Rational design of a highly efficient catalytic system for the production of 3'-phosphoadenosine-5'-phosphosulfate from ATP

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Abstract

The compound 3'-phosphoadenosine-5'-phosphosulfate (PAPS) serves as a sulfate group donor in the production of valuable sulfated compounds, such as glycosaminoglycan and oxamniquine. However, elevated costs and low conversion efficiency limit the industrial applicability of PAPS. Here, we designed and constructed an efficient and controllable catalytic system for the conversion of ATP (disodium salt) into PAPS without inhibition from by-products. In vitro and in vivo testing in Escherichia coli identified adenosine-5'-phosphosulfate kinase from Penicillium chrysogenum (PcAPSK) as the rate-limiting enzyme. Based on analysis of the catalytic steps and molecular dynamics simulations, a mechanism-guided "ADP expulsion" strategy was developed to generate an improved PcAPSK variant (L7), with a specific activity of 48.94 U·mg-1 and 73.27-fold higher catalytic efficiency (kcat/Km) that of the wild-type enzyme. The improvement was attained chiefly by reducing the ADP-binding affinity of PcAPSK, as well as by changing the enzyme's flexibility and lid structure to a more open conformation. By introducing PcAPSK L7 in an in vivo catalytic system, 73.59 mM (37.32 g·L-1) PAPS was produced from 150 mM ATP in 18.5 h using a 3-L bioreactor. The achieved titer is the highest reported to date and corresponds to a 98.13% conversion rate. The proposed strategy will facilitate industrial production of PAPS as well as the engineering of similar enzymes.

Introduction

The compound 3'-phosphoadenosine-5'-phosphosulfate (PAPS) acts as a sulfate group donor in the production of glucosinolate, heparin, chondroitin sulfate, and oxamniquine (Ji et al., 2020; Zhang, Lin, Huang, & Linhardt, 2020). At present, PAPS can be produced either via metabolic or enzymatic synthesis. In the metabolic biosynthetic pathway (Harjes, Bayer, & Scheidig, 2005; Sekulic, Dietrich, et al., 2007), ATP is converted to PAPS by ATP sulfurylase (ATPS) and adenosine-5'-phosphosulfate (APS) kinase (APSK). However, because ATP is an energy-rich compound with limited capacity to accumulate in cells, only 0.8–1.2 μmol PAPS is obtained per gram of cells (Badri et al., 2021; Badri, Williams, Xia, Linhardt, & Koffas, 2019). Enzymatic synthesis (Burkart, Izumi, Chapman, Lin, & Wong, 2000), which includes single-enzyme catalysis and dual-enzyme cascade catalysis, has been developed to further enhance PAPS output.

In single-enzyme catalysis, aryl sulfotransferase (ASST) converts 3'-phosphoadenosine-5'-phosphate (PAP) and *p* -nitrophenyl sulfate (PNPS) to PAPS (Jin et al., 2020; Z. Zhou et al., 2019). Over the past decade, various ASSTs from mammalian species and bacteria, such as*Streptomyces* sp. (Kaysser et al., 2010) and *Escherichia coli* (Malojcic, Owen, & Glockshuber, 2014; Malojcic et al., 2008), have been identified and used to synthesize PAPS. However, ASST expression and affinity for PAP need to be improved (Berger, Guttman, Amar, Zarivach, & Aharoni, 2011). To increase yields, fusion with the signal peptides Cex, YebF, and PelB has been applied, allowing for ASST secretion and fourfold higher expression (89.67 U·mL^{-1}) (Z. Zhou et al., 2019). To improve affinity for PAP, random mutagenesis and molecular evolution of the PAP-binding pocket

gate loop were applied based on the enzyme's crystal structure. PAP affinity and ASST catalytic efficiency were thus increased by 2.48 times and 12.50 times, respectively (Z. Zhou et al., 2019). Still, some hurdles remain, as the regeneration of PAPS requires coupling to sulfotransferases and prevents PAPS accumulation (An, Zhao, Wei, & Zhou, 2017; Bao et al., 2015), while the elevated cost of PAP (38 \$·mg^{-1}, Sigma) lowers its commercial appeal.

A relatively cheap dual-enzyme cascade catalysis using ATP as the substrate was developed. Given that the price of ATP is only 1/25 that of PAP, this strategy offers an appealing alternative (Datta et al., 2020). The catalysis process includes two steps: first, sulfate and ATP are converted by ATPS to adenosine-5'-phosphosulfate (APS) and the by-product pyrophosphate (PPi) (Kang et al., 2018; Schmidt, 1977); second, APS kinase catalyzes the conversion of APS and ATP to PAPS and the by-product ADP (Badri et al., 2019). At present, this method has been used to synthesize PAPS at gram-level in the preparation of bioengineered heparin and chondroitin sulfate (Jian, Liu, Robert, & reports, 2014; Jin et al., 2020). However, by-product inhibition and low enzymatic activity compel a substrate conversion rate of only 47% (An et al., 2017). To alleviate inhibition, by-product degradation and recycling have been attempted (Michael D. Burkart, 2000; X. Zhou, Chandarajoti, Pham, Liu, & Liu, 2011). In one such example, the conversion rate was increased by 50%, and 5.0 g*L^{-1}PAPS was generated when ADP was transformed back to ATP, using phosphoenolpyruvate (PEP) as a phosphate donor (An et al., 2017).

APSK is a key factor affecting catalytic efficiency of the dual-enzyme cascade and is characterized by a typical "lid structure" (Gay, Segel, & Fisher, 2009). It catalyzes a sequential reaction, whereby ATP binds ahead of APS, and then PAPS leaves before ADP is released (Lansdon, Segel, & Fisher, 2002). If ADP is not released in time, an APS–enzyme–ADP termination complex forms, resulting in a decrease in enzymatic activity (Lansdon et al., 2002; Mueller & Shafqat, 2013). Protein crystallization (Ian J. MacRae, 2000; Poyraz et al., 2015), site-directed mutagenesis (Wang D. Z et al., 2016), and truncated mutagenesis (Ravilious, Westfall, & Jez, 2013; Sekulic, Konrad, & Lavie, 2007) have been used to study APSK. Secondary structure analysis of APSK from *Arabidopsis thaliana* showed that Arg^{93} was necessary for substrate recognition, and affinity for ADP was 217-times lower in the APSK^{R93A}mutant (Ravilious et al., 2013). Site-directed mutagenesis produced an*Oryza sativa Os* APSK$^{C36A/C69A}$ mutant, whose *kcat* was 43% lower than that in the wild-type, while the*Km* for APS was 1.6-fold higher (Wang D. Z et al., 2016). These studies focused mainly on APSK structure and its catalytic mechanism; however, attempts to improve catalytic performance of the enzyme have yielded only limited success.

In this study, a PAPS-producing catalytic system composed of a main module and an auxiliary module was designed and assessed *in vivo* . The main module converted ATP to PAPS, and the auxiliary module effectively eliminated by-product inhibition by hydrolyzing PPi to regenerate ATP from ADP. APSK was identified as the limiting step in this catalytic system due to delayed release of ADP. To further increase conversion efficiency, a mechanism-guided "ADP expulsion" strategy was developed to weaken the binding affinity of APSK for ADP and expand the bottleneck caused by its U-shaped release channel. Finally, by integrating the best variant in the cascade pathway, 73.6 mM (37.3 g*L^{-1}) PAPS was synthesized, using a 3-L fermenter, with 98.1% conversion.

Materials and methods

Strains, plasmids, and media

All strains and plasmids used in this study are listed in **Table S1** . The variants were constructed by whole plasmid PCR protocol with the main primers listed in **Table S2** . The PCR system (100 μl) was composed of template (100-150 ng), corresponding primers (20 μM with 2 μl), PrimeSTAR polymerase (1 μl; Takara Biomedical Technology, Dalian, China), 5 × PrimeSTAR Buffer (20 μl), dNTP mix (8 μl), and sterilized water. Next, DpnI was added to the PCR reaction mixture and incubated for 3 h at 37°C to eliminate the template plasmid. The digested product was transformed into *Escherichia coli* BL21(DE3) cells for the following screening or DNA sequencing (Genewiz, China). Luria-Bertani (LB) medium containing 5 g·L^{-1} yeast extract, 10 g·L^{-1} tryptone, and 10 g·L^{-1} NaCl was used for strain selection and propagation. Terrific

Broth (TB) medium containing 4 g·L^{-1} glycerin, 24 g·L^{-1} yeast extract, 12 g·L^{-1}tryptone, 2.31 g·$L^{-1}$$KH_2PO_4$, and 16.43 g·$L^{-1}$ K_2HPO_4 was used for protein expression. Chloramphenicol (30 μg·mL^{-1}), kanamycin (50 μg·mL^{-1}), and ampicillin (100 μg·mL^{-1}), and IPTG (0.4 mM) were added at the appropriate time.

Homology modeling and molecular dynamics (MD) simulations

The 3D structural of *Pc* APSK and its mutants were constructed based on its X-ray crystal structures (PDB ID: 1M7H) (Lansdon et al., 2002). The 3D structure of ADP was obtained from the ChemSpider Ultra 11.0 program (*http://www.chemspider.com/*). Nonpolar hydrogen atoms were added to the enzyme using the GROMACS 4.5.5 simulation package (*http://www.gromacs.org/*). The whole system was immersed in an explicit TIP 3P water box and extended with a thickness of at least 10 A from the dissolved atoms in each dimension. MD simulations were performed with GROMACS 4.5.5 and an AMBER03 force field following the three main steps of energy minimization, system equilibration, and production protocols. The 50 ns MD simulations were performed using NAMD 2.12 (derived from "Not Another Molecular Dynamics program", by University of Illinois) with the Charmm27 force field with a 2-fs time step at the temperature of 310 K. The enzyme and product were assigned to separate energy groups, and the binding energies between them were calculated as total intergroup potential energy. The MD simulations results were analyzed in Visual Molecular Dynamics software (VMD 1.9.3, by University of Illinois). All images of the structures shown here were generated using PyMol and CAVER. The two flexible regions (A and B) identified by MD simulations were as follows: region A comprised residues from L33 to V43, and B region included residues from Y137 to H181.

Analytical methods

The optical density at 600 nm (OD_{600}) was measured using a spectrophotometer. ATP, APS, and PAPS were quantified by high-performance liquid chromatography (HPLC). The analysis was performed with an HPLC system equipped with an polyamine column (YMC-Pack PolyamineII, 250 mmx4.6 mm) and 0.7 mM of KH_2PO_4 as the mobile phase. The flow rate of the mobile phase was 0.6 mL/min and oven temperature was maintained at 30 throughout the operation with an UV detector at 254 nm. Meanwhile, the product APS and PAPS were also analyzed by ESI-MS and the molecular weight was in accordance with that of standard.

More materials and methods are shown in Supporting Information.

Results

1 Cascade design and *in vitro* construction of the PAPS biosynthesis pathway

To efficiently produce PAPS, a sequential enzymatic cascade composed of a main module and an auxiliary module was designed (**Figure 1A**). In the main module, ATP and sulfate were first converted to APS and PPi by ATPS. Thereafter, APS and ATP were converted to PAPS and ADP by APSK. In the auxiliary module, a pyrophosphatase (PPA) was introduced to hydrolyze PPi to Pi, and a polyphosphate kinase (PPK) was introduced to regenerate ATP from ADP; in this way, the inhibitory effect of the by-products (PPi and ADP) was eliminated. Using the BRENDA database, four different microorganisms were selected as the sources of each enzyme: ATPS from *Kluyveromyces lactis* (*Kl* ATPS), APSK from*Penicillium chrysogenum* (*Pc* APSK), PPA from *E. coli*(*Ec* PPA), and PPK from *Rhodobacter sphaeroides*(*Rs* PPK). Following purification and *in vitro* evaluation, their optimal specific activity was determined (**Table 1** and**Table S3**).

To confirm the effectiveness of the main module *in vitro* ,*Kl* ATPS and *Pc* APSK were mixed at a 1:1 molar ratio in the presence of 20 mM ATP. After 10 h, formation of 4.56 mM PAPS as the final product was confirmed by nuclear magnetic resonance and mass spectrometry analyses (**Figure S1**), demonstrating the efficacy of using *Kl* ATPS and *Pc* APSK for converting ATP to PAPS. The effect of varying the *Kl* ATPS:*Pc* APSK ratio on PAPS titer was investigated with *Kl* ATPS activity fixed at 3.0 U*mL^{-1}. The highest PAPS titer (6.06 mM, conversion rate 59.6%) was achieved when *Pc* APSK activity was 4.5 U*mL^{-1} (**Figure 1B**). This result indicated an optimal *in vitro Kl* ATPS:*Pc* APSK ratio of 1:1.5. Next, the effect of adding *Ec*

PPA at 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 U*mL^{-1} on PAPS titer was examined (**Figure 1C**). Addition of 4.0 U*mL^{-1} *Ec* PPA to the main module led to a PAPS titer of 7.18 mM from 20 mM ATP, with conversion rate of 81.8%. Furthermore, when 7.0 U*mL^{-1} *Rs* PPK was added to the *in vitro*conversion broth, the PAPS titer increased to 8.05 mM, with a conversion rate of 92.5% (**Figure 1D**). Taken together, the optimal*Kl* ATPS:*Pc* APSK:*Ec* PPA:*Rs* PPK ratio was approximately 3:4.5:4:7.

2 *In vivo* construction of the PAPS biosynthesis pathway

To prove the effectiveness of the proposed catalytic pathway *in vivo* , the genes encoding *Kl* ATPS and *Pc* APSK in the main enzyme module and *Ec* PPA and *Rs* PPK in the auxiliary module were inserted into pACYCDuet-1 and pETDuet-1 plasmids, respectively. These were then used to transform *E. coli* BL21 (DE3), resulting in *E. coli* strain 01. Co-expression of the four enzymes was confirmed by protein gel electrophoresis of cell-free lysates. Then, the conversion performance of strain *E. coli* 01 was investigated with 30 g*L^{-1} wet cell (treated with 1g*L^{-1} Tween 80) at 30degC, it was found that the PAPS titer increased from 6.03 to 11.59 mM as the substrate ATP concentration was increased from 20 to 60 mM (**Figure 2A**). When the ATP concentration was increased >60 mM, the PAPS titer did not augment any further. In contrast, the conversion rate of PAPS decreased from 60.13% to 25.62%, when the ATP concentration increased from 20 to 80 mM. This drop was due to APS, an intermediate of the enzymatic cascade, which accumulated at increasing amounts (from 3.86 to 16.07 mM) in the conversion broth (**Figure 2A**). Intracellular enzymatic activity of *Kl* ATPS, *Pc* APSK, *Ec* PPA, and *Rs* PPK in *E. coli* 01 was 135.06, 12.75, 188.19, and 243.31 U*g^{-1} wet cells, respectively (the ratio was 3:0.28:4.18:5.41) (**Figure 2B**). This finding revealed the need to increase the intracellular enzymatic activity of *Rs* PPK and*Pc* APSK.

Previous studies had shown that *Rs* PPK could easily form inclusion bodies in cells. Therefore, four molecular chaperones (SUMO, MBP, TrxA, and GST) involved in expression of soluble proteins were co-expressed with *Rs* PPK to increase its activity in the cytoplasm. TrxA alone caused the intracellular activity of *Rs* PPK to increase from 243.31 (*E. coli* 01) to 317.38 U*mg^{-1} wet cells (*E. coli* 04) (**Figure 2C**), while *Kl* ATPS,*Pc* APSK, and *Ec* PPA reached 132.61, 12.85, and 186.63 U*g^{-1} wet cells, respectively (corresponding to a 3:0.29:4.22:7.18 ratio) (**Figure 2A**). To improve *Pc* APSK intracellular activity, its expression was increased by adding six ribosome-binding site (RBS) sequences (RBS1–RBS6) of different intensities (**Table S4**). RBS5 produced the best results and increased *Pc* APSK activity from 10.75 (*E. coli* 01) to 22.47 U*g^{-1} wet cells (*E. coli* 06) (**Figure 2D**). However, increasing the expression of *Pc* APSK decreased the transformation efficiency of the system (**Figure 2D**). Moreover, the intracellular activity of the other three enzymes to drop significantly to 95.07 (*Kl* ATPS), 87.35 (*Ec* PPA), and 194.05 (*Rs* PPK) U*g^{-1} wet cells (**Figure 2A**). These results suggested that increasing enzyme expression alone had limited efficacy on improving overall performance and might lead to an imbalance in intracellular resource allocation. To overcome this limitation, it is necessary to improve the inherent properties of*Pc* APSK through protein engineering.

3 ADP expulsion increases *Pc*APSK activity

To increase *Pc* APSK activity, the enzyme's catalytic mechanism and structural characteristics were first studied. The catalytic process of*Pc* APSK can be divided into three steps (**Figure 3A**): (i) ATP binds to the enzyme, causing the lid structure to close; (ii) APS reaches the binding site and reacts with ATP; and (iii) the lid structure is opened to first release PAPS and then ADP. If ADP is not released in time, a complex (Enzyme-ADP-APS) will be formed and hinders the catalysis progress. To study the interaction between *Pc* APSK and ADP, ADP was docked into the *Pc* APSK based on its crystal structure (PDB ID: 1M7H) (**Figure 3B**), and a bottleneck of 6.4 A in the release channel was identified using CAVER (**Figure 3C**). Then, molecular dynamics (MD) simulation was performed to calculate the root mean square fluctuation (RMSF) values and conformation of*Pc* APSK. As shown in **Figure 3D** , two regions (A and B) displayed higher RMSF values, suggesting that these two motifs could undergo noticeable movement and influence protein conformation. In addition, MD simulations revealed a conformational change of the lid, which regulated the release of ADP by controlling the "closed-open" movement (**Figure 3E**). ADP was immobilized by residues, whose binding free energy reached -55.81 kcal*mol^{-1} in the closed conformation.

These results proved that the release of ADP was unfavorable; hence, promoting it might speed up the catalytic reaction.

Based on the structure of ADP-APSK and MD simulations, an "ADP expulsion" strategy was designed to promote the release of ADP by (i) reducing the binding affinity of target residues, (ii) changing the conformation of the lid to widen the release tunnel. Thirty-four candidate residues in regions A and B were selected for alanine scanning (**Figure 4A**). Six residues whose activity was [?]30% higher were identified: binding residues S36A (35.2%), K38A (74.7%), and T40A135.17 (57.2%) in region A, and lid constituent residues K151A (107.2%), D139A (43.6%), and G167A (67.3%) in region B. Then, two smart mutation libraries (A and B) were constructed and screened for NNK-based site-saturation mutagenesis (**Figure 4B**). In library A, saturation mutation of K38 increased APSK activity by 5.31-fold in variant $L1_{K38G}$, whereas iterative saturation mutation of K38 and T40 produced mutant $L2_{K38G/T40S}$, which presented 12.12-fold higher activity. Notably, when mutation S36 was introduced into L2, resulting in variant $L3_{S36G/K38G/T40S}$, a significant drop in activity, amounting to only 4.99-fold of the wild-type (L0), was observed (**Figure 4B**). In library B, saturation mutation of K151 increased the activity by 4.50-fold in variant $L4_{K151V}$, and saturation mutation of G167 increased it by 7.80-fold in variant $L5_{K151V/G167I}$. Iterative saturation mutation of K151, G167, and D139 produced mutant $L6_{D139V/K151V/G167I}$, which presented 19.65-fold higher APSK activity. Finally, the best mutants from the two libraries were combined to generate variant $L7_{K38G/T40S/D139V/K151V/G167I}$. As shown in **Table 2** , variant L7 exhibited a 31.89-fold increase in *kcat* , from 0.61 to 19.45 $s^{-1}*mM^{-1}$, and a 45.74-fold higher specific activity (48.94 $U*mg^{-1}$) compared to L0.

To explain the improved *Pc* APSK activity from a mechanism point of view, binding affinity and release channel width were compared between variants L0 and L7. In the latter, binding affinity for ADP was reduced due to an increase in binding energy from -55.82 to -21.57 $kcal*mol^{-1}$, as calculated by MD simulations. As shown in **Figure 4C** , the average number of hydrogen bonds decreased from 31.1 to 27.3, which might have led to a weaker ADP–enzyme interaction. As a result, the root mean square distance increased from 0.55 to 0.63 A (**Figure 4D**), suggesting a reduced stability of*Pc* APSK. Analysis of the release channels revealed that the bottleneck in L7 increased by nearly 0.73 A to 7.13 A (**Figure 4E**) and was more conducive to the release of ADP. In addition, the lid of the L7 variant changed to a more open state following engineering of the lid and hinge residues (**Figure 4F**). These, caused a kink in the loop (from I162 to A170) that shifted two α-helix moieties to a more open state (0.52 and 0.55 Å, respectively). In particular, compared to the L0 variant, L7 showed an increase in RMSF of nearly 0.18 and 0.25 Å around regions A and B, respectively (**Figure 4G**). These results indicate that the change in hinge residues led to a more flexible channel, which favored ADP release. In summary, the mechanism promoting the release of ADP might have resulted from a lower binding affinity due to weaker inner interactions, as well as increased flexibility of hinge residues, leading to a more open conformation of the lid and a wider release channel.

4 One-pot production of PAPS at a 3-L scale

To demonstrate the *in vivo* applicability of the proposed strategy, the L7 variant was introduced in a whole-cell catalytic system, resulting in strain*E. coli* 11. Finally, the intracellular activity of *Kl* ATPS, *Pc* APSK, *Ec* PPA and*Rs* PPK could be controlled to 166.32, 253.08, 225.91, and 403.26 $U*g^{-1}$ wet cells, respectively (**Figure 5A**). Optimal operational conditions were determined as pH 8.0 and 35 degC. As shown in **Figure S2** , increasing substrate concentration from 100 to 150 mM achieved a conversion rate >97%, but a further increase to 200 mM caused the conversion rate to drop to 80.3%. Therefore, 150 mM substrate was considered optimal for conversion. The strains*E. coli* 04 (L0) and 11 (L7) were each grown in a 3-L bioreactor under optimal conditions, with air flow of 4.5 $L*min^{-1}$and shaking speed of 210 $r*min^{-1}$ (**Figure 5B**).*Escherichia coli* 11 produced 73.59 mM (37.32 $g*L^{-1}$) PAPS after incubation for 18.5 h, corresponding to a 98.1% conversion rate and space-time yield of 1.8 $g*L^{-1}*h^{-1}$. In comparison, *E. coli* 04 could produce only 37.94 mM (19.25 $g*L^{-1}$) PAPS after 20.5 h, corresponding to a 50.6% conversion efficiency and a space-time yield of 0.94 $g*L^{-1}*h^{-1}$. In addition, almost no APS could be detected in the reaction solution of*E. coli* 11, whereas 22.35 mM APS was found in the reaction solution of *E. coli* 04. These results showed that protein engineering of *Pc* APSK in the dual-enzyme catalytic cascade had successfully eliminated the rate-limiting

step in PAPS production.

Discussion

In this study, a new dual-enzyme catalytic system for *in vivo*synthesis of PAPS was developed that comprised a main enzyme module for the conversion of ATP into PAPS and an auxiliary module for the removal of by-product inhibition. *Pc* APSK was identified as the rate-limiting enzyme in this cascade due to the delayed release of ADP. To overcome this limitation and improve catalytic efficiency, a rational "ADP expulsion" strategy was applied. Accordingly, the ADP-binding affinity was weakened, and the binding channel was expanded to promote the release of ADP, resulting in a 45.74-fold higher activity in the APSK L7 variant. The latter was introduced in an *E. coli*catalytic system, whereby it successfully converted ATP to PAPS. This study describes not only a feasible method for the industrial production of PAPS but also a valuable strategy for engineering similar enzymes.

A synthetic and controllable catalytic system was designed for the synthesis of PAPS. The proposed catalytic system eliminates pathway inhibition from by-products by converting these by-products into substrates for reuse. During the conversion of ATP to PAPS, two by-products, PPi and ADP, inhibit the performance of ATPS and APSK (Bao et al., 2015). To eliminate such by-product inhibition, PPA and Nudix hydrolase have been used as PPi and ADP hydrolases, respectively (Hong et al., 2014; W. Xu, Dunn, O, Handley, Smith, & Bessman, 2006). Previously, a complex ATP conversion system was constructed using 3-bromopyruvic acid as a cheap substrate for PEP-K^+, which then acted as a phosphate donor for ADP (An et al., 2017). In this study, an auxiliary module comprising PPA and PPK was designed. PPA hydrolyzed PPi to phosphate, and PPK phosphorylated ADP to ATP through a one-step reaction that employed low-cost short-chain polyphosphate $PolyP_{(6)}$ as phosphate donor. In contrast to other methods for producing PAPs via the one-pot process, the present catalytic system is composed of two independent modules, whose strength can be adjusted by controlling their intracellular expression to balance the production process.

An effective protein engineering strategy was developed to improve*Pc* APSK activity. To date, protein crystallization (Ian J. MacRae, 2000; Poyraz et al., 2015), site directed mutagenesis Wang D. Z et al., 2016), and truncated mutagenesis (Ravilious et al., 2013; Sekulic, Konrad, et al., 2007) have been used to characterize APSK. However, these studies focused mainly on the structure and catalytic mechanism of APSK, and no effective strategies for improving its catalytic performance have been proposed. Here, a mechanism-guided "ADP expulsion" strategy was developed by combining information about conformational dynamics and crystal structure of the protein (Ian J. MacRae, 2000; Lansdon et al., 2002). The strategy included three steps: (i) computer- and protein structure-assisted binding energy calculation and release channel identification; (ii) identification of six key mutation hotspots affecting binding energy and release channels; and (iii) construction of two mutation libraries aimed at weakening the binding energy through looser inner interactions and an expanded release channel. As a result, the specific activity and $kcat\ /Km$ of the optimal L7 variant were 46.39-fold and 73.27-fold higher than those in wild-type *Pc* APSK, respectively. This approach is in line with a recent trend in protein engineering that focuses on construction of small and smart libraries while reducing the size of the mutation library and improving evolution efficiency (Li, Qu, Sun, & Reetz, 2019; Sun, Lonsdale, Ilie, Li, & Reetz, 2016). Compared to traditional mutagenesis, this method is more rational as it relies on tunnel identification (Song et al., 2020; Yuan et al., 2019), conformational dynamics (Yang et al., 2017), specific hotspot scanning (Xu , Cen, Singh, Fan, & Wu, 2019; J. Xu et al., 2019), and saturation mutagenesis. Overall, this protein engineering strategy could greatly improve the performance of enzymes with a release channel or lid structure.

The study provides a simple and efficient method for *in vivo* ATP conversion to PAPS. Efficient PAPS production has relied on the use of either PAP or ATP as the substrate (Kang et al., 2018). In the first case, PAP and PNPS are converted to PAPS by ASST (T. Wang, Liu, & Voglmeir, 2020); however, this method does not allow for large-scale PAPS production because of the high cost of PAP and the coupling with sulfotransferases (Xiong et al., 2013). ATP is a much cheaper substrate and, therefore, has been applied in the present study together with ATPS, APSK, PPA, and pyruvate kinase II as catalysts. The phospho-donor PEP-K^+ was generated by organic synthesis. Using 0.05 $mg*mL^{-1}$ of each purified enzyme and PEP-K^+, 5

g*L^{-1} of PAPS was generated in 6 h with a productivity of 0.83 g*L^{-1}*h^{-1} (An et al., 2017). In this study, the main module and auxiliary module were assembled and transformed into an *E. coli* strain, the rate-limiting enzyme and catalytic conditions were optimized, and a stable whole-cell PAPS catalytic synthesis system was constructed. Finally, 73.59 mM (37.32 g*L^{-1}) PAPS was produced in 18.5 h with a conversion rate of 98.1% and productivity of 1.75 g*L^{-1}*h^{-1} at a 3-L scale. Therefore, the biocatalytic process used in this study provides an attractive strategy for the transformation of ATP into high-value PAPS at a fraction (1/5000) of the cost of commercial sources and thus may remarkably facilitate the industrial production of PAPS.

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Tables

Table 1 Kinetic constants of *Kl* ATPS, *Pc* APSK,*Ec* PPA and *Rs* PPK

Parameters	*Km* (mM)	*kcat* (s^{-1})	*kcat/Km* (s^{-1}·mM^{-1})	Specific activity (U·mg^{-1} protein)
*Kl*ATPS	0.62	6.85	11.05	10.73
*Pc*APSK	1.35	0.61	0.45	1.07
*Ec*PPA	0.32	27.06	84.56	50.47
*Rs*PPK	0.41	38.42	93.71	63.85

Table 2 Results of site-directed mutagenesis experiments

Mutant	Mutant	mutation	*Km* (mM)	*Kcat* (s^{-1})	*kcat/Km* (s^{-1}·mM^{-1})	Specific activity (U·mg^{-1} protein)
L0	none	none	1.35	0.61	0.45	1.07
L1	K38G	K38G	0.93	4.37	4.70	5.68
L2	K38G /T40S	K38G /T40S	1.49	8.56	5.74	12.97
L3	S36A /K38G /T40S	S36A /K38G /T40S	0.77	4.04	5.25	5.34
L4	K151V	K151V	0.76	3.16	4.16	4.81
L5	K151V/ G167I	K151V/ G167I	0.68	5.76	8.47	8.35
L6	D139V/ K151V /G167I	D139V/ K151V /G167I	0.62	9.18	14.81	21.03
L7	K38G/ T40S / D139V/K151V /G167I	K38G/ T40S / D139V/K151V /G167I	0.59	19.45	32.97	48.94

Figures Legends

Figure 1 Design and reconstruction of main enzyme module and auxiliary module for the synthesis of PAPS. (A) Schematic representation of PAPS biosynthesis from ATP by the main enzymes module (ATPS and APSK) and auxiliary module (PPA and PPK); (B) Effect of different activity ratio of *Kl* ATPS to *Pc* APSK on PAPS production. The dual-enzyme system was supplemented with ATP, with *Kl* ATPS activity fixed at 3.0 U·mL^{-1}. The ratio of*Kl* ATPS to *Pc* APSK was changed from 1:1 to 1:3; (C) Effect of the amount of *Ec* PPA on the conversion rate of PAPS; (D) Effect of the amount of *Rs* PPK on the conversion rate of PAPS.

Figure 2 Construction and optimization of catalytic system*in vivo* . (A) Effect of substrate loading on PAPS production by strain *E. coli* 01; The reactions were supplemented with varying concentrations of ATP

from 20 to 80 mM added at a fixed whole-cell biocatalyst (wet) 30 g·L^{-1} at 30°C; (B) Intracellular enzyme activities of three recombinant strains (*E. coli* 01,*E. coli* 04, and *E. coli* 10); (C) Intracellular enzyme activity of *Rc* PPK in recombinant strains with different chaperones; (D) Intracellular enzyme activity of *Pc* APSK in recombinant strains with different RBS and the effect on the conversion rate of PAPS.

Figure 3 Computer-assisted identification of enzyme structure. (A) The catalytic progress of *Pc* APSK; (B) Molecular docking and highlighted the predicted hot spots as green sticks; (C) U-shaped release tunnel of ADP and its bottleneck; (D) Root-mean-square fluctuation (RMSF) of *Pc* APSK; (E) Open and Closed conformation of the lid, and the structure comprises lid constituent residues (red), hinge residues (blue), and binding residues (yellow).

Figure 4 Protein engineering of *Pc* APSK to accelerate ADP release and mechanism analysis. (A) Alanine scanning of selected hot spots; (B) Engineering scheme of *Pc* APSK; Libraries A and B were constructed using the iterative saturation mutation (ISM) strategies, respectively; Library A had three residues, K38, T40, S36, ISM was used to build the library with an NNK codon; There were three residues, D139, K151, and G167 in library B, which are not close to each other in the structure, and therefore, ISM was used to build the library with an NNK codon. The order is based on the increased enzyme activity (from high to low) of alanine scanning; (C) Hydrogen bonds calculated from MD simulations of L0 and L7; (D) RMSD from MD simulations of L0 and L7; (E) Bottleneck changes in U-shaped tunnel of L0 and L7; (F) Structure alignment of L0 (brown) and L7 (green); (G) Root-mean-square fluctuation (RMSF) value calculated from MD simulations of L0 and L7.

Figure 5 Intracellular enzyme activity and whole cell catalysis. (A) Determination of four intracellular enzyme activities of*E. coli* 11; (B) Conversion experiments of *E. coli* 04 and*E. coli* 11 at the 3-L scale with 150 mM ATP. The *E. coli*04 and *E. coli* 11 were cultivated in a 5-L bioreactor (with the 3-L working volume). Then, the cells were harvested by centrifugation at 6,000g for 8 min and then placed at 35degC for 22 hr for fully self-processing. Moreover, 30 g cells were resuspended into a 3-L of bioconversion mixture (150 Mm ATP, pH was adjusted to 8.0 with NaOH). Finally, a total of 3-L volume conversion broth was obtained.

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