capable of catalyzing the reduction reaction, we used a three-step screening approach. The first step was a BLAST-based sequence comparison and systematic sequence analysis screening for evaluating the potential of CO2 or formate-reducing homologues using the EcFDH or PpFaldDH sequences, respectively, as the search driver. Then, based on the phenazine methosulfate (PMS)-nitroblue tetrazolium (NBT) assay,24 a library of 20 FDHs (Table S1) and 26 FaldDHs (Table S2) was constructed. Finally, the libraries of 20 FDHs and 26 FaldDHs were analyzed for CO2 or formate reduction, respectively (see the Supporting Information for details).

**Cloning, Expression, and Purification of CIFDH, BmFaldDH, and PTDH.** The Clfdh (UniProt D8GN53;
RefSeq WP_013237369), Bmfalddh (UniProt J4QK49), and Ptdh genes from pUC57 vector were PCR-amplified using specific primers. The amplified fragments of Clfdh and Bmfalddh were digested by Ndel and Xhol, and BamHI and Xhol, respectively, and ligated with pET 28(a) to construct recombinant plasmids pET 28(a)-Clfdh and pET 28(a)-Bmfalddh. pET 28(a) is under the control of a T7 promoter and expresses a hexa-histidine tag fused to the N terminus of the protein of interest. The amplified PTDH gene was cloned into vector pQE-80L. The cloned Clfdh, Bmfalddh, and PTDH genes were confirmed to be free of point mutations by DNA sequencing at Macrogen Inc. (Seoul, Korea). The recombinant plasmids were used to transform competent Escherichia coli BL21 (DE3).

Expression and purification of the FDHs were performed as previously described.25 The recombinant CIFDH, BmFaldDH, and PTDH were expressed using 0.1, 0.1, and 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) at 16 °C, respectively. Ni-NTA resin (3.4 × 13.5 cm, Qiagen) was used to purify the enzymes CIFDH, BmFaldDH, and PTDH. To obtain pure CIFDH, BmFaldDH, and PTDH enzymes, bound Ni-NTA resin was washed with 5 mL of wash buffer (50 mM NaH2PO4, pH 7.0, 300 mM NaCl, 50 mM imidazole). Purity of the enzymes was determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie blue R250 (BioShop, Burlington, Canada).

Protein Quantification and Enzyme Assay. Protein concentration was estimated by the Bradford protein assay method using bovine serum albumin as a reference protein.26 CO2 reduction activity of FDH was measured in sodium phosphate buffer (100 mM; pH 7.0), at 37 °C. Reaction mixtures (10 mM NaHCO3, 2 mM NADH, and enzyme) were incubated at 37 °C for 15 min. Each reaction mixture was placed on ice in a sealed tube, and the product was estimated instantaneously following the Lang and Lang method.27,28 NaHCO3 was used as a substrate instead of CO2 because the concentration of gaseous CO2 could not be accurately determined.29 Therefore, CO2 concentration was represented as that of NaHCO3. The initial velocity of enzyme catalysis for CO2 reduction was analyzed by estimating absorbance of formate at 515 nm. An excess of bicarbonate and NADH were provided to ensure that the assays were initiated far from equilibrium. One unit of reduction activity was defined as the amount of enzyme required to produce 1 μmol of formate per minute under standard conditions. For FaldDH, the standard assay was carried out using NaHCO3 (0.2 mM) and sodium formate (HCOONa, 5 mM) in sodium phosphate buffer (100 mM; pH 7.0) at 25 °C. For the alcohol dehydrogenase (YADH), the standard assay was performed with NADH (0.2 mM) and formaldehyde (CH5O H2) in sodium phosphate buffer (100 mM; pH 7.0) at 25 °C. FaldDH and YADH activity were determined by following the decrease in the absorbance of NADH at 340 nm by UV–visible spectroscopy.

Reduction of CO2 into Methanol. Reduction of CO2 by cascade reactions was performed using CIFDH, BmFaldDH, and YADH enzymes with and without NADH regeneration. A 50 mM sodium phosphate (Na2HPO4) was used as substrate for the recycling of NADH using PTDH in 100 mM phosphate buffer at pH 7. CIFDH, BmFaldDH, and YADH ratios and experimental conditions such as pH, temperature, and substrate concentration were optimized using response surface methodology (RSM).30 The statistical software package Design-Expert (Stat-Ease Inc., Minneapolis, MN, USA) was used for regression analysis of experimental data and to plot response surfaces. One-way analysis of variance (ANOVA) was used to estimate the statistical parameters. The CIFDH-BmFaldDH, BmFaldDH-YADH, CbFDH-PpFaldDH, and PpFaldDH-YADH coupled enzyme systems were assayed under standard assay conditions (30 °C, pH 7.0) using stopped-flow spectroscopy by monitoring the decrease in NADH fluorescence or absorbance at 460 or 340 nm, respectively. The transient time was determined as described previously.51,52 Methanol was produced from CO2 by purging the gas into a sodium phosphate buffer (100 mM; pH 7.0) for 1 h. The pH was adjusted to 7.0 using NaOH. The reaction mixture was then purged with CO2 at the flow rate of 50 mL/min during the reduction to methanol. The flow rate of the gas was controlled by means of a pressure valve. The reaction solution was continuously stirred at 100 rpm with a magnetic bar and the pressure was maintained below 2 bar.

Screening of Cosolvents. To identify a suitable cosolvent capable of enhancing the enzymatic conversion of CO2 to methanol by using CIFDH, BmFaldDH, and YADH along with PTDH for NADH regeneration, the optimized ratio of the amounts of CIFDH to BmFaldDH to YADH was employed for NaHCO3 as the starting substrate under optimum reaction conditions. Different types of cosolvents were tested to analyze their effect on methanol production. The initial screening of cosolvents was performed by mixing 100 mM of NaHCO3 with 5% of the cosolvents (v/v); then, they were properly mixed to dissolve the substrate at 30 °C. After 1 h of mixing, the pH was appropriately adjusted to 7 and the reaction was initiated by adding the enzymes and NADH. After 6 h of the reaction, the methanol concentration was determined.

Analytical Methods. Formate concentration was measured following the method described by Lang and Lang.27,28,33 Samples (100 μL) containing formate were mixed with 0.2 mL of solution A, 10 μL of solution B, and 0.7 mL of 100% acetic anhydride and incubated at 50 °C for 2 h with occasional mixing. A red color developed which was measured photographically at 515 nm. Solution A was prepared by dissolving 0.5 g of citric acid and 10 g of acetamide in 100 mL of isopropanol; solution B was prepared by dissolving 30 g of sodium acetate in 100 mL of water. Sodium formate dissolved in potassium phosphate buffer (100 mM; pH 7.0) was used for standard calibration. For the detection and quantification of methanol, aliquots at various time points were taken and analyzed for methanol content by using an Agilent 7890A gas chromatograph with an Agilent J&W DB-1 nonpolar column (60 m × 0.32 mm × 2.0 μm) and a FID detector, with ethyl acetate as the internal standard. A calibration curve was prepared by employing the known concentrations of methanol that ranged from 0.05 to 10.0 mM. To estimate the methanol produced as a result of the enzyme-catalyzed reaction, 1.0 μL of the final reaction solution was used for the GC measurements while the injector temperature was maintained at 200 °C. The concentration of methanol was calculated based on the area corresponding to the characteristic methanol peak observed in the chromatogram.

## RESULTS AND DISCUSSION

Novel Formate Reducing FaldDH, BmFaldDH. To date, an FaldDH reducing formate to formaldehyde has not been reported. Recently, Ma and colleagues concluded that the reduction of formate to formaldehyde by PpFaldDH was
The reduction of CO₂ to a chemical state in multienzymatic reactions is a bottleneck for carbon capture and storage. ClFDH and BmFaldDH are the most effective catalysts for this reaction. We used bioinformatics tools and the Braunschweig Enzyme Database to identify suitable FaldDH candidates. A library of 26 putative FaldDHs was constructed based on their formate reduction activities in crude cell lysates of 75 different microorganisms. We limited our search to FaldDHs capable of catalyzing the reduction of CO₂ to formate in a cascade reaction. We screened a number of FaldDHs and evaluated their formate reduction capabilities. We used a cascade reaction approach to evaluate the formate reduction activities of ClFDH and BmFaldDH, the optimum pH and temperature were 7.0 and 40 °C, respectively (Figure S2). The results presented in Table S2 strongly suggest that ClFDH and BmFaldDH are the most effective catalysts for the reduction of CO₂ to a chemical state in multienzymatic cascade reactions.

Although BmFaldDH and PpFaldDH showed high sequence identity (80%), computational analysis revealed significant differences in the formate orientation in terms of distance and angle with the cofactor NADH (Figure S3). Inductively coupled plasma-optical emission spectrometry (ICP-OES) of BmFaldDH revealed the presence of two zinc atoms per monomer, which are designated as the catalytic and structural metal ions (Table S3). Before hydrogen transfer occurs, the carbonyl oxygen needs to be first activated via the catalytic zinc ion. The average distance between the Zn and carbonyl oxygen should be less than 2.5 Å, and the Zn—O—C₁ angle should be in the range 142 ± 16° for activation. Since the formate can dock with a delocalized negative charge, the distances between the two oxygens of the formate and the Zn were calculated. The distances Zn—O₁ (Zn—O₂) for BmFaldDH and PpFaldDH are 1.9 (2.0) and 4.2 (2.1) Å, respectively. Based on the molecular dynamics simulation results (Figure S4), it has been further confirmed that the distance between both the oxygens and Zn in BmFaldDH is very well conserved (1.9 to 2.0 Å), but in PpFaldDH, the distance is highly fluctuating, ranging from 2.1 to 4.2 Å, which offers a significant clue about the difference in the reduction activity of the two enzymes. Similarly, the distances between the carbonyl carbon and NADH (C₄) are 2.4 and 3.2 Å, respectively (Figure S3D and B). To make the hydride transfer effective, the substrate and cofactor angle (C₄—H—C₁) should be close to 160°. In BmFaldDH and PpFaldDH, the above angle was observed as 153° and 91°, respectively. A shorter distance between the carbonyl carbon (C₁) and NADH (C₄) is favorable for hydride transfer during the reduction of formate by BmFaldDH.

**Table 1. Comparison of Kinetic Parameters of Various FDHs and FaldDHs**

<table>
<thead>
<tr>
<th>enzyme</th>
<th>$K_{m_{\text{NaHCO}_3}}$ (mM)</th>
<th>$K_{m_{\text{NADH}}}$ (mM)</th>
<th>$k_{cat}/K_{m_{\text{HCO}_3^-}}$ (1/s)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CfFDH</td>
<td>31.3 ± 8.03</td>
<td>0.512 ± 0.186</td>
<td>0.0004</td>
<td>29</td>
</tr>
<tr>
<td>CbFDH</td>
<td>30–50*</td>
<td>ND</td>
<td>ND</td>
<td>7</td>
</tr>
<tr>
<td>TsFDH</td>
<td>9.23 ± 3.98</td>
<td>0.264 ± 0.076</td>
<td>0.034</td>
<td>29</td>
</tr>
<tr>
<td>CcFDH</td>
<td>0.37 ± 0.02</td>
<td>0.050</td>
<td>7.45</td>
<td>25</td>
</tr>
<tr>
<td>ClFDH</td>
<td>0.03 ± 0.01</td>
<td>0.003 ± 0.001</td>
<td>183</td>
<td>this study</td>
</tr>
<tr>
<td>BmFaldDH</td>
<td>0.12 ± 0.02*</td>
<td>0.003 ± 0.001</td>
<td>39.2</td>
<td>this study</td>
</tr>
<tr>
<td>PpFaldDH</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>7</td>
</tr>
</tbody>
</table>

* CO₂ gas was used as substrate instead of NaHCO₃. ** Sodium formate was used as substrate; ND, not detected.

**Table 2. Thermodynamic Parameters Determined for FDH and FaldDH**

<table>
<thead>
<tr>
<th>enzyme</th>
<th>ligand</th>
<th>N</th>
<th>$K_d$ (µM)</th>
<th>$\Delta G^\circ$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ClFDH</td>
<td>NADH</td>
<td>1.85 ± 0.45</td>
<td>0.21 ± 0.01</td>
<td>−38.67</td>
</tr>
<tr>
<td></td>
<td>NAD⁺</td>
<td>2.10 ± 0.32</td>
<td>0.84 ± 0.03</td>
<td>−35.22</td>
</tr>
<tr>
<td></td>
<td>CO₂</td>
<td>1.82 ± 0.28</td>
<td>1.92 ± 0.06</td>
<td>−33.15</td>
</tr>
<tr>
<td></td>
<td>formate</td>
<td>2.00 ± 0.33</td>
<td>7.55 ± 0.40</td>
<td>−29.69</td>
</tr>
<tr>
<td></td>
<td>CO₂ + EMIM-Ac</td>
<td>1.79 ± 0.19</td>
<td>0.44 ± 0.05</td>
<td>−36.85</td>
</tr>
<tr>
<td>CbFDH</td>
<td>NADH</td>
<td>4.12 ± 0.52</td>
<td>1.04 ± 0.07</td>
<td>−34.67</td>
</tr>
<tr>
<td></td>
<td>NAD⁺</td>
<td>3.97 ± 0.30</td>
<td>0.07 ± 0.01</td>
<td>−41.47</td>
</tr>
<tr>
<td></td>
<td>CO₂</td>
<td>3.94 ± 0.51</td>
<td>9.95 ± 0.80</td>
<td>−29.00</td>
</tr>
<tr>
<td></td>
<td>formate</td>
<td>4.21 ± 0.34</td>
<td>0.89 ± 0.07</td>
<td>−35.07</td>
</tr>
<tr>
<td>BmFaldDH</td>
<td>NADH</td>
<td>3.85 ± 0.22</td>
<td>0.67 ± 0.05</td>
<td>−35.2</td>
</tr>
<tr>
<td></td>
<td>NAD⁺</td>
<td>3.91 ± 0.36</td>
<td>0.37 ± 0.03</td>
<td>−36.7</td>
</tr>
<tr>
<td></td>
<td>HCHO</td>
<td>3.98 ± 0.50</td>
<td>3.70 ± 0.24</td>
<td>−75.7</td>
</tr>
<tr>
<td></td>
<td>formate</td>
<td>3.80 ± 0.46</td>
<td>6.20 ± 0.81</td>
<td>−29.7</td>
</tr>
</tbody>
</table>

* LIGAND binding to proteins was measured by ITC. Titration experiments were performed at 25 °C and consisted of 20 2.0-µL injection volumes and 120-s time intervals between consecutive injections.
efficiently catalyze reactions in both directions. In contrast to PpFaldDH, BmFaldDH showed formate reduction to formaldehyde. The ΔG value for BmFaldDH (Table 2) which resulted from BmFaldDH and formate interaction suggests that formate reduction is favored when it is present in excess in the reaction mixture. The ΔG, along with the favorable Kd value for NADH, makes BmFaldDH a more efficient catalyst for enzymatic reduction of formate to formaldehyde. When the ΔG for ClFDH is compared with respect to NAD+ and NADH (Figure S6), it is evident that ClFDH facilitates the reduction of CO2 to formate under controlled experimental conditions (Table 2).

**Construction of the Multienzymatic Cascade Reaction for Reduction of CO2 to Methanol.** *Overcoming the Bottleneck for CO2 Conversion to Chemicals.* Several groups have reported reduction of CO2 into methanol using CbFDH, PpFaldDH, and YADH as cascade enzymes.7,13–15 The biocatalytic reduction of CO2 is thermodynamically unfavorable13,16 and the multienzymatic conversion of CO2 to methanol is quite inefficient owing to the low activities of CbFDH and PpFaldDH. To overcome this limitation, novel ClFDH and BmFaldDH were employed in place of CbFDH and PpFaldDH, respectively. Our system overcomes the bottleneck of the multienzymatic cascade reaction using formate-reducing BmFaldDH. To the best of our knowledge, this is the first report of the enzymatic reduction of CO2 to formaldehyde. ClFDH and BmFaldDH converted CO2 and formate to their products, formate and formaldehyde, respectively, confirming the cascade reaction pathway (Figure S7). We used a statistical RSM technique to optimize the various factors simultaneously (Figure S8). For maximum production of methanol (0.44 mM), the optimum ratios of ClFDH, BmFaldDH, and YADH were 2.5, 2.5, and 50 U, respectively. Optimum pH and temperature for the cascade reaction were found to be 7.0 and 30 °C, respectively. Optimum pH was found to be well within the range for all three dehydrogenases.

**NADH Cofactor Regeneration.** During the complete reduction of one molecule of CO2 into one molecule of methanol, three molecules of NADH have to be spent, making the whole process inefficient without NADH regeneration. The oxidation of NADH leads to the accumulation of NAD+ in the reaction mixture, which can result in product inhibition of CO2 reduction and stimulate the reverse oxidative reaction. The Kd values of ClFDH, BmFaldDH, and YADH for NAD+ were determined to be 0.182, 0.225, and 0.764 mM, respectively (Figure S9). At the end of the cascade reaction consisting of ClFDH, BmFaldDH, and YADH, 1.37 mM of NAD+ was accumulated. Therefore, to maintain the initial amount of NADH in the multienzymatic cascade reaction and to drive the reaction toward the formation of methanol, regeneration and recycling of NADH is essential. In this work, PTDH was used for the regeneration of NADH, along with the cascade enzymes (ClFDH, BmFaldDH, and YADH) for methanol production from CO2. PTDH shows optimum activity at pH 6.0–8.0, which is well within the optimized catalytic conditions for the conversion of CO2 into methanol (pH 7.0, 30 °C) (Figure S10). The optimum initial concentration of NADH was observed to be 50 mM for the production of methanol in the absence of the NADH regeneration system (Figure 2). The conversion of CO2 to methanol showed the highest methanol productivity with 3.5 mg mL−1 PTDH (Figure S11).

Figure 2. Methanol production as a function of initial NADH amount. (A) Influence of initial NADH concentration on the conversion of CO2 to methanol for ClFDH + BmFaldDH + YADH (red ■) without PTDH, after 6 h of reaction. Similarly, for CbFDH + PpFaldDH + YADH (red ●) without PTDH. Productivity of CbFDH + BmFaldDH + YADH (blue ■) and CbFDH + PpFaldDH + YADH (blue ●) as a function of initial NADH concentration. All the reactions were carried out at 30 °C in phosphate buffer (100 mM, pH 7.0) with NaHCO3 (100 mM). (B) Methanol production by various cascade reaction systems indicating the initial NADH concentrations. 1, 2, 3, and 4 represent ClFDH + PpFaldDH + YADH + 100 mM NADH, CbFDH + PpFaldDH + YADH + 100 mM NADH with PT DH, CbFDH + BmFaldDH + YADH + 50 mM NADH, and ClFDH + BmFaldDH + YADH + 1.5 mM NADH with PT DH, respectively.

from 50 to 1.5 mM for the reduction of CO2 to methanol. In a previous study, 100 mM of NADH was used for enzymatic reduction of CO2 into methanol (0.9 mM; after 48 h) via three enzyme cascade reactions (CbFDH-PpFaldDH-YADH) in the presence of an NADH regeneration system.7 In the current study, however, a 66-fold lower NADH concentration was used and the methanol concentration obtained was 3.28 mM.

**Conversion of CO2 into Formaldehyde and Methanol.** CO2 was reduced to methanol using the newly constructed multienzymatic cascade system (ClFDH, BmFaldDH, and YADH) with NADH regeneration (PTDH) and NaHCO3 as the starting substrate. The time course of methanol production was examined and methanol concentration was estimated by GC (Figure S12A). Methanol reached its highest concentration of 3.28 mM after 6 h. However, the amount of methanol produced by enzymatic reduction of CO2 using CbFDH, PpFaldDH, and YADH without and with the NADH regeneration system was only 0.023 mM and 0.26 mM after 6 h, respectively.

CbFDH and PpFaldDH were not able to reduce CO2 to formaldehyde within 6 h of the reaction, but ClFDH and BmFaldDH could reduce CO2 to formaldehyde (Figure 3B), resulting in the subsequent efficient reduction of CO2 to methanol. To further identify and quantify the amount of formaldehyde produced, Nash’s reagent-derivatized formaldehyde was analyzed by high-performance liquid chromatography (Figure S12B). These analyses showed a novel time-dependent formaldehyde production from CO2. It has been reported that formaldehyde cannot be produced from CO2 or formate by enzymatic action.34 Because formaldehyde is an important intermediate used to synthesize a variety of value-added chemicals, this result provides a new platform that can be used to synthesize value-added chemicals from CO2 feedstock.

When PpFaldDH was replaced with BmFaldDH, the cascade reaction consisting of ClFDH, BmFaldDH, and YADH could achieve a methanol concentration of 1.69 mM. Here, BmFaldDH overcame the limitation of PpFaldDH for the
Figure 3. Methanol and formaldehyde production from CO₂ by cascade reaction. (A) Time profile of methanol production by multienzymatic cascade reactions. (black ▲) CbFDH + PpFaldDH + YADH with PTDH, (green ■) CbFDH + PpFaldDH + YADH with GDH, (purple ◦) CbFDH + BmFaldDH + YADH with PTDH, (red ●) CbFDH + BmFaldDH + YADH with PTDH and (blue ▼) CbFDH + PpFaldDH + YADH with PTDH and EMIM-Ac (1%). All reactions were carried out at 30 °C in 100 mM phosphate buffer (pH 7.0) with 100 mM NaHCO₃. (B) Time profile of formaldehyde production using FDH and FaldDH with NADH regeneration. CbFDH + PpFaldDH with PTDH (green ▲), CbFDH + BmFaldDH without PTDH (blue ▼), and CbFDH + BmFaldDH with PTDH (red ▲). The reaction mixture consisted of 1.5 mM NADH and 100 mM NaHCO₃ in 100 mM phosphate buffer at pH 7 with 2.5 U/mL of CbFDH and 2.5 U/mL BmFaldDH.

Table 3 summarizes the current state of CO₂ conversion into methanol using an enzymatic cascade reaction and highlights the superiority of the CbFDH, PpFaldDH, and YADH system. The methanol yield of the CbFDH-PpFaldDH-YADH cascade system was estimated to be 1.59 × 10⁸%, which is 529-fold higher than that of the previously reported CbFDH-PpFaldDH-YADH cascade system.7 The lower cofactor requirement and higher productivity makes this cascade system efficient.

We also tested CO₂ gas as a substrate. Figure 4 shows that 7.86 mM of methanol was produced by the CbFDH-BmFaldDH-YADH cascade reaction in the presence of a NADH regeneration system and EMIM-Ac (1%) when CO₂ gas was used as the starting substrate. However, the CbFDH-PpFaldDH-YADH cascade produced only 0.20 mM of methanol. Here, the methanol yield of the CbFDH-BmFaldDH-YADH cascade system in the presence of the NADH regeneration system was estimated to be 1.59 × 10⁸%, which is 529-fold higher than that of the previously reported CbFDH-PpFaldDH-YADH system with NADH regeneration.13

Protein–Ligand Interactions in the Presence of Cosolvent. A multienzyme cascade reaction is a complex reaction that involves several enzymes and their respective substrates, along with cofactors. Owing to differences in their solubility and behavior in aqueous media, cosolvents are often introduced in biochemical reactions to enhance their efficacy. As shown in Table S4, we screened a wide spectrum of cosolvents to achieve higher methanol concentrations. Although cosolvents have been used in many enzyme-catalyzed reactions, this is the first report where the effects of cosolvents were investigated for multienzymatic reduction of CO₂ into methanol. Among all the screened cosolvents, EMIM-Ac exhibited the highest enhancing effect on the reduction of CO₂ to methanol (Table S4). The EMIM-Ac concentration was further optimized (ranging from 0.1 to 10% (v/v)) to achieve

<table>
<thead>
<tr>
<th>enzymes</th>
<th>methanol (mM)</th>
<th>system</th>
<th>productivity (mM/h)</th>
<th>methanol/NADH</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbFDH + PpFaldDH + YADH + GDH</td>
<td>0.02 ± 0.003</td>
<td>co-immobilized</td>
<td>0.040</td>
<td>0.046</td>
<td>40</td>
</tr>
<tr>
<td>CbFDH + PpFaldDH + YADH</td>
<td>0.015 ± 0.002</td>
<td>immobilized</td>
<td>0.002</td>
<td>0.075</td>
<td>41</td>
</tr>
<tr>
<td>CbFDH + PpFaldDH + YADH</td>
<td>0.023 ± 0.004</td>
<td>free enzyme</td>
<td>0.007</td>
<td>0.001</td>
<td>7</td>
</tr>
<tr>
<td>CbFDH + PpFaldDH + YADH + PTDH</td>
<td>0.20 ± 0.03</td>
<td>free enzyme</td>
<td>0.014</td>
<td>0.009</td>
<td>7</td>
</tr>
<tr>
<td>CbFDH + PpFaldDH + YADH</td>
<td>0.011</td>
<td>free enzyme</td>
<td>0.007</td>
<td>0.028</td>
<td>17</td>
</tr>
<tr>
<td>CbFDH + PpFaldDH + YADH + GDH</td>
<td>0.10 ± 0.02</td>
<td>free</td>
<td>0.20</td>
<td>0.010</td>
<td>13</td>
</tr>
<tr>
<td>CbFDH + PpFaldDH + YADH + GDH</td>
<td>0.10 ± 0.01</td>
<td>immobilization</td>
<td>0.20</td>
<td>0.010</td>
<td>13</td>
</tr>
<tr>
<td>CbFDH + BmFaldDH + YADH + PTDH</td>
<td>3.28 ± 0.20</td>
<td>free enzyme</td>
<td>0.55</td>
<td>2.19</td>
<td>this report</td>
</tr>
<tr>
<td>CbFDH + BmFaldDH + YADH + PTDH</td>
<td>2.95 ± 0.34</td>
<td>free enzyme</td>
<td>0.49</td>
<td>1.97</td>
<td>this report</td>
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<tr>
<td>CbFDH + BmFaldDH + YADH + PTDH + EMIM-Ac</td>
<td>6.75 ± 0.24</td>
<td>free enzyme</td>
<td>1.13</td>
<td>4.50</td>
<td>this report</td>
</tr>
<tr>
<td>CbFDH + BmFaldDH + YADH + PTDH + EMIM-Ac</td>
<td>7.86 ± 0.41</td>
<td>free enzyme</td>
<td>1.31</td>
<td>5.24</td>
<td>this report</td>
</tr>
</tbody>
</table>

*CO₂ gas used as substrate. Productivity, average methanol formation rate per hour.
maximum methanol production. Methanol production by the cascade reaction was the highest (6.75 mM) at 1.0% EMIM-Ac concentration when NaHCO₃ was used as the starting substrate.

As discussed above, EMIM-Ac enhanced methanol production from 3.28 mM to 6.75 mM. Here, the optimized concentration of EMIM-Ac (1.0%) was added to the cascade reaction along with NADH regeneration (PTDH). CO₂ interacts with electronegative nitrogen and oxygen atoms. This causes the solubility of CO₂ to be higher in EMIM-Ac.⁹ The highest methanol concentration was found to be 6.75 mM after 6 h of reaction, as presented in Figure 3A. This indicates that EMIM-Ac plays crucial roles during CO₂ reduction in the cascade reaction. To date, aqueous medium was quite popular among researchers for enzymatic reduction of CO₂. EMIM-Ac is a green solvent, completely miscible with water at all concentrations and is extremely hygroscopic. The solubility of CO₂ is reported to be higher in EMIM-Ac than in water, predominantly due to anion—CO₂ interactions.⁹ Effects of EMIM-Ac on enzyme activity was evaluated by performing enzyme assay in the presence of EMIM-Ac (Table S5). The results presented in Table S5 clearly indicate that EMIM-Ac does not increase the activity of ClFDH, BmFaldDH, or YADH.

To investigate the role of EMIM-Ac in the enhancement of methanol production, ITC was performed to determine the thermodynamics of ClFDH and CO₂ interactions in the presence (Figure S13) and absence of EMIM-Ac. The ΔG value of ClFDH for CO₂ in the presence of EMIM-Ac was 4.4-fold lower than that of ClFDH for CO₂ in the absence of EMIM-Ac (Table 2). The ΔG value of CO₂ interactions with ClFDH in the absence of EMIM-Ac was −33.15 kJ mol⁻¹, which is larger than that (−36.85 kJ mol⁻¹) of CO₂ and ClFDH interaction in the presence of EMIM-Ac. Here, the lower binding enthalpy (ΔH value) reflects the increased strength of interaction between CO₂ and ClFDH in the presence of EMIM-Ac. It has been reported that the presence of EMIM⁺ cations around CO₂ molecules can reduce the reaction barrier for electrons passing into CO₂.⁹,¹⁰ Thus, the observed lower Kₚ and ΔG values of CO₂ in the presence of EMIM-Ac supports the enhanced methanol production by the cascade reaction.

**Transient Time of Multienzymatic Cascade Reaction.**

Multienzyme cascade reactions are well coordinated and possess systematic pathways to reach the final metabolite. During the whole process of CO₂ reduction to methanol, the transfer of a reaction intermediate from the ClFDH active site to BmFaldDH and subsequently to YADH is an important factor. To gain insight into the stepwise reduction of CO₂ to methanol by the newly constructed cascade reaction (ClFDH, BmFaldDH, and YADH), transient time (τ) was determined for each coupled enzyme step (Figure 5). Transient time is the time required to reach steady state when CO₂ is converted to formaldehyde by ClFDH and BmFaldDH via formate. Similarly, τ₁ is the time prior to reaching steady state while the formate is being converted to methanol via formaldehyde. Transient time was measured over the course of the reaction using stopped-flow spectroscopy. τ₁ for the ClFDH-BmFaldDH coupled reaction was observed to be 19 s (Figure 5C). In comparison, τ₁ was found to be 14 s for a BmFaldDH-YADH coupled reaction (Figure 5D). Under the same experimental conditions, the τ₁ and τ₂ for a commercial enzyme cascade reaction construct (CbFDH, PpFaldDH, and YADH) could not be determined due to the extended lag phase (>60 s). A decrease in transient time indicates a decreased lag phase between the two consecutive reactions catalyzed by two consecutive enzymes and highlights the efficiency of a multienzyme (ClFDH, BmFaldDH, and YADH) cascade reaction.

**CONCLUSIONS**

We have demonstrated that the newly constructed multienzyme cascade system using a novel formate-reducing BmFaldDH in the presence of EMIM-Ac can overcome the bottleneck (reduction of formate to formaldehyde) of the previously reported cascade systems for enzymatic conversion of CO₂ to chemicals. This is the first report where BmFaldDH and EMIM-Ac were incorporated in the cascade reaction for the reduction of CO₂ to chemicals. We also demonstrated biocatalytic production of formaldehyde from CO₂ or formate...
The concentration of the cascade reaction intermediate, [I], is shown with a green line. Figure 5. Determination of the transient time (τ) of a coupled enzyme reaction. (A and B) Raw data image of FDH-FaldDH and FaldDH-YADH catalyzed reactions using a stopped-flow spectrophotometer at 25 °C and pH 7.0. CIfDH-BmFaldDH and BmFaldDH-YADH are shown with blue lines; CbFDH-PpFaldDH and PpFaldDH-YADH are shown with green lines. Black lines show the control reactions. (C) CIfDH-BmFaldDH (blue ▲) coupled reaction is shown with a blue line; CbFDH-PpFaldDH (green ●) coupled reaction is shown with a green line. (D) BmFaldDH-YADH (blue ▲) coupled reaction is shown with a blue line; PpFaldDH-YADH (green ●) coupled reaction is shown with a green line. A linear fit to the product concentration as a function of time at steady state crosses the y-axis at τ. The y-intercept is equal to the negative of the steady state product concentration, [P]ss.

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