Kinetic compartmentalization by unnatural reaction for itaconate production

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Abstract

Physical compartmentalization of metabolisms using membranous organelles in eukaryotes is helpful for chemical biosynthesis to ensure the availability of substrates from competitive metabolic reactions. Bacterial hosts lack such a membranous system, which is one of the major limitations for efficient metabolic engineering. Here, we introduced kinetic compartmentalization as an alternative strategy to enable substrate availability from competitive reactions. This method utilizes a non-natural biochemical reaction performed by an engineered enzyme to kinetically isolate the metabolic pathways and ensure substrate availability for the desired reaction. As a proof of concept, we could successfully demonstrate kinetic separation for efficient itaconate production from acetate in *Escherichia coli*, mimicking the native mitochondrial membrane system in *Aspergillus* species. Despite the utilization of the non-preferred carbon source, kinetic compartmentalization could lead to substantial increases of itaconate in both yield and titer, suggesting enough potential of our strategy for broad applications in diverse engineering.

Background

Diverse intracellular biochemical reactions have evolved to efficiently supply energy and synthesize essential metabolites required for organism survival^{1–4}. For example, highly orchestrated enzymatic reactions involving substrate channeling by multienzyme complex or consecutive enzyme reactions, have evolved to rapidly consume intermediates, avoiding the formation of byproducts that undermine the efficiency of a desired pathway^{5–9}. However, such an efficient metabolic reaction chain may limit metabolic engineering when the newly introduced metabolic pathway must use the metabolic intermediate as a substrate, as the accessibility of the intermediate is limited by kinetic competition with the native reaction chain¹⁰. For example, in itaconate biosynthesis, substrate availability can limit the biotechnological production of valuable compounds¹¹. Itaconate is a dicarboxylic acid used in the resin and plastic industry¹² and can be synthesized from a decarboxylation reaction of *cis*-aconitate by *cis*-aconitate decarboxylase^{11,13}, with *cis*-aconitate as an intermediate molecule that is transiently generated in the tricarboxylic acid (TCA) cycle via the enzyme aconitase, which converts citrate into isocitrate⁸. In addition, the higher affinity for *cis*-aconitate leads aconitase to rapidly convert this compound into isocitrate⁸, reducing the *cis*-aconitate availability for itaconate biosynthesis catalyzed by *cis*-aconitate decarboxylase^{11,13,14}.

One solution used by nature to avoid competing reactions is spatial compartmentalization^{13,15,16}, which utilizes the physical separation of an intermediate to block kinetic competition between diverse chemical reactions^{13,16}. For example, *Aspergillus terreus*, a native itaconate producer, facilitates spatial compartmentalization of *cis*-aconitate by pumping out mitochondrial *cis*-aconitate into the cytosol by exchanging cytosolic oxaloacetate via an antiporter, MttA (Supplementary Fig. 1)¹³. This physical separation allows the fungus to accumulate *cis*-aconitate in the cytosol and efficiently produce itaconate by avoiding competition with the TCA cycle. Further, itaconate production can be efficiently regulated via MttA activity¹⁷. However, because of the insufficiently implemented genetic engineering tools and

complicated culture conditions of *A. terreus*, research focused on itaconate production has been performed in well-known workhorse cells such as *Escherichia coli*^{11,14}. Mimicking the spatial compartmentalization in *E. coli* would be an efficient approach for itaconate production; however, prokaryotic hosts lack a membranous system, leading to challenges in building a compartmentalized system^{11,14,15}.

To overcome the inapplicability of spatial compartmentalization in bacteria, we propose kinetic compartmentalization for kinetically separating competitive reactions by releasing the intermediate from the native reaction chain without creating a physical barrier. Particularly, we kinetically compartmentalized the itaconate production reaction from the TCA cycle by introducing a non-natural enzyme that can separate consecutive biochemical reactions catalyzed by aconitase. We hypothesized that introducing a non-natural biochemical reaction into *E. coli* cells, which can effectively synthesize *cis*-aconitate from citrate, would increase the intracellular *cis*-aconitate level, thus triggering its conversion into itaconate through catalysis by *cis*-aconitate decarboxylase.

In this study, we successfully engineered an endogenous 2-methylcitrate dehydratase, PrpD¹⁸, to catalyze the conversion of citrate into *cis*-aconitate. PrpD was evolutionarily engineered to have high specificity toward citrate by computational simulation design and high-throughput screening^{19–21}. Based on the non-natural enzyme, we constructed a novel itaconate production pathway that was kinetically isolated from the TCA cycle (Fig. 1). Using our approach, we obtained a significantly increased itaconate yield of up to 9.81-fold compared to the parental strain at 43.0% of the theoretical maximum. This kinetic compartmentalization concept allows bacteria to overcome the limitation of being a spatially inseparable host by introducing a new biochemical reaction to efficiently synthesize an intermediate molecule. This method shows potential for kinetic compartmentalization in prokaryotes, considering the substantial number of promiscuous enzymes that can be engineered using this approach.

Results

Selection of target enzyme for compartmentalization

To separate *cis*-aconitate production from the TCA cycle, we first reviewed the detailed reaction mechanisms of *cis*-aconitate synthesis in *E. coli*. The precursor of itaconate, *cis*-aconitate, can be synthesized by aconitase encoded by *acnA* and *acnB* in *E. coli*. Dehydration of citrate is sequentially coupled to rehydration of *cis*-aconitate by the same enzymes, which appear to catalyze the isomerization of citrate to isocitrate. Thus, *cis*-aconitate was temporarily synthesized as a reaction intermediate (Supplementary Fig. 2a). Furthermore, the catalytic efficiency (k_{cat}/K_m) of aconitase is much higher for *cis*-aconitate than for citrate⁸. Consequently, *cis*-aconitate accumulates at very low levels in native *E. coli*^{11,14}, resulting in low itaconate production. Aconitase may only conduct a dehydration reaction. However, the dehydration and rehydration reactions are coupled with two main catalytic residues, H444 and S244²². H444 functions as a proton donor, whereas S244 acts as a proton acceptor in the first

dehydration reaction²², after which these residues switch roles. In the rehydration reaction, H444 acts as a proton acceptor and S244 functions as a proton donor²². After these two reactions, the catalytic residues return to the initial state to conduct another enzyme reaction. Thus, it was difficult to isolate the dehydration reaction.

Thus, we screened enzymes with dehydration activity; their known substrates are similar to those of citrate structures. Among all enzyme candidates, the endogenous enzyme of *E. coli*, PrpD, converts 2-methylcitrate into 2-methyl-*cis*-aconitate in a one-step dehydration reaction without rehydration to 2-methylisocitrate (Supplementary Fig. 2b)¹⁸. This enzyme has a much lower affinity and catalytic efficiency for citrate and *cis*-aconitate compared to aconitases¹⁸. Engineering PrpD to alternatively catalyze citrate may be a useful strategy for enhancing the pool of *cis*-aconitate for itaconate production.

Development of itaconate-specific screening system

Recently, a itaconate-responsive LysR-type transcription factor, ItcR, from *Yersinia pseudotuberculosis* was shown to regulate the expression of the itaconate degradation pathway based on the presence and amount of itaconate²³. We exploited ItcR and its cognate promoter, P_{ccI}, to construct an itaconate-responsive screening system. The screening system was designed to regulate the expression of the antibiotic resistance gene^{24,25} according to itaconate concentration. Specifically, ItcR was constitutively expressed using a synthetic promoter (P_{BBa_J23106}), and the tetracycline resistance gene (*tetA*) was controlled under P_{ccI}. Collectively, the system was intended to provide a growth advantage under tetracycline pressure in environments with high itaconate concentrations or in high-producing strains (Fig. 2a).

To validate the screening system, itaconate-responsive screening system was transformed into an acid-tolerant *E. coli* W strain¹⁴ to produce the WS strain (Supplementary Table 1). We demonstrated the effectiveness of the screening system and further improvement in itaconate production using a previously developed itaconate-producing *E. coli* strain with acetate as the sole carbon source¹⁴. The tetracycline concentration as the selection pressure was varied up to 15 mg/L and growth retardation were confirmed in accordance with the increased tetracycline concentration, indicating tight regulation of ItcR/P_{ccI} (Fig. 2b). In addition, the gradual increase in the specific growth rate was validated by extracellular addition of itaconate under selection pressure. Specifically, the specific growth rate increased as the concentration of itaconate increased to 2 g/L. Additionally, the growth rate according to itaconate showed different tendencies depending on the tetracycline selection pressure (Fig. 2b). Collectively, these results indicate that the itaconate-responsive screening system was successfully constructed and is widely applicable for itaconate production.

Screening PrpD mutant with altered substrate specificity

To select enzyme variants that can produce *cis*-aconitate, PrpD protein engineering was conducted using the itaconate-responsive screening system. Target residues for mutagenesis were selected based on structural analysis of tartrate-bound MmgE (PDB code: 5MUX), the homologue of PrpD derived from *Bacillus subtilis*²⁶ (Supplementary table 2). To enhance the catalytic activity toward citrate rather than 2-methylcitrate, which has no methyl group on the second carbon, residues near the methyl group position of 2-methylcitrate were selected as targets: W110, G111, and I331. A mutant library of PrpD (W110, G111, and I311 residues) was constructed for expression in moderate strength (BBa_P_{J23108}) based on structural analysis (see **Methods**) with theoretical 20³ variants numbers in size and transformed into the WAICS strain.

To increase the intracellular level of citrate and ensure the activity of PrpD mutants, the catalytic efficiency of the competing enzyme, AcnB, was decreased through site-directed mutagenesis (AcnB^{W482R}, Table 1)²⁷. Mutagenesis of AcnB efficiently lowered the catalytic efficiency to citrate by 3.75-fold (Table 1). Notably, the glyoxylate shunt pathway was activated to increase anaplerosis and enhance itaconate production by inactivating the IclR-encoding transcriptional repressor^{14,28}. The resulting WAIC strain with AcnB^{W482R} showed a lower cell biomass (2.16 g DCW/L) and itaconate production (0.26 g/L) by 1.17- and 1.22-fold, respectively, compared to the WCI strain (Supplementary Fig. 3). Nevertheless, the accumulation of citrate was significantly enhanced by up to 0.51 g/L, as expected. Overall, an environment was created in which the PrpD mutant with altered substrate specificity produced increased itaconate and showed a sufficient growth advantage with the screening system. Screening was conducted by increasing the selection pressure from 7–15 mg/L of tetracycline over four rounds.

Characterization of enriched PrpD mutants

After enrichment, 10 isolated mutants were analyzed, and five types of mutants were characterized (Supplementary Table 3). We initially validated itaconate production for each mutant (WAICP^{NNN} strains, Supplementary Table 1) at the test tube scale (Supplementary Fig. 4). The WAICP^{VTL} strain (PrpD^{VTL} with W110V, G111T, and I331L) showed a 1.50-fold increase in itaconate production, whereas most mutants showed a decreased level of itaconate production compared to the WAICP strain with wild-type PrpD.

An additional culture was conducted at the flask-scale to validate the WAICP^{VTL} strain (Fig. 3a and 3b). Interestingly, the cell biomass was remarkably reduced in the WAICP^{VTL} strain (Fig. 3b) compared to that of the WAICP strain (Fig. 3a) by 1.80-fold (1.01 g DCW/L), and citrate accumulation was reduced by 2.00-fold (0.15 g/L), similar to that of the WCI strain (Fig. 3b and Supplementary Fig. 3a). These results indicate that PrpD^{VTL} exhibits increased catalytic efficiency toward the citrate to produce *cis*-aconitate, which induced kinetic separation and redirected more citrate for use in itaconate production. Indeed, the WAICP^{VTL} strain showed a 2.56-fold increase in itaconate production to 0.86 g/L compared to the WAICP strain. Moreover, the itaconate yield was significantly enhanced in the WAICP^{VTL} strain by up to 4.76-fold

(0.27 g/g), indicating that kinetic compartmentalization strategy is a powerful approach for enhancing the *cis*-aconitate availability and itaconate production.

To validate the effect of each residue on PrpD^{VTL}, mutants at single residues and a combination of double mutants were generated as the WAICP^V, WAICP^T, WAICP^L, and WAICP^{TL} strains (Supplementary Table 1); these mutants showed no significant difference in itaconate production compared to the WAICP strain (Supplementary Fig. 5). In contrast, the WAICP^{VT} and WAICP^{VL} strains showed 3.55- (1.19 g/L) and 3.84-fold (1.28 g/L) increases in the itaconate titer at 48 h (Supplementary Fig. 5 and Fig. 3c), respectively, which are even higher than that of WAICP^{VTL} (Fig. 3b). These results agree with those of structural analysis, as W110 is the closest residue among the target residues to the methyl group of the second carbon on 2-methylcitrate (Supplementary Table 2), combined with the rationale for the initial multi-residue library design.

Kinetic and structural analysis of PrpD mutants

The enzyme kinetics of wild-type PrpD, PrpD^{VTL}, PrpD^{VT}, and PrpD^{VL} were characterized to evaluate the enhancement of itaconate production (Table 2). As demonstrated previously, wild-type PrpD shows promiscuity for catalyzing citrate¹⁸. However, this enzyme showed 12.21-fold lower catalytic efficiency compared to AcnB (Tables 1 and 2), supporting the slight decrease in citrate and increase in itaconate of the WAICP strain (Fig. 3a) compared to the WAIC strain (Supplementary Fig. 3b). In contrast, the higher affinity for citrate (15.81 mM of K_m) was confirmed in PrpD^{VL} compared to wild-type PrpD (66.39 mM), as expected. However, the mutant displayed a 1.35-fold lower turnover rate (k_{cat}) for citrate (7,804.72 s⁻¹), improving the catalytic efficiency by 3.11-fold (Table 2).

A noticeable decrease in the affinity for *cis*-aconitate was also observed in PrpD^{VL} (1.52 mM of K_m) compared to wild-type PrpD (0.70 mM, Table 2). The change in the binding pocket following mutagenesis may have altered the affinity for *cis*-aconitate and citrate^{25,29}. In addition, the turnover rate of PrpD^{VL} was reduced (96.34 s⁻¹), leading to a 2.68-fold decrease in catalytic efficiency. Collectively, these results indicate that PrpD^{VL} efficiently extracted citrate from the TCA flux and induced kinetic compartmentalization by converting it into *cis*-aconitate to facilitate itaconate production (Fig. 1).

Computational simulations of 2-methylcitrate into PrpD enzyme showed that in the wild-type, single mutated (PrpD^V, PrpD^T, and PrpD^L), and double mutant (PrpD^{TL}) enzymes, the methyl group faced the residues W110 and G111 (wild type or mutated), and the conformation shifted in the opposite direction (Supplementary Fig. 6). Moreover, docking simulations revealed a slight decrease in hydrogen bonds between 2-methylcitrate and PrpD residues in the double mutants PrpD^{VT} and PrpD^{VL} and triple mutant PrpD^{VTL} compared to simulation into the active pocket of wild-type (Table 3). In contrast, docking predictions of citrate interactions showed an increased number of hydrogen bonds between this ligand and PrpD residues in all mutants compared to in the wild-type enzyme (Supplementary Fig. 7, Table 3). Interestingly, mutants showing a higher substrate specificity for citrate (PrpD^{VT}, PrpD^{VL}, and PrpD^{VTL})

exhibited a greater increase in hydrogen bonds between citrate and PrpD residues, with W110V potentially acting as the most important residue determining the substrate shift, which is supported by the fact that mutating this single amino acid increased the number of hydrogen bonds, as for the previously mentioned double and triple mutants (Table 3). Finally, docking on the double mutant PrpD^{TL} gave the same amount of hydrogen bonds with 2-methylcitrate and citrate, in accordance with the data showing the lowest enzymatic activity of this mutant towards citrate.

Further flux optimization for increasing production

The WAICP^{VL} strain was further optimized. First, PrpD^{VL} expression was optimized by employing constitutive promoters (Supplementary Fig. 8)³⁰. Itaconate production was significantly affected by PrpDVL expression, as expected, indicating that efficient kinetic compartmentalization can allow metabolic flux to be regulated by changing the activity of PrpD^{VL} in a manner similar to spatial compartmentalization in *A. terreus*, where the precise flux distribution is regulated by MttA activity. The WAICP100^{VL} strain with the highest PrpD^{VL} expression showed the highest itaconate production (1.35 g/L) after 48 h of cultivation. Next, the TCA cycle and glyoxylate shunt were additionally activated by overexpression of citrate synthase and isocitrate lyase encoded by *gltA* and *aceA* for further flux amplification and to facilitate the anaplerotic reaction to maximize itaconate production, respectively^{14,31} (Fig. 4a and b). The expression of *aceA* was varied to determine the optimized flux distribution between the glyoxylate shunt and TCA cycle^{14,31}. The itaconate titer was increased along with increased expression of *aceA* (Fig. 4a), and the WAICPG5 strain with the highest expression level of *aceA* showed a 1.52-fold increase in itaconate production (2.03 g/L) compared to the WAICP100^{VL} strain after 48 h of cultivation. Finally, itaconate production reached up to 3.13 g/L at 96 h; the yield was maintained at a significant level (0.31 g/g, 43.0% of theoretical maximum yield) throughout cultivation and was driven by the substantially increased acetate uptake rate (Fig. 4b)¹⁴. These results indicate that in addition to the strongly kinetically compartmentalized flux toward the itaconate, the flux distribution could be optimized for itaconate production.

Discussion

In nature, efficiently designed spatial compartmentalization systems have been introduced to preserve substrate availability¹³ or prevent damage caused by toxic intermediates^{15,32}. Naturally assembled proteinaceous organelles including carboxysomes³³ and metabolosomes³⁴ have been detected even in some prokaryotes. However, in most prokaryotes, simultaneous reactions involving numerous substrates within a single space are determined only by the kinetic properties of the enzymes, which have evolved to be well-coordinated to maximize cell growth with high precision. Based on these characteristics, we achieved kinetic compartmentalization in prokaryotes by developing a non-natural enzymatic reaction. Given that around 40–50% of enzymes with known functions have multiple substrates and 10–20% of these multi-substrate specific enzymes can mediate consecutive reactions (Supplementary Fig. 9), our

approach can be applied to other consecutive reactions to enable metabolic engineering of unreachable intermediates.

Numerous studies of the heterologous production of itaconate have consistently focused on substrate availability^{11,14,35} but could not imitate or recapitulate the spatial compartmentalization strategy of its native producer, *A. terreus*, pumping out *cis*-aconitate into an independent space¹³. Therefore, we introduced kinetic compartmentalization to provide an efficient supply of *cis*-aconitate. Rather than using existing aconitase which is highly reactive to *cis*-aconitate, the promiscuous enzyme PrpD was semi-rationally engineered and applied in itaconate production. Especially, we applied the itaconate production from acetate, which is a non-preferred carbon source, and generally, intensive engineering should be essential for its efficient conversion¹⁴. The efficient itaconate production using PrpD mutant is also expected to be widely applicable to production systems from glucose¹¹ or glycerol³⁶.

In addition to the modified catalytic characteristic of PrpD, the non-natural *cis*-aconitate synthesis reaction can be regulated by the expression level of the PrpD mutant. Generally, the itaconate titer was increased when a stronger promoter was used to express PrpD^{VL} (Supplementary Fig. 8), indicating that more carbon flux was kinetically compartmentalized according to PrpD^{VL} expression. Surprisingly, the resulting strain WAICPG5 produced up to 3.13 g/L itaconate at 43.0% of the theoretical maximum yield from acetate (Fig. 4b), which showed a 9.81-fold increase in yield compared to the parental strain (WCI strain, Table 1), indicating the efficient kinetic compartmentalization.

An itaconate-responsive screening system was successfully constructed in this study. The initial library was constructed to sufficiently cover all combinations; however, while determining the effect of each residue, double mutants with more desired characteristics were identified. In addition, the enriched population clearly contained mutants that improved itaconate production; however, not all mutants showed the desired phenotype. After the PrpD^{VTL} mutant dominates during the initial enrichment, hitchhikers may have been enriched together through crosstalk effects³⁷. Nonetheless, we screened for effective mutant in four rounds of enrichment and observed an unintended decrease in catalytic efficiency toward *cis*-aconitate, supporting the effectiveness of the high-throughput screening system^{21,24,25,38,39}. This method can be further applied in diverse strategies for itaconate production, including adaptive laboratory evolution, the evolution of *cis*-aconitate decarboxylase, and even the *de novo* itaconate production pathway.

Methods

Oligonucleotides and reagents

Oligonucleotides were obtained from Cosmo Genetech (Seoul, South Korea). For routine DNA manipulation, Takara PrimeSTAR™ HS DNA Polymerase (Shiga, Japan) was used for DNA amplification. Plasmid DNA and genomic DNA were extracted using the AccuPrep^R Nano-Plus

Plasmid Mini Extraction Kit (Bioneer, Daejeon, Korea) and GeneAll[®] Exgene[™] Cell SV Kit (GeneAll, Seoul, Korea), respectively. Enzymes, including ligase and restriction enzymes, and Gibson assembly were purchased from New England Biolabs (Ipswich, MA, USA). Other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in Supplementary Table 1. *Escherichia coli* Mach1-T1^R (Thermo Fisher Scientific, Waltham, MA, USA) was utilized as a cloning host, and the acid-tolerant *E. coli* W strain was utilized as an expression host¹⁴ and source of wild-type *prpD*. The WA and WAI strains were constructed using the Lambda-Red recombination system with plasmids pM_FKF, pKD46, and pCP20⁴⁰. The pCDF_CAD plasmid was used to construct pCAD, and variants of the pCOGA plasmid (pCOGA, pCOGA1,3,4,5) were used to amplify the glyoxylate shunt from our previous study¹⁴. The taconate-responsive screening system, a codon-optimized *itcR* fragment from *Y. pseudotuberculosis*, was synthesized and assembled with amplified pETduet-1 and *tetA* fragments^{23,24}. pPRPD was constructed by assembling amplified pACYCduet-1 and *prpD* from *E. coli* W. Genetic variants of PrpD were obtained by site-directed mutagenesis and the Gibson assembly method⁴¹. The terminators and promoters for all vectors were obtained from the Registry of Standard Biological Parts (<http://parts.igem.org>). Synthetic 5' untranslated regions were computationally designed using UTR Designer (http://sbi.postech.ac.kr/utr_designer)⁴².

Cell cultivation and enrichment for screening

Cells were cultivated in modified minimal acetate medium containing 0.5 g/L MgSO₄·7H₂O, 2.0 g/L NH₄Cl, 1.0 g/L NaCl, 2.0 g/L yeast extract, and 100 mM potassium phosphate buffer (pH 7.0)¹⁴. As a carbon source, 10 g/L neutralized acetate (pH 7.0) was added to the medium. To maintain the plasmids, antibiotics were added to the medium (50 µg/mL streptomycin, 50 µg/mL kanamycin, 34 µg/mL chloramphenicol, and 100 µg/mL ampicillin).

For itaconate production, single colonies of each strain were inoculated into 3 mL of medium in a 15 mL test tube. After 12 h, the cell cultures were inoculated into 3 or 20 mL of fresh medium in the test tube or 300-mL Erlenmeyer flasks, respectively, to an optical density at 600 nm (OD₆₀₀) of 0.05. Isopropyl-β-D-thiogalactopyranoside was initially added to a final concentration of 0.1 mM for induction. Cultures were performed in biological triplicate with continuous shaking (200 rpm) at 30°C¹⁴. The pH was adjusted to

7.0 by adding an appropriate amount of 5 M HCl solution. Culture samples were periodically collected and stored at -80°C for analysis.

To validate the screening system and enrichment for screening PrpD mutants, we exerted selection pressure using tetracycline. The concentration of tetracycline was varied from 0–50 mg/L to determine the initial selection pressure. The toxicity of itaconate was determined using various concentrations of itaconate from 0–2 g/L. The mutant library was initially enriched with 7 mg/L tetracycline and increased to 15 mg/L over four rounds of enrichment. All cultures except for the enrichment culture were conducted in triplicate.

Analytical methods to detect cellular metabolites

Cell biomass (OD_{600}) was measured using a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan), and the dry cell weight (DCW) was calculated by converting 1 unit of OD_{600} to 0.31 g/L⁴³. Metabolites were measured using an Ultimate 3000 high-performance liquid chromatography system (Dionex, Sunnyvale, CA, USA). Filtered samples were analyzed using an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). As the mobile phase, 5 mM H_2SO_4 was used at a flow rate of 0.6 mL/min; the temperature of the column oven was maintained at 14°C ²⁸. The refractive index and absorbance at a UV wavelength of 210 nm were monitored using a Shodex RI-101 detector (Shodex, Klokkefaldet, Denmark) and variable wavelength detector (Dionex).

Characterization of enzyme kinetics

Cells were cultivated for 9 h after induction, and cell pellets were resuspended in 40 mM Tris-HCl buffer (pH 8.0). The cells were lysed using a Qsonica sonicator (Sonics & Materials, Newtown, CT, USA) for 3 min. To avoid oxidation of an iron-sulfur cluster of enzymes, dithiothreitol and $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ were added to final concentrations of 2.5 and 0.25 mM, respectively⁸. The cell lysates were centrifuged for 10 min at $13,000 \times g$ at 4°C . The supernatants were utilized to purify the 6X His-tagged enzymes with a MagListoTM His-tagged protein purification kit (Bioneer) under anaerobic conditions to prevent inactivation of aconitase activity⁸. The elutes were treated with 1 mM dithiothreitol, 0.14 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, and 0.12 mM Na_2S to prevent oxidization of iron-sulfur clusters. The amount of purified enzyme was quantified and adjusted to the same amount using the Bradford assay.

The enzyme assay was conducted in 2 mM Tris-HCl buffer with varying amounts of substrate: 1, 2, 5, 10, and 20 mM for citrate; 0.05, 0.1, 0.2, 0.5, and 1.0 mM for *cis*-aconitate. This reaction was conducted at 37°C for 10 min followed by inactivation of the enzyme at 90°C for 5 min. The samples were analyzed

using a high-performance liquid chromatography system with an XTerra^R RP18 column. H₂SO₄ (5 mM) was utilized as the mobile phase at a flow rate of 0.6 mL/min. Absorbance was monitored at a UV wavelength of 215 nm. All assays were conducted in triplicate.

Structural analysis for PrpD mutant library design

To identify the catalytic sites of PrpD, a structural model of PrpD was first generated using the structure of the homologous protein MmgE (PDB code: 5MUX)²⁶ from *B. subtilis*. From the ligand of 5MUX, the catalytic site of MmgE was identified, and well-conserved residues were found in both PrpD and MmgE (Supplementary Fig. 10)⁴⁴. A 3D model of 2-methylcitrate was generated from the downloaded SDF format of (2*S*,3*S*)-2-methylcitrate from PubChem. The SDF file was converted to PDB format using the online SMILES translator and structure file generator (<https://cactus.nci.nih.gov/translate/>). Finally, the 3D model of 2-methylcitrate was aligned with the ligand of 5MUX using LS-Align. From this final model structure, the distance between the catalytic sites of PrpD and nearby residue from the methyl group of 2-methylcitrate was calculated (Supplementary Table 3).

Among the residues in the catalytic site, those key for catalytic reactions and important for the interaction with the carboxyl group of citrates were preserved to maintain catalytic activity. For example, histidine often acts as a proton donor and acceptor, which is critical in the catalytic reaction, and arginine is an open-form salt bridge with the anion form of a carboxyl group. Hydrophobic and small residues near the methyl group of 2-methylcitrate were selected as target residues for the mutant library.

Docking simulations of the mutants

Both the PrpD natural substrate 2-methylcitrate (PubChem ID: 5460420) and citrate (PubChem ID: 31348) were docked into the active pocket of the PrpD enzyme using the crystallographic structure of apo-protein 2-methylcitrate dehydratase from *E. coli* (PDB: 1SZQ)²⁶ using Chimera software⁴⁵. This protein structure included two chains, one of which was deleted before docking simulation. The protein was prepared by eliminating water molecules and adding hydrogen and charge. The ligands were also charged before analysis. As the presence of the methyl group of 2-methylcitrate is an important factor in the substrate specificity of PrpD, the orientation of this group was evaluated in all docking simulations along with the number of hydrogen bonds between the ligand and protein residues resulting from each analysis.

Characterization of multi-substrate and consecutive enzymes

We collected the enzyme reactions of *E. coli* from KEGG (<https://pubmed.ncbi.nlm.nih.gov/10592173/>) and BioCyc (<https://pubmed.ncbi.nlm.nih.gov/29447345/>). To characterize enzymes, have multiple

substrate specificities, we counted the number of known reactions for each enzyme. If the enzyme is involved in more than one enzymatic reaction, we categorized them as multiple substrate-specific enzymes. Then, we further characterize enzymes that can conduct consecutive reactions by themselves by checking if the same enzyme can take their product in one reaction as substrate in the other reactions. During the analysis we excluded the following molecules because they can work as cofactors or common substrates: "H₂O", "H⁺", "ATP", "GTP", "CTP", "TTP", "ADP", "GDP", "CDP", "TDP", "NADH", "NADH⁺", "NADPH", "CO₂", "NAD", "NADP⁺", "AMP", "GMP", "CMP", "TMP", "Fe²⁺", "Fe³⁺", and "phosphate".

Declarations

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Author Contributions

D.Y., M.H.N., J.M., M.K., A.M., J.W.L., J-S.Y., and G.Y.J. conceived the project. D.Y., M.H.N., and J.M. designed and conducted the experiments. D.Y., M.H.N., J.M., M.K., A.M., J.W.L., J-S.Y., and G.Y.J. conducted data analysis and interpretation and wrote the manuscript. J-S.Y. and G.Y.J. supervised the project. All the authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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Tables

Table 1. Kinetic parameters of AcnB and AcnB^{W482R}

Enzyme	Substrate	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (mM ⁻¹ s ⁻¹)
AcnB	Citrate	4,882.62 ± 696.56	3.32 ± 1.03	1,402.61 ± 249.94
AcnB ^{W482R}	Citrate	2,232.31 ± 758.05	6.34 ± 3.12	374.31 ± 67.74

Table 2. Kinetic parameters of PrpD, PrpD^{VTL}, PrpD^{VT}, and PrpD^{VL}

Enzyme	Substrate	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (mM ⁻¹ s ⁻¹)
PrpD	Citrate	10,562.00 ± 3,427.82	66.39 ± 22.03	159.37 ± 2.36
	<i>cis</i> -Aconitate	119.03 ± 13.42	0.70 ± 0.10	170.61 ± 5.65
PrpD ^{VTL}	Citrate	9,586.41 ± 1,193.47	35.32 ± 5.17	272.17 ± 11.72
	<i>cis</i> -Aconitate	81.83 ± 14.14	0.72 ± 0.10	113.60 ± 14.61
PrpD ^{VT}	Citrate	6,093.22 ± 1,122.92	14.82 ± 2.38	410.08 ± 22.85
	<i>cis</i> -Aconitate	172.08 ± 19.98	1.72 ± 0.25	100.52 ± 3.18
PrpD ^{VL}	Citrate	7,804.72 ± 693.10	15.81 ± 1.89	494.97 ± 15.22
	<i>cis</i> -Aconitate	96.34 ± 4.55	1.52 ± 0.08	63.63 ± 3.33

Table 3. Docking simulations summary

Receptor	Ligand	Binding energy (kJmol ⁻¹)	H-bonds
PrpD	2-Methylcitrate	-5.0	4
	Citrate	-5.0	4
PrpD ^V	2-Methylcitrate	-4.9	4
	Citrate	-4.8	6
PrpD ^T	2-Methylcitrate	-5.2	4
	Citrate	-5.1	5
PrpD ^L	2-Methylcitrate	-5.1	4
	Citrate	-5.0	5
PrpD ^{VT}	2-Methylcitrate	-4.9	3
	Citrate	-4.8	6
PrpD ^{VL}	2-Methylcitrate	-4.9	3
	Citrate	-4.8	6
PrpD ^{TL}	2-Methylcitrate	-5.1	5
	Citrate	-5.0	5
PrpD ^{VTL}	2-Methylcitrate	-4.9	3
	Citrate	-4.8	6

Figures

Figure 1

Schematic diagram of overall strategies for kinetic compartmentalization using the non-natural enzyme reaction. 2-Methylcitrate dehydratase (PrpD) was evolutionarily engineered to alter the substrate preference and catalytic efficiency towards citrate. The desired PrpD mutant has higher activity towards citrate to secure cis-aconitate, resulting in increased itaconate production. The non-natural enzyme exhibits a kinetically compartmentalized effect without actual spatial separation. Citrate (blue); isocitrate (orange); cis-aconitate (green); itaconate (red).

Figure 2

Design and validation of itaconate-responsive screening system. (a) Schematic diagram of itaconate-responsive screening system based on the tetracycline resistance gene. (b) Specific growth rates of WS strains were plotted in accordance with itaconate concentration under varied tetracycline concentrations. Error bars indicate the standard deviations from experiments conducted in triplicate.

Figure 3

Fermentation profiles of itaconate production (a) WAICP, (b) WAICPVTL, and (c) WAICPVL strains. The left y-axis and y-offset represent the cell biomass (g DCW/L) and acetate (g/L), respectively. The right y-axis indicates the production of itaconate and citrate (g/L). The x-axis denotes time (h). Symbols: circles, cell biomass; up-triangles, acetate; squares, itaconate; down-triangles, citrate. Error bars indicate the standard deviations from three biological replicates.

a **b** **Figure 4**

Further optimization of itaconate production (a) Comparison of the itaconate titer of strains with varied *aceA* expression for 48 h. (b) Fermentation profile of WAICPG5 strain. The left y-axis and y-offset represent the cell biomass (g DCW/L) and acetate (g/L), respectively. The right y-axis indicates the production of itaconate (g/L). The x-axis denotes time (h). Error bars indicate the standard deviations from three biological replicates.

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