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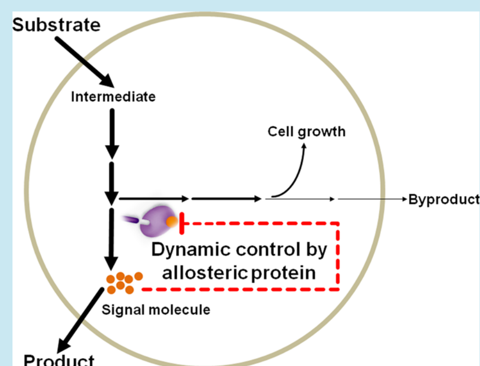
Rational Design of Allosteric Regulation of Homoserine Dehydrogenase by a Nonnatural Inhibitor L-Lysine

Zhen Chen, Sugima Rappert, and An-Ping Zeng*

Institute of Bioprocess and Biosystems Engineering, Hamburg University of Technology Denickestrasse 15, D-21073 Hamburg, Germany

Supporting Information

ABSTRACT: Allosteric proteins, which can sense different signals, are interesting biological parts for synthetic biology. In particular, the design of an artificial allosteric enzyme to sense an unnatural signal is both challenging and highly desired, for example, for a precise and dynamical control of fluxes of growth-essential but byproduct pathways in metabolic engineering of industrial microorganisms. In this work, we used homoserine dehydrogenase (HSDH) of *Corynebacterium glutamicum*, which is naturally allosterically regulated by threonine and isoleucine, as an example to demonstrate the feasibility of reengineering an allosteric enzyme to respond to an unnatural inhibitor L-lysine. For this purpose, the natural threonine binding sites of HSD were first predicted and verified by mutagenesis experiments. The threonine binding sites were then engineered to a lysine binding pocket. The reengineered HSD only responds to lysine inhibition but not to threonine. This is a significant step toward the construction of artificial molecular circuits for dynamic control of growth-essential byproduct formation pathway for lysine biosynthesis.



KEYWORDS: *dynamic flux control, allosteric regulation, homoserine dehydrogenase, protein design, lysine*

The development of metabolic engineering in the past decades has achieved impressive success in the bioproduction of chemicals such as 1,3-propanediol[1] and amino acids.[2] However, the process of strain development is still relatively slow. Thus, the toolboxes of metabolic engineering need to be expanded in order to improve its throughput and enhance its ability to design and control natural and non-natural metabolic pathways.[3,4] Toward this goal, synthetic biology has provided a general framework to design and assemble different biological parts that can be integrated into biological systems.[5,6]

In comparison to DNA and RNA as biological parts in synthetic biology, the design and application of allosteric proteins has not been well explored so far. Allosteric proteins, which can sense desired signals, can be used for different purposes. For example, allosteric proteins are promising biosensor for high-throughput screening.[7,8] Another potential use of allosteric proteins is to design biological circuits to dynamically control cellular metabolism. Actually, this strategy has been widely used by nature. For example, the first enzyme in branch metabolic pathways is often allosterically controlled by its end-products.[9−12] Cells use this mechanism to prevent the overflow of the pathway and to optimize biological efficiency. By employing the same principle, we may design artificial allosteric regulation of enzymes to dynamically control enzyme activities and to adjust the corresponding metabolic fluxes of pathways which are essential for cell growth, but not for the formation of the desired product. This can be a promising alternative to traditional gene modifications such as gene knockout, which often results in metabolic disturbance between cell growth and product synthesis.

In the study, we chose lysine biosynthesis as a model system because of the widespread allosteric regulations in amino acid biosynthesis pathways. For lysine bioproduction, the homoserine dehydrogenase (HSD)[13] and isocitrate dehydrogenase[14] are two key enzymes that direct the metabolic intermediates and their fluxes to the major competing pathways (Figure 1). Knockout or knockdown of these genes resulted in strain auxotroph for amino acids and impaired cell growth. As a proof of concept, we attempted to design a lysine-sensitive HSD that can use lysine as a signal molecule to control its activity (Figure 1). We expect that by engineering such a artificial allosteric regulation, the flux to the threonine/methionine pathways would be adjusted according to the intracellular concentration of lysine, leading to an enhanced lysine production.

The homoserine dehydrogenase from *Corynebacterium glutamicum* (CgHSD) belongs to the ACT domain-containing protein family[15] and is strongly inhibited by its end-products threonine and isoleucine.[11] Unfortunately, no 3-D crystal structure of this protein is available for the intended redesign. Here, we first predicted the potential threonine binding sites and deregulated the native allosteric inhibition by site-directed

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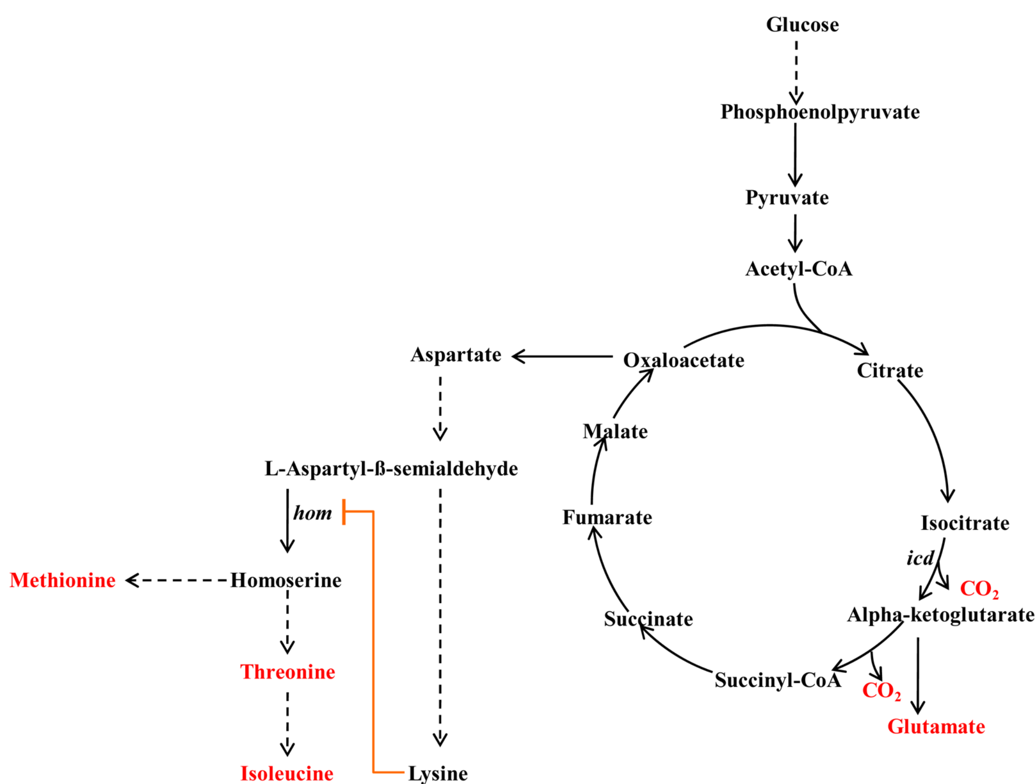

Figure 1. Simplified metabolic pathways and strategy for dynamic control of the byproduct synthesis for increased lysine production in bacteria. Homoserine dehydrogenase (*hom*) and isocitrate dehydrogenase (*icd*) direct the fluxes to the main competing pathways to lysine synthesis: the threonine/methionine synthesis pathway and carbon dioxide generation in the tricarboxylic acid cycle. A non-natural allosteric regulation of homoserine dehydrogenase in response to lysine could be constructed to dynamically reduce the flux to homoserine and its derived pathways. The major byproducts of lysine synthesis are illustrated with red colors. Dotted lines indicate lumped multiple bioreactions.

mutagenesis. A lysine-sensitive HSD was then constructed by reconstruction of an artificial lysine binding pocket in the native ACT domain. This paved the way to design an artificial allosteric control system for lysine bioproduction.

RESULTS AND DISCUSSION

Prediction of Native Threonine/Isoleucine Binding Sites of HSD. Since no crystal structure of CgHSD is available, we first generated homology models of CgHSD by using the software MODELLER[16] and the crystal structure of HSD from *Thiobacillus denitrificans* (PDB code 3MTJ). CgHSD shared 37.2% identity with the amino acid sequence of *T. denitrificans* HSD. A homotetrameric structure was generated and is shown in Figure 2A. As illustrated in Figure 2B, CgHSD is predicted to have an N-terminus catalytic domain and a C-terminus regulatory domain (ACT domain), which are connected by a long linker. The ACT domains from different subunits interact with each other, building the basis for the tetrameric structure.

So far, there is no information about the potential threonine and isoleucine binding sites in CgHSD. The ACT domain was previously shown to be able to bind different small molecules, especially amino acids.[9−11] Thus, we supposed that both threonine and isoleucine are bound at the same pocket located within the interface between two ACT domains (Figure 2C). As threonine can also bind to the interface of ACT domains in aspartokinase from *C. glutamicum* (CgAK), we performed a detailed structure alignment between the ACT domains of CgHSD and CgAK. The structural alignment illustrated two asymmetry threonine binding sites within a dimer of CgHSD (Figure 2B). Combing with a subsequent docking simulation, we predicted the detailed binding pose of threonine, as shown in Figure 2C. According to the prediction, the carboxyl group of threonine is stabilized through a hydrogen-bond network with interaction with G378, V379, and L380 from one subunit and I392 and S393 from another subunit. The amino group of threonine is fixed by interactions with L394 and D375. Q399 plays the most important role in the recognition of threonine by stabilizing the hydroxyl group of its side chain through hydrogen bonds. Interestingly, it has been reported that a mutation of G378 to glutamate desensitize CgHSD from the feedback inhibition by threonine.[17] This was consistent with our prediction, as the mutation of G378E would introduce a strong repulsion with the carboxyl group of threonine. Since the structures of threonine and isoleucine are quite similar, we suppose that isoleucine is bound in the same binding pocket with similar binding mode but not for residue Q399, which is specific for threonine.

Deregulation of the Native Feedback Inhibitions of CgHSD by Threonine and Isoleucine. To verify the predictions described, the corresponding residues were mutated. The wildtype and mutant CgHSDs were attached with an N-terminal His-tag, overexpressed, and purified. The enzyme activities and the inhibition profiles of the enzymes were determined.

As illustrated in Figure 3, the wildtype CgHSD was highly sensitive to the feedback inhibition of threonine with a $K_{0.5}$ value (half maximal inhibitory concentration) equal to 0.095 mM. The native enzyme loosed about 95% of its original activity when 0.5 mM of threonine was added into the medium. All of the designed mutations could significantly deregulate the

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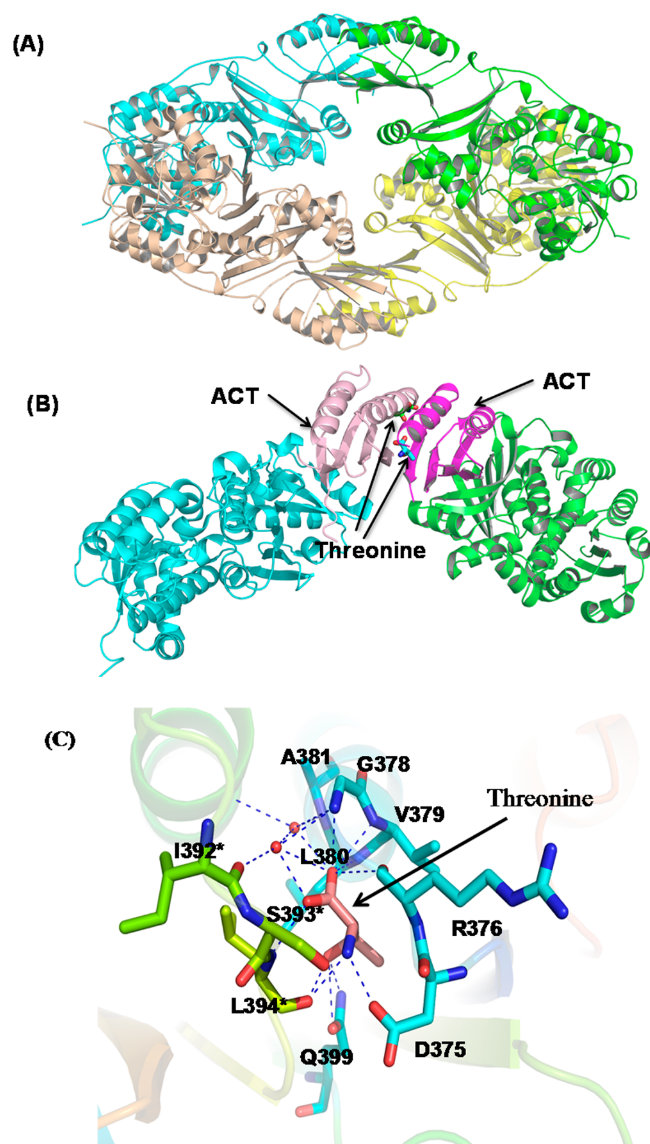

Figure 2. Homology modeling of homoserine dehydrogenase from *C. glutamicum* (CgHSD). (A) The whole tetrameric structure. Different subunits are shown with different colors. (B) The interface of ACT domains in the dimer structure and the potential threonine binding site. The ACT domains were illustrated as pink and magenta. (C) The threonine binding site. Asterisks indicate residues from a different subunit. Hydrogen bonds are represented by dotted lines.

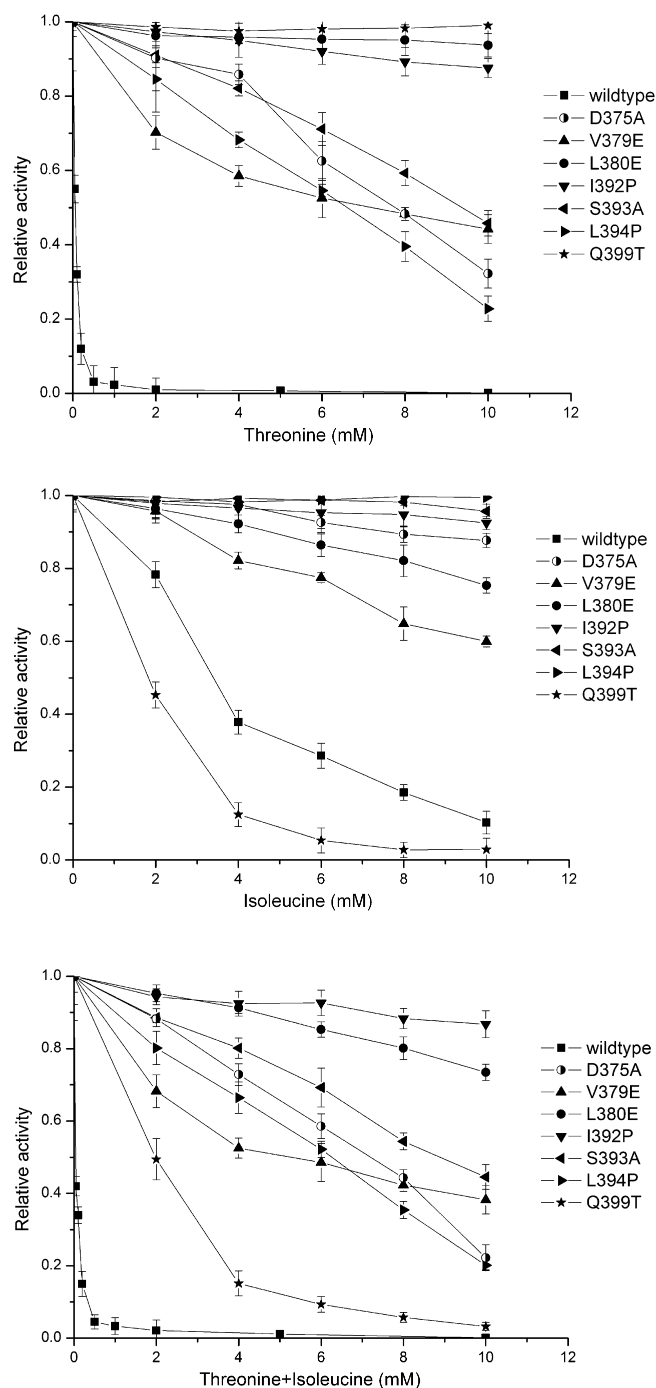

Figure 3. Inhibition profiles of homoserine dehydrogenase and its mutants by threonine (up) and isoleucine (down). The specific enzyme activities without inhibitor for the wild-type and the mutants D375A, V379E, L380E, I392P, S393A, L394P, and Q399T were 105 ± 14, 5.4 ± 2.2, 98 ± 12, 7.8 ± 1.7, 2.2 ± 1.1, 75 ± 15, 72 ± 12, and 57 ± 14 U/mg protein, respectively.

feedback inhibition by threonine, showing that the predicted threonine binding sites are correct. CgHSD with a point mutation Q399T can keep more than 98% of its activity even at 20 mM threonine, indicating that Q399 is a critical residue for threonine binding.

Isoleucine also inhibited the activity of the wildtype CgHSD with a $K_{0.5}$ value of 1.8 mM (Figure 3). Except for Q399T, all of the other designed mutations also significantly released CgHSD from the feedback inhibition of isoleucine, indicating that isoleucine almost binds to the same sites as threonine. Interestingly, the mutation of Q399 to threonine did not reduce but enhance the feedback inhibition of CgHSD by isoleucine. This is consistent with our prediction that Q399 is not involved in isoleucine binding. The mutation of Q399T could provide more space for isoleucine binding and thus enhances the feedback inhibition. An addition of threonine and isoleucine at the same time did not show obvious concerted effect (Figure 3C), confirming that threonine and isoleucine may use similar binding sites.

Design of a Nonnatural Lysine-Sensitive CgHSD. In this study, we set out to use lysine as a signal molecule for controlling the activity of CgHSD, which consequently control the flux to homoserine-derived byproduct synthesis pathway. There is no natural HSD that is sensitive to lysine regulation. Because of the structural similarity between lysine and

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threonine, we attempted to construct a non-natural lysine-sensitive HSD by reengineering the native threonine binding sites to accept lysine.

Both threonine and lysine have been previously reported to be able to bind to specific ACT domains of enzymes, such as aspartokinases.[9,10] A careful structure alignment between the ACT domain of CgHSD and the ACT domain of *E. coli* aspartokinase III (AK3, a lysine-sensitive enzyme) indicated that the binding pockets of threonine and lysine are structurally similar (Figure 4). The binding pocket can be generally divided

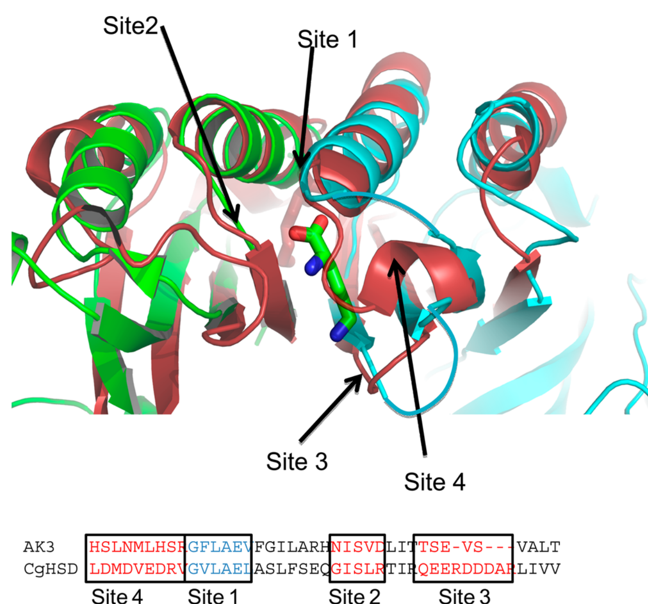

Figure 4. Structural (up) and sequential (down) comparison of threonine binding sites of *C. glutamicum* homoserine dehydrogenase (CgHSD) and lysine binding sites of *E. coli* aspartokinase III (AK3). The ACT domains from the different subunits of CgHSD are shown as green and cyan. The ACT domains from AK3 are shown as firebrick. Lysine in AK3 is shown as stick.

into four sections. The sequences and structures of site 1 (Figure 4), which interact with the backbone of threonine or lysine are highly conserved. The discrimination between the bindings of threonine and lysine mainly rely on three loop-regions illustrated as site 2, site 3, and site 4 in Figure 4, which interact specifically with the carboxyl group of the side chain of lysine or the hydroxyl group of the side chain of threonine. Generally, the lysine binding sites show a larger steric space and a more negatively charged environment. For example, loop 2 of AK3 has a negatively charged aspartate instead of positively charged arginine of CgHSD, which could facilitate the binding of positively charged lysine. The loop in site 3 of AK3 is shorter than that of CgHSD, providing larger space for the side chain of lysine. The structure of site 4 of AK3 is also more beneficial for lysine binding by steric effect and van der Waals interaction. Initially, efforts to introduce specific mutations into the three sites have been made independently; however, all of the designed mutants were not inhibited by lysine (data not shown). Thus, we attempted to substitute all of the three loop-regions of CgHSD (loop 2, loop 3, and loop 4) by the corresponding sequences of AK3. A designed sequence of hybridized CgHSD (Supporting Information 1) with all of three loops swapped from AK3 was synthesized and designated as HSD-S1.

The gene fragment of HSD-S1 was inserted into pET-28a(+) and transformed into *E. coli* BL21-CodonPlus (DE3)-RIL. The recombinant protein was overexpressed, purified, and characterized. As shown in Figure 5, the wildtype CgHSD was not

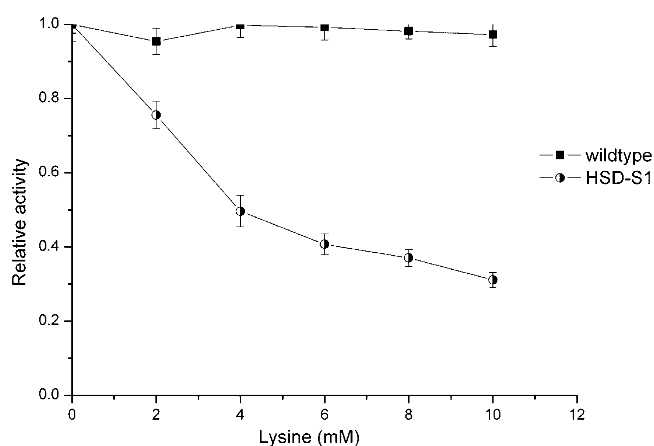

Figure 5. Inhibition profiles of homoserine dehydrogenase and its mutant by lysine. The specific enzyme activities without inhibitor for wild-type, HSD-S1 were 105 ± 14 and 17 ± 3 U/mg protein, respectively.

allosterically regulated by lysine. The HSD-S1 enzyme was inhibited by lysine with a $K_{0.5}$ value of 3.8 mM. The HSD-S1 enzyme also loosed the allosteric regulation by threonine, indicating that the construction was successful. Although the construction of HSD-S1 does not involve in any modification of the active site of HSD, the enzyme activity of HSD-S1 is significantly lower than that of the wildtype HSD. The enzyme kinetic analysis showed that the reduced enzyme activity resulted from the lower k_{cat} and increased K_m of NAD(P)H (Table 1), indicating that the modification of selected loops may affect the binding of NAD(P)H and the catalysis.

Table 1. Enzyme Kinetics of HSD and Its Mutant

enzyme	K_m (mM) aspartate semialdehyde	K_m (mM) NADPH (NADH)	k_{cat} (s^{-1})
wild-type	0.94 ± 0.11 (4.43 ± 0.32[a])	0.11 ± 0.02 (0.57 ± 0.06[a])	14.5 ± 1.2 (10.7 ± 0.2[a])
HSD-S1	1.22 ± 0.11 (5.64 ± 0.77[a])	0.45 ± 0.04 (1.32 ± 0.12[a])	3.2 ± 0.5 (2.2 ± 0.2[a])

[a]The values in parentheses were the kinetic parameters using aspartate semialdehyde and NADH as substrates.

DISCUSSION

Reduction of byproduct synthesis is an important strategy for increasing the yield of targeted metabolites. In general, the deletion of genes is a permanent and abrupt manipulation of cellular function and can only be applied to bioreactions or pathways which are not essential for biosynthesis and cell growth. Leaky mutations of enzymes represent a somewhat less dramatic but unfortunately uncontrolled manipulation of cellular metabolism. A tunable manipulation of the reaction rate is highly desired for systems metabolic engineering.[11] Using a specific metabolite as signal to control the metabolic reaction in response to the cells physiology is an appealing idea. To this end, genetic circuits or allosteric regulation could be

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integrated into the byproduct or anabolic synthesis pathways to dynamically control their metabolic fluxes. A key challenge toward such a goal is to find a suitable transcriptional factor or enzyme that can sense the desired signal.[5] This kind of proteins, however, may not exist in nature. Understanding the mechanism of allostery evolution is an important step toward the construction of artificial allosteric proteins. One possible event during the evolution of protein allostery is the so-called domain recruitment. This strategy has been recently used to design artificial allsoteric proteins. For example, by examining the diversity of allosteric regulations within the family of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAH7PS), Cross et al. were able to rebuild a functional allostery to an nonallosteric DAH7PS by transplanting the regulatory domain from a regulated DAH7PS.[21] We also demonstrated a successful switch of the allosteric regulation within aspartokinase family by regulatory domain swapping.[22] On the other hand, the construction of a completely new allosteric regulation, for example, by a new effector, still represents a major challenge. Protein engineering is an important tool to this end.

In this study, CgHSD was chosen as an example for the design of artificial allosteric regulation with a new effector. CgHSD is of interest for metabolic engineering, since it leads to the formation of homoserine-derived byproducts, which are essential for cell growth but not desired for the formation of the main product in lysine bioproduction process. The mechanism of natural allosteric control of CgHSD by its downstream products, threonine and isoleucine, was first clarified in this work by structural modeling and site-directed mutagenesis. Based on these results, different strategies have been tried for introducing non-native lysine inhibition into CgHSD. It should be mentioned that differently from the deregulation of feedback inhibition, which can be achieved by point mutation(s), the introduction of a new alloertic regulation (in this case inhibition of the enzyme by lysine) is much more complicated. It requires considering both the ligand binding and allosteric signal transduction. In our initial attempts, we attempted to introduce different point mutations in loop 2 of CgHSD to provide a larger space and a more negatively charged environment for lysine binding. All of our point mutations and their combinations (e.g., Q399T, Q399T/R395D, Q399T/R402 V, Q399T/E400S, and Q399T/R395D/E400S) for these purposes were not successful. A further deletion of residues R402 and D403 in the constructed mutants to shorten the length of loop 3 also did not achieve the desired lysine inhibition. The finally successful construction of a non-natural CgHSD inhibited by lysine was achieved by the transplantation of all three loops (loop 2, loop 3, and loop 4). The results indicated that the binding of lysine is coordinated by different contributions of the three loops. In comparison to the natural allosteric regulation of the widetype CgHSD by threonine, the artificial HSD-S1 showed a relatively lower sensitivity to the feedback inhibition of lysine. The feedback inhibition may be strongly dependent on the transduction of allosteric signal from the lysine binding site(s) to the active sites. Some recently developed methods such as energy dissipation model[18] may be employed to improve the design process. Compared to the wild-type enzyme, the artificial HSD-S1 also showed a relatively lower enzyme activity. More efforts are needed to address these two issues. Nevertheless, this work demonstrated the feasibility of designing a new allosteric regulation with a new effector. This represents an important step toward the construction of artificial dynamic control systems for metabolic engineering of industrial microorganisms.

■ METHODS

Homology Modeling. Homology models of *C. glutamicum* HSD were generated based on the structure of HSD from *Thiobacillus denitrificans* (Protein Data Bank code 3MTJ) using the molecular modeling software MODELLER.[16] Conformations of the substrate and the residues located within the binding sites were refined by using the molecular docking software Autodock.[19]

Plasmids Construction. *hom* gene encoding the wild-type HSD was amplified by PCR from genomic DNA of *C. glutamicum* ATCC 13032 using primers 5′-ATCGTA<u>CATATG</u>ACCTCAGCATCTGCCCCAAG-3′ and 5′-TATA<u>GTCGAC</u>TTAGTCCCTTTCGAGGCGGATC-3′ (restriction sites are underlined). The PCR fragment was double-digested with NdeI and *Sal*I, and subsequently inserted into the corresponding sites of plasmid pET-28a (+) (Novagen), designated as pET-hom. Point mutations within the *hom* gene were introduced by using QuikChange site-directed mutagenesis kits (Strategene) according to the standard protocol. The *hom* mutant with three swapped loops was synthesized by Invitrogen (Germany).

Protein Expression and Purification. *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene) was used as host cells for the overexpression of HSD and its mutants. The recombinant cells containing pET-hom or its derived plasmids were initially grown in 100 mL of LB media (supplemented with 50 μg/mL Kanamycin) at 37 °C until the OD_{600} reached 0.6. 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was then added to induce the gene expression and the culture was continued for an additional 12 h at 20 °C before harvest. The harvested cells were washed twice with 20 mM Tris-HCl buffer (pH 7.5) and suspended in 5 mL of 50 mM K_2HPO_4 (pH 7.5), 0.2 mM EDTA, and 0.1 mM dithiothreitol. Suspended cells were disrupted by sonication and centrifuged at 10 000 × g for 30 min. The enzyme was purified by Ni^{2+}-NTA column (GE Healthcare Bio-Sciences, Piscataway, NJ) according to the protocol. The purity of the protein was checked by SDS-PAGE and the protein concentrations were quantified at 595 nm with a Bio-Rad protein assay kit (Bio-Rad Laboratories).

Enzyme Assay. The enzyme activity of HSD was determined at 25 °C by monitoring the change of NADPH in absorbance at 340 nm using a Multiskan spectrophotometer (Thermo Scientific, Germany). The standard reaction mixture contained 100 mM Tris-HCl (pH 8.0), 150 mM KCl, 0.2 mM NADPH, and 2 mM aspartate semialdehyde.[20] One unit of enzyme activity corresponds to the reduction of one micromole of NADPH per minute. Kinetic constants were obtained from nonlinear regression data analysis and expressed as the mean ± SD.

■ ASSOCIATED CONTENT

Supporting Information

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■ AUTHOR INFORMATION

Corresponding Author

*Tel: +49-40-42878-4183. Fax: +49-40-42878-2909. E-mail: aze@tu-harburg.de.

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Notes

The authors declare no competing financial interest.

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