The α-aminoadipate pathway of lysine biosynthesis is modulated at the transcriptional and biochemical levels by feedback inhibition. The first enzyme in the α-aminoadipate pathway, homocitrinate synthase (HCS), is the target of the feedback regulation and is strongly inhibited by L-lysine. Here we report the structure of Schizosaccharomyces pombe HCS (SpHCS) in complex with L-lysine. The structure illustrates that the amino acid directly competes with the substrate 2-oxoglutarate for binding within the active site of HCS. Differential recognition of the substrate and inhibitor is achieved via a switch position within the (α/β)8 TIM barrel of the enzyme that can distinguish between the C5-carboxylate group of 2-oxoglutarate and the ε-ammonium group of L-lysine. *In vitro* and *in vivo* assays demonstrate that mutations of the switch residues, which interact with the L-lysine ε-ammonium group, abrogate feedback inhibition, as do substitutions of residues within the C-terminal domain that were identified in a previous study of L-lysine-insensitive HCS mutants in *Saccharomyces cerevisiae*. Together, these results yield new insights into the mechanism of feedback regulation of an enzyme central to lysine biosynthesis.

Among the 20 basic amino acids, lysine is unique in that two distinct pathways can synthesize it. Most bacteria and plants synthesize lysine via the diaminopimelate pathway, whereas lysine biosynthesis in many fungi and certain archaeabacteria occurs through the α-aminoadipate (AAA)*4* pathway (1, 2). The AAA pathway consists of eight enzymatic steps with homocitrinate synthase (HCS) catalyzing the first and committed reaction by condensing 2-oxoglutarate (2-OG) and acetyl-coenzyme A (AcCoA) to form homocitrinate and coenzyme A (CoA) (1). Due to its absence in mammals and its importance in the AAA pathway, HCS has been proposed as a candidate for antifungal drug design.

Genetic and biochemical studies of *Saccharomyces cerevisiae* and other fungal species have demonstrated that the AAA pathway is regulated at both levels (3). Biochemical regulation is mediated predominantly through feedback inhibition of HCS by L-lysine, thus limiting the metabolic flux through the pathway. In contrast, transcriptional regulation occurs via two distinct mechanisms. As with other amino acid biosynthetic pathways in fungi, the AAA pathway is regulated by general control of amino acid biosynthesis, particularly the enzymes composing the latter half of the path (4). In addition, the expression of several AAA pathway enzymes is regulated by the transcription factor Lys14 that is activated upon its binding to the pathway intermediate α-aminoadipate semialdehyde (5). Conversely, transcription of six of the seven AAA pathway enzymes in *S. cerevisiae*, including HCS, is repressed by L-lysine (6). This repression has been attributed to decreased production of α-aminoadipate semialdehyde due to inhibition of HCS by L-lysine that results in diminished transcriptional activation by Lys14 (7, 8). Thus, feedback regulation of HCS plays a pivotal role in regulating lysine biosynthesis at the biochemical and transcriptional levels *in vivo*.

Feedback inhibition of HCS has been characterized in the AAA pathway. Based on these studies, two models have been proposed for the kinetic mechanism by which L-lysine inhibits HCSs. Biochemical studies of HCSs from *S. cerevisiae* (9), *Penicillium chrysogenum* (10), and *Thermus thermophilus* (11, 12) report that L-lysine is a competitive inhibitor of 2-OG, implying that the amino acid binds within the active site of the enzyme. This model leads to a paradox in understanding how HCS can accommodate 2-OG, a dicarboxylic acid, and lysine, a basic amino acid, within the same site. In contrast to the competitive model, an allosteric mechanism for L-lysine inhibition has been proposed for the HCS from *Yarrowia lipolytica* (13), suggesting that an alternative effector-binding site exists within the enzyme. Further support for the allosteric model emerged from a genetic study of the *S. cerevisiae* HCS Lys20 and Lys21 isozymes by Feller et al. (7) in which point mutations were identified distal to the active sites of the enzymes that conferred resistance to feedback regulation. Due to the data supporting

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**Structural Basis for L-Lysine Feedback Inhibition of Homocitrinate Synthase**

Stacie L. Bufer1, Erin M. Scott1,2, Lorraine Pillus1, and Raymond C. Trievel1,3

From the 1Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109 and the 2Department of Biological Sciences and Moores UCSD Cancer Center, University of California San Diego, La Jolla, California 92039-0347

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these two competing models, the mechanism of feedback inhibition of HCS has remained unresolved.

To define the molecular basis for feedback regulation, we have determined the crystal structure of Schizosaccharomyces pombe HCS (SpHCS) bound to l-lysine and compared it with the structures of the free enzyme and its binary complex with 2-OG that we recently reported (14). The structure of the SpHCS-l-lysine complex reveals that the inhibitor binds within the 2-OG-binding site in the (α/β)₈ TIM barrel domain, in agreement with steady state kinetic analysis demonstrating that the amino acid is a competitive inhibitor of 2-OG. Differential recognition of 2-OG and l-lysine is achieved through a switch position within the active site in which acidic residues interact with the ε-ammonium group of the inhibitor and basic residues coordinate the C5 carboxylate group of the substrate. Kinetic and yeast growth assays of SpHCS mutants demonstrated that the inhibition patterns for l-lysine versus 2-OG for the SpHCS mutants were also determined using this assay. Initial velocities were measured in triplicate at room temperature using the reported assay conditions (14) by varying the concentration of one substrate (2-OG concentrations of 0–2 or 0–100 mM for the E222Q mutant and 0–100 μM for AcCoA) at various fixed concentrations of inhibitor with the other substrate held at a saturating level to determine the inhibition patterns. To correct for the increased fluorescence due to l-lysine concentrations over 1 mM, coenzyme A calibration curves were performed at every combination of the l-lysine and 2-OG concentrations used in each assay.

Data were analyzed using the program Prism (GraphPad Software). Double reciprocal plots of the initial velocity versus substrate concentration for each l-lysine concentration and Lineweaver-Burk lines using $K_m$ and $V_{max}$ calculated from hyperbolic fits to the untransformed data were plotted to determine the pattern of inhibition. To determine $K_I$ values for l-lysine versus the substrates 2-OG and AcCoA, the Michaelis-Menten equation was fit to the data using a nonlinear regression for a competitive inhibitor (Equation 1) or for a mixed inhibitor (Equation 2), where $K_I$ and $K_P$ represent the equilibrium constants for competitive and uncompetitive inhibition, respectively.

$$v = \frac{v_0 [S]}{[S] + K_m(1 + [I]/K_I)}$$  

(Eq. 1)

$$v = \frac{v_0 [S]}{[K_m(1 + [I]/K_I) + [S](1 + [I]/K_I)]}$$  

(Eq. 2)

In Vivo Growth Assay—Viability of the lys4⁴ mutants in the absence of l-lysine was determined as described previously (14). Cells were assayed for growth on synthetic medium lacking l-lysine (lys⁻) and uracil (ura⁻) to maintain selection of the vector, WT, or mutant lys4⁴ plasmids. Where indicated, plates contained 5-aminoethyl-l-cystine (AEC). Protein immunoblot analysis showed that the steady state levels of the mutant proteins were comparable with WT SpHCS (data not shown).

RESULTS

Crystal Structure of SpHCS Bound to l-Lysine—The crystal structure of the Zn(II)-bound form of full-length SpHCS (residues 1–418) in complex with l-lysine was determined to 2.38 Å resolution by molecular replacement (Table 1). A superimposition of the coordinates of the SpHCS-l-lysine complex with those of the Co(II)-bound free enzyme reveals that the structures are highly similar (root mean square difference of 0.66 Å for all aligned atoms) (Fig. 1A), whereas an alignment with the Zn(II)-bound SpHCS-2-OG complex reveals that the l-lysine-bound structure lacks the β-hairpin motif and domain-swapped N-terminal extension present in the substrate complex (Fig. 1B). In addition, the domain-swapped lid motif (residues 320–333), which regulates substrate access to the active site of the neighboring catalytic domain, adopts a closed conformation, consistent with the structures of the free enzyme and the 2-OG binary complex displaying a closed lid motif (14).

An inspection of the initial electron density maps within the active site of the inhibitor complex revealed strong density corresponding to a bound l-lysine molecule that was subsequently confirmed in a simulated annealing $F_o - F_c$ omit map (Fig. 1C). 

L-Lysine Inhibition of Homocitrate Synthase
L-Lysine Inhibition of Homocitrate Synthase

alignment of the structures of the lysine complex and the free enzyme illustrates that the residues within the active site are virtually superimposable, with the exception of Arg-43, which adopts an alternative side chain conformation to accommodate inhibitor binding (see below). In the free enzyme, a Mg(II) ion occupies the approximate position of the ε-ammonium group of l-lysine and is coordinated in an octahedral geometry by five water molecules and the carboxylate of Glu-222, suggesting that this pocket can function as a general cation-binding site (Fig. 1A). Inter-}

A comparison of the ligand-bound complexes of SpHCS illustrates that the coordinates of the substrate and inhibitor are nearly superimposable, providing direct structural evidence that l-lysine is a competitive inhibitor of 2-OG (Fig. 2A). Interestingly, the substrate and inhibitor adopt similar binding conformations with the active site of the enzyme despite their lack of structural and chemical homology. The divalent metal coordination sites of the α-amino group and the carboxylate of l-lysine coincide with the coordination sites of the C1 carboxylate and 2-oxo moiety of 2-OG, respectively (14). In addition, the carboxylate group of the inhibitor forms hydrogen bonds with the hydroxyl group of Thr-197, analogous to its interactions with the C1 carboxylate of 2-OG. The lysine side chain adopts an extended all-trans conformation that spans the diameter of the interior of the (α/β)₈ TIM barrel, positioning its ε-ammonium for the salt bridge interactions with Asp-123 and Glu-222 and water-mediated hydrogen bonds with Glu-74 and Asp-248 (Fig. 2B). Asp-123 and Glu-222 are located adjacent to Arg-163, which forms a salt bridge interaction with the C5 carboxylate of 2-OG. Glu-222 assists in positioning the guanidinium group of Arg-163 for interactions with the substrate (Fig. 2C).

An alignment of the l-lysine and 2-OG complexes furnishes insights into the plasticity of the active site in accommodating the binding of the inhibitor and substrate (Fig. 2A). This plasticity manifests itself through conformational changes in key active site residues that are intimately coupled to ligand recognition. Specifically, His-103, Asp-123, Arg-163, and Glu-222 have key roles in distinguishing between 2-OG and l-lysine.

These four residues cluster along the interior wall of the (α/β)₈ TIM barrel opposite the metal ion-binding site in a locus that we refer to as the switch position. In the SpHCS 2-OG complex, the basic residues His-103 and Arg-163 rotate toward the substrate to form hydrogen bonds or salt bridge interactions with the C5 carboxylate group of the substrate (Fig. 2C). Our previous studies have shown that these interactions are important for homocitrate synthesis in vitro and in vivo (14). However, in the l-lysine complex, these residues are oriented away from the inhibitor, and the acidic residues Asp-123 and Glu-222 rotate inward to engage in salt bridge interactions with its ε-ammonium group (Fig. 2B). A comparison of the free enzyme and the ligand-bound complexes of SpHCS indicates that the conformational changes of the residues composing the switch position appear to be coordinated due to their close juxtaposition and the salt bridge interaction between Arg-163 and Glu-222 that is maintained in each of the structures (Fig. 2). The conformational changes in the switch position in the 2-OG complex may also explain the ordering of the adjacent β-hairpin motif that is disordered in the structures of the free enzyme and the l-lysine complex (Fig. 1, A and B) (14). An analysis of the crystal contacts of the various SpHCS structures indicates that the β-hairpin is solvent-exposed and that packing interactions do not contribute to the ordering of this motif. Thus, it appears that conformational changes in the switch region that occur during 2-OG binding are coupled to ordering of the β-hairpin, as described previously (14).

In addition to conformational changes within the active site, perturbations in the lid motif are observed in the free enzyme and ligand-bound complexes. In the free enzyme, Arg-43 participates in maintaining the closed state of the lid through bifurcated hydrogen bonds between its guanidinium group and the carboxyl oxygen of Ala-324 (the prime denotes residues from the neighboring subunit in the homodimer) (supplemental Fig. S1A). However in the l-lysine complex, inhibitor binding induces a conformational change in the Arg-43 side chain, resulting in a direct and a water-mediated hydrogen bond from the guanidinium group to the Ala-324 carboxyl oxygen. The conformational change in Arg-43 and the altered hydrogen pattern contribute to a subtle shift in the lid motif away from the active site. A third conformation is observed for the Arg-43 side chain in the 2-OG complex in which its guanidinium groups form hydrogen bonds to the 2-oxo moiety of the substrate (supplemental Fig. S1B). This rearrangement abrogates hydrogen bonding between Arg-43 and Ala-324, resulting in a further shift of the lid motif away from the active site. These observations illustrate that the conformation adopted by the lid motif is sensitive to the ligand bound within the active site of the enzyme and thus
may influence the equilibrium between the open and closed states adopted by the lid motif (14).

**Kinetic Analysis of L-Lysine Inhibition**—Our characterization of the SpHCS l-lysine complex provides structural evidence that l-lysine is a competitive inhibitor of 2-OG through binding within the active site. To corroborate this finding, we analyzed the kinetic mechanism by which l-lysine inhibits SpHCS. Double reciprocal plots of velocity versus substrate concentration illustrate that l-lysine is a competitive inhibitor toward 2-OG and a mixed inhibitor of AcCoA (Fig. 3, A and B). This inhibition pattern is consistent with the ordered Bi-Bi kinetic reaction mechanism of HCS in which 2-OG binding precedes the association of AcCoA (21) and concurs with the structural studies illustrating that 2-OG and l-lysine compete for binding to the same position within the active site (Fig. 2A). L-Lysine competitively inhibited 2-OG binding to SpHCS with a $K_i$ value of 3.56 ± 0.66 µM (Table 2), in agreement with the values reported for the HCSs from *T. thermophilus* ($K_i = 9.4$ µM) (22) and from *P. chrysochromulina* ($K_i = 8$ µM) (10). In addition, we examined the mixed inhibition by l-lysine with respect to AcCoA and measured $K_i$ and $K_f$ values of 63.2 ± 9.1 and 191 ± 60 µM, respectively. Finally, we probed the stereospecificity of inhibition by d- and l-lysine. d-Lysine was unable to inhibit SpHCS at concentrations up to 2 mM (data not shown), demonstrating that the enzyme specifically recognizes the l-stereoisomer of the amino acid. In summary, the kinetic data substantiate our structural studies of SpHCS illustrating that l-lysine is a competitive inhibitor of 2-OG.

**Analysis of l-Lysine-interacting Residues**—After characterizing the biochemical basis for inhibition, we probed the functions of the l-lysine-interacting residues in SpHCS that mediate feedback regulation. The interactions that confer specificity for l-lysine as opposed to 2-OG involve specific recognition of the lysine ε-ammonium group, which occurs through salt bridge interactions with the switch residues Asp-123 and Glu-222 (Fig. 2, A and B). To investigate the importance of these residues in l-lysine binding, we engineered conservative D123N and E222Q substitutions in SpHCS. The D123N mutant had kinetic parameters similar to the WT enzyme (Table 2). The E222Q mutant also exhibited a turnover number comparable with the WT enzyme but displayed a substantially elevated $K_m$ value for 2-OG (>60-fold), presumably due to a disruption of the salt bridge interaction between its carboxylate group and the guanidinium group of Arg-163 that forms a salt bridge interaction with the substrate (Fig. 2C) (14). Kinetic analysis of the E222Q mutant revealed that l-lysine inhibition is severely compromised ($K_i = 44.4$ mM), whereas no inhibition could be detected in assays with the D123N mutant with l-lysine concentrations up to 100 mM (Table 2). Given that...
the intracellular concentrations of L-lysine in yeast range from 1 to 10 mM (23, 24), these data suggest that the SpHCS D123N and E222Q mutants would be insensitive to feedback regulation in vivo.

To examine this possibility, we evaluated growth of these mutants in an S. cerevisiae lys20Δ lys21Δ double deletion strain that was generated in our previous studies (14). This strain lacks genes encoding HCS in S. cerevisiae and exhibits lysine auxotrophy that can be rescued by the heterologous expression of the mutants in an in vivo and E222Q mutants would be insensitive to feedback regulation of the active site of the SpHCS-L-lysine complex (Fig. 1). Residues composing the switch position are labeled in red. B, stereo view of the active site of the SpHCS-L-lysine complex (pink carbons with green carbons). Coordination to the Zn(II) ion (gray) is shown as green dashes, whereas potential hydrogen bonds to the inhibitor lysine are represented by blue dashes. Hydrogen bonds within the protein and to solvent molecules are displayed as red dashes. C, stereo view of the active site of the SpHCS-2-OG complex (blue carbons with 2-OG shown with yellow carbons). Coordination to the Zn(II) ion (gray), hydrogen bonds to 2-OG, and interactions between the protein and solvent are as depicted as in panel B. For clarity, only certain residues that form hydrogen bonds to 2-OG are shown.

After establishing that the D123N and E222Q mutants were viable in the absence of lysine in the lys20Δ lys21Δ strain, we then tested their response to feedback inhibition in vivo using AEC. This toxic L-lysine analog was previously used to identify ScHCS mutants that are defective in feedback regulation (7). As expected, the lys20Δ lys21Δ strain transformed with WT SpHCS exhibited weak growth at 5 μg/ml AEC that was completely abolished at 50 μg/ml AEC (Fig. 4). Similar to the WT enzyme, the E222Q mutation could not overcome AEC toxicity at either concentration, primarily due to its defect in catalytic efficiency (Table 2) and reduced growth phenotype on ura lys media. Conversely, the D123N mutation displayed strong resistance to AEC at concentrations up to 50 μg/ml (Fig. 4), in agreement with the complete insensitivity of this mutant to L-lysine inhibition in vitro (Table 2). These findings also suggest that the D123N mutant overproduced lysine in vivo, enabling cells to circumvent the toxicity of AEC. Taken together, our in vitro and in vivo data demonstrate that the salt bridge interactions between the L-lysine ε-ammonium group and the Asp-123 and Glu-222 carboxylate groups in the switch position are critical to feedback regulation in vitro and in vivo.

Location and Function of the Original L-Lysine-insensitive Mutants—In addition to investigating the roles of Asp-123 and Glu-222 in L-lysine inhibition, we examined the role of the residues for which mutations were previously reported to suppress feedback regulation of the ScHCS Lys20 and Lys21 isozymes. These point mutants include R276K and S385F in ScHCS Lys20 and Q366R in ScHCS Lys21 that were identified through a genetic screen for AEC resistance (7). A sequence alignment of the C-terminal domains of SpHCS and the ScHCS isozymes demonstrates that Arg-276 in ScHCS Lys20 and Gln-366 in ScHCS Lys21 are conserved in SpHCS (homologous to Arg-288 and Gln-354, respectively), whereas Ser-385 in ScHCS Lys20 is not conserved in SpHCS and thus was not included in our studies (supplemental Fig. S2).

Mapping of Arg-288 and Gln-354 onto the structure of SpHCS illustrates that they are located over 25 Å away from the active site. Arg-288 resides in the α10 helix in the N-terminal domain of the enzyme (Fig. 5) and adopts different conforma-

![Image](Image-50x349-to-407x733)

**FIGURE 2.** The switch position in SpHCS active site mediates differential ligand recognition. A, stereo view of the active site of the SpHCS L-lysine complex (pink carbons) superimposed with the SpHCS 2-OG closed lid complex (blue carbons). Orange and yellow dashes represent coordination to the Zn(II) ion and hydrogen bonding to 2-OG, respectively, in the 2-OG closed loop complex, whereas coordination to the Zn(II) ion and hydrogen bonding to L-lysine are depicted as described in the legend for Fig. 1. Residues composing the switch position are labeled in red. B, stereo view of the active site of the SpHCS-L-lysine complex (pink carbons with green carbons). Coordination to the Zn(II) ion (gray) is shown as green dashes, whereas potential hydrogen bonds to the inhibitor lysine are represented by blue dashes. Hydrogen bonds within the protein and to solvent molecules are displayed as red dashes. C, stereo view of the active site of the SpHCS-2-OG complex (blue carbons with 2-OG shown with yellow carbons). Coordination to the Zn(II) ion (gray), hydrogen bonds to 2-OG, and interactions between the protein and solvent are as depicted as in panel B. For clarity, only certain residues that form hydrogen bonds to 2-OG are shown.
tions in the free enzyme and its ligand-bound structures. In the \( \alpha \)-lysine complex, the arginine side chain extends toward the end of \( \alpha \)-helix in subdomain II of the C-terminal domain and forms hydrogen bonds to the backbone carbonyl groups of Leu-399 and Tyr-402 (Fig. 5A). Similar interactions are observed in the SpHCS-2-OG complex (data not shown). However, in the free enzyme, the Arg-288 side chain adopts two different conformations (Fig. 5B). One conformation mimics that seen in the ligand-bound complexes, whereas the other forms a salt bridge interaction with the carboxylate group of Asp-396 in the \( \alpha \)-13 helix. In contrast, Gln-364, which is located within the \( \alpha \)-1 helix in C-terminal subdomain II, adopts a similar conformation in the free enzyme and ligand-bound structures wherein its amide group forms hydrogen bonds to the backbone carbonyl groups of Phe-314 and Arg-344 and to the guanidinium group of the neighboring Arg-361 in an \( \alpha \)-11 (Fig. 5A and B). Notably, the Arg-288 and Gln-364 in each monomer are located on opposite faces in the SpHCS homodimer (Fig. 5C) and only participate in intramonomer interactions (Fig. 5, A and B), indicating that these residues do not directly participate in interactions or conformational changes at the subunit interface of the dimer. Moreover, Arg-288 and Gln-364 do not contact each other (Fig. 5C), implying that they do not form a surface that binds \( \alpha \)-lysine.

To assess the effects of the R288K and Q364R mutations in SpHCS on feedback regulation, we assayed their activity in vitro and in vivo (Table 2 and Fig. 4). The SpHCS R288K and Q364R mutants displayed kinetic parameters comparable with the WT enzyme (Table 2). Consistent with the previously reported effects of the R288K and Q364R ScHCS mutants, \( \alpha \)-lysine inhibition was impaired by \( \sim 150\) and \( \sim 300\)-fold, respectively, when compared with the \( K_{i} \) value for competitive inhibition toward 2-OG for the WT enzyme. Although these mutants exhibited a substantial decrease in sensitivity to feedback regulation, their \( K_{i} \) values are >45-fold lower than those determined for the D123N and E222Q mutants. This disparity may reflect the fact that the latter mutants directly abrogate interactions with \( \alpha \)-lysine (Table 2 and Fig. 2B), whereas the R288K and Q364R mutants may attenuate inhibition via an indirect mechanism, given their distance from the active site.

We also examined the effects of the SpHCS R288K and Q364R mutants in vivo. The R288K mutant and the Q364R mutant both complemented the lysine auxotrophy of the \( S. \) cer- visiae \( \text{lys20} \Delta \text{lys21} \Delta \) strain (Fig. 4). In agreement with the previous studies by Feller et al. (7), both the R288K and the Q364R mutants exhibited decreased sensitivity to AEC when compared with WT SpHCS and the E222Q mutant (Fig. 4). However, their resistance to AEC was not as pronounced as that of the D123N mutant, consistent with the kinetic data demonstrating that this mutation abolished feedback inhibition (Table 2). In summary, the diminished \( \alpha \)-lysine sensitivity of the SpHCS R288K and Q364R mutants corroborates the prior studies of the homologous mutations identified in the ScHCS isozymes (7).

**DISCUSSION**

Feedback inhibition of HCS represents an important mechanism by which the metabolic flux through the AAA pathway is regulated in vivo. Collectively, our structural and functional data demonstrate that \( \alpha \)-lysine competes with 2-OG for binding to SpHCS, in agreement with prior studies of HCS from \( \text{P. chryso-}
ogena} \) (10), \( \text{T. thermophilus} \) (11, 12), and \( S. \) cer- visiae (9).

The inherent plasticity of the active site of the enzyme enables differential recognition of 2-OG and \( \alpha \)-lysine. This recognition is achieved predominantly through conformational rearrangements in the acidic and basic residues comprising the switch position that form salt bridge interactions with the \( \alpha \)-lysine \( \epsilon \)-ammonium group or the C5 carbonylate of 2-OG, respectively (Fig. 2). In contrast to the conformational differences between the \( \alpha \)-lysine and 2-OG-bound forms of SpHCS, the overall structures of the \( \alpha \)-lysine complex and free enzyme are remarkably similar, particularly within the active site (Fig. 1, A and C). This structural homology may

**TABLE 2**

Kinetic analysis and lysine inhibition studies of WT SpHCS and mutants implicated in lysine inhibition

| Mutant | \( K_{m} \) AcCoA | \( K_{m} \) varying AcCoA | \( k_{cat}/K_{m} \) | \( V_{max} \) 2-OG | \( k_{cat} \) varying 2-OG | \( K_{m} \) 2-OG | \( K_{cat}/K_{m} \) | 1/Lys vs. 2-OG | 2-OG |
|--------|------------------|-------------------------|-----------------|----------------|-------------------|----------------|----------------|----------------|
| WT     | 10.7 ± 0.6*      | 299 ± 7                 | 28.0 ± 1.7      | 0.159 ± 0.015  | 308 ± 4           | 1940 ± 190     | 3.56 ± 0.66    | >100,000       |
| D123N  | 13.2 ± 1.7       | 217 ± 7                 | 16.4 ± 2.1      | 0.284 ± 0.033  | 217 ± 7           | 197 ± 3.2      | 44,400 ± 9700  | >100,000       |
| E222Q  | 47.7 ± 5.6       | 215 ± 9                 | 4.51 ± 0.56     | 0.966 ± 1.46   | 191 ± 7           | 30 ± 0.06      | 55 ± 5.7       | >100,000       |
| R288K  | 140 ± 1.6        | 194 ± 7                 | 13.9 ± 1.7      | 0.247 ± 0.032  | 200 ± 6           | 810 ± 102      | 558 ± 57       | >100,000       |
| Q364R  | 13.3 ± 1.2       | 170 ± 4                 | 11.1 ± 0.9      | 0.296 ± 0.028  | 165 ± 4           | 557 ± 58       | 921 ± 120      | >100,000       |

* Errors are reported as the error in curve fitting.

**FIGURE 3.** Kinetic mechanism of \( \alpha \)-lysine inhibition of SpHCS. A, double reciprocal plots of initial velocity versus 2-OG concentration at different concentrations of \( \alpha \)-lysine and a fixed saturating concentration of AcCoA. B, double reciprocal plots of initial velocity versus AcCoA concentration at different concentrations of \( \alpha \)-lysine and a fixed saturating concentration of 2-OG. Data points are the average of triplicate measurements, and the error bars represent one S.D. The lines represent Lineweaver-Burk fits using the \( V_{max} \) and \( K_{m} \) values derived from hyperbolic fits to the untransformed data.
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**FIGURE 4.** In vivo analysis of L-lysine-insensitive SpHCS mutants. The yeast HCS null strain was transformed with empty vector, wild-type lys4−, and lys4 mutants engineered at positions D123N, E222Q, R288K, and Q364R (pLP2387–pLP2390, respectively). Growth was compared on medium containing lysine (ura−) to select for the plasmids, on medium lacking lysine to evaluate HCS function (ura−) and on (ura−, lys−) medium containing AEC, a toxic L-lysine analog, at the indicated concentrations. Cell cultures were grown under permissive conditions (ura−), normalized to an A600 of 1, and serially diluted 5-fold. Plates were incubated at 30 °C for 4 days (ura−, lys−) or 10 days (AEC).

**FIGURE 5.** Location of the L-lysine-insensitive mutants in the structure of SpHCS. A, ribbon diagram of the N-terminal (α/β)8 TIM barrel catalytic domain (pink) and C-terminal domain (violet) of the SpHCS structure in complex with L-lysine. Arg-288 and Gln-364, along with their interacting partners, are depicted as sticks with hydrogen bonds depicted as green dashes. B, ribbon diagram of the N-terminal (α/β)8 TIM barrel active site domain (gray) and C-terminal domain (teal) of the SpHCS free enzyme. Arg-288 and Gln-364 and adjacent interacting residues are depicted as sticks with hydrogen bonds illustrated in sticks. C, ribbon diagram of the SpHCS-L-lysine homodimer depicting the location of Arg-288 and Gln-364 (rendered as spheres). The N- and C-terminal domain of monomer A is rendered as in panel A and is depicted in yellow and blue, respectively, in monomer B. L-lysine (green carbons) and the active site Zn(II) (gray) metal ion are represented as spheres.

predispose HCS for feedback inhibition by L-lysine, providing a molecular explanation for the relatively high affinity that the inhibitor displays for the enzyme (Table 2). The salt bridge interactions between the L-lysine ε-ammonium cation and the carboxylate groups of Asp123 and Glu-222 in the switch position are critical to this tight binding as mutations of these residues to asparagine and glutamine, respectively, severely impair feedback inhibition in vitro (Table 2) and in vivo (Fig. 4). In very recent studies, the structure of T. thermophilus HCS in complex with L-lysine was reported (12). This enzyme displays an inhibitor binding mode that is essentially identical to that of SpHCS, illustrating structural conservation throughout evolution. In summary, these results provide strong evidence for a competitive model of L-lysine feedback inhibition of HCS.

Although our results do not support an allosteric mechanism of inhibition, they do not exclude the involvement of residues outside the active site in mediating feedback regulation. Indeed, the R288K and Q364R mutations in SpHCS confer diminished sensitivity to feedback inhibition by L-lysine in vitro and in vivo (Table 2 and Fig. 4) despite being located over 25 Å from the active site. Because these residues do not participate in subunit interactions in the SpHCS homodimer (Fig. 5), it is conceivable that these mutations alter the conformational dynamics of the enzyme by disrupting hydrogen bonding within its C-terminal domain, thereby perturbing the equilibrium between the conformations adopted by the free enzyme and the L-lysine complex versus the 2-OG complex (Figs. 1 and 2). These changes in protein dynamics may disproportionately affect the binding of L-lysine that preferentially recognizes the conformation adopted by the free enzyme (Fig. 1, A and C). Alternatively, HCS may exhibit half-site reactivity between its two active sites in the homodimer in which L-lysine competitively inhibits the subunit to which it binds and concomitantly induces an allosteric signal that results in inhibition of the neighboring subunit. This type of inhibition need not exhibit allosterity, as shown in other homodimeric enzymes, such as prostaglandin endoperoxide H synthases (25–27), which possess half-site reactivity but lack conventional allosteric kinetics. Further studies are needed to determine whether HCS functions in a similar manner.

A comparison of SpHCS and the related enzyme Mycobacterium tuberculosis α-isopropylmalate synthase (α-IPMS) highlights the different regulatory mechanisms employed by these enzymes. α-IPMS catalyzes the first committed step in leucine biosynthesis by condensing AcCoA and 2-oxoisovalerate to yield α-isopropylmalate, analogous to the homocitrate synthase.
sis reaction of HCS (28, 29). The catalytic domains of HCS and α-IPMS share a structurally homologous (α/β)$_8$ TIM barrel fold, and the active site residues involved in metal coordination and implicated in substrate binding and catalysis are generally conserved between the two enzymes (14, 29). Both enzymes are also subject to feedback regulation by the end products of their respective pathways, although the mechanisms underlying this inhibition are distinct. Our data illustrate that HCS feedback regulation occurs via a competitive mechanism, whereas the activity of α-IPMS is allosterically regulated by L-leucine (29, 30). These differences are due to the presence of a regulatory module in the C-terminal domain of α-IPMS that is absent in HCS. L-Leucine binds in a hydrophobic pocket formed by the interface between the two regulatory modules that is located ~50 Å from the active site of α-IPMS (29). Kinetic studies have demonstrated that L-leucine exhibits slow-onset noncompetitive inhibition versus the substrate 2-oxoisovalerate (30). This mode of inhibition contrasts with the rapid competitive inhibition by L-lysine illuminated in our structural and functional studies of SpHCS. These differences may reflect distinct physiological requirements for modulating the metabolic flux of the leucine and lysine biosynthetic pathways via feedback regulation.

HCS has been proposed as a novel target for antifungal drug design due to its conservation in pathogenic fungal species, such as Aspergillus fumigatus, Candida albicans, and Crypto- coccus neoformans (31). Our structural characterization of the SpHCS-L-lysine complex and accompanying functional studies yield important insights that are applicable to inhibitor design. Most notably, our results underscore that small molecule competitive inhibitors, such as L-lysine, effectively inhibit HCS activity in vitro and in vivo (Table 2 and Fig. 4). The intrinsic plasticity displayed by the residues in the switch position (Fig. 2) may furnish opportunities to identify or design small molecule inhibitors that target specific conformations adopted by the HCS active site. This strategy may ultimately yield a diverse set of lead compounds in the development of HCS-specific inhibitors.

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