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Glutarate L-2-Hydroxylase (CsiD/GlaH) is an Archetype Fe(II)/2-Oxoglutarate-Dependent Dioxygenase

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Abstract

The *Escherichia coli* gene initially named *ygaT* is located adjacent to *lhgO*, encoding L-2-hydroxyglutarate oxidase/dehydrogenase, and the *gabDTP* gene cluster, utilized for γ -aminobutyric acid (GABA) metabolism. Because this gene is transcribed specifically during periods of carbon starvation, it was renamed *csiD* for carbon starvation induced. The CsiD protein was structurally characterized and shown to possess a double-stranded β -helix fold, characteristic of a large family of non-heme Fe(II)- and 2-oxoglutarate (2OG)-dependent oxygenases. Consistent with a role in producing the substrate for LhgO, CsiD was shown to be a glutarate L-2-hydroxylase. We review the kinetic and structural properties of glutarate L-2-hydroxylase from *E. coli* and other species, and we propose a catalytic mechanism for this archetype 2OG-dependent hydroxylase. Glutarate can be derived from L-lysine within the cell, with the *gabDT* genes exhibiting expanded reactivities beyond those known for GABA metabolism. The complete CsiD-containing pathway provides a means for the cell to obtain energy from the metabolism of L-lysine during periods of carbon starvation. To reflect the role of this protein in the cell, a renaming of *csiD* to *glaH* has been proposed.

Keywords

oxygenase; hydroxylase; 2-oxoglutarate; alpha-ketoglutarate; non-heme iron; ferrous ion

1. INTRODUCTION TO CSID

In 1993, a series of carbon starvation-induced genes was identified in *Escherichia coli* on the basis of induction studies using *lacZ* fusions created with a λ placMu system (Weichart, Lange, Henneberg, & Hengge-Aronis, 1993). One of these genes, initially termed *csi-12* or *ygaT* and later designated *csiD*, was shown to require the general stress sigma factor RpoS (σ^S) (Hengge, 2008), 3',5'-cyclic adenosine monophosphate (cAMP), and the cAMP-receptor protein (CRP) for its transcription (Marshall et al., 1998). Subsequent investigation

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(Metzner, Germer, & Hengge, 2004) revealed that this gene initiates a six-gene cluster: *csiD*, *ygaF* (later renamed *lhgO*), *gabD*, *gabT*, *gabP*, and *csiR* (Fig. 1). Regulation of these genes is complex and includes action by the CsiR repressor (Metzner et al., 2004) and at least three promoters: *csiD*_p is used during carbon starvation or stationary phase and dependent on cAMP, CRP, the leucine regulatory protein (Lrp), and σ^S ; *gabD*_{p1} is governed by σ^S ; and *gabD*_{p2} is controlled by σ^S and Nac (Marschall et al., 1998; Metzner et al., 2004; Schneider et al., 2002).

Biochemical roles have been identified for the *lhgO* and *gabDTP* gene products (Fig. 2). The name *lhgO* derives from its function; it encodes L-2-hydroxyglutarate (2HG) oxidase, a soluble flavoprotein that reduces dioxygen to hydrogen peroxide as it converts 2HG to 2-oxoglutarate (2OG) (Kalliri & Hausinger, 2008). A soluble L-2HG oxidase activity also was demonstrated for the corresponding LhgO from *Pseudomonas putida* KT2440 (Zhang et al., 2018). Additional studies of the *E. coli* protein found it to be a membrane-bound, ubiquinone-linked dehydrogenase (Knorr et al., 2018). It is unclear why the two studies of *E. coli* LhgO report differences in cellular localization and function, but the discrepancies may relate to subtle differences associated with the pET42b versus pET16b plasmid constructs. The *gabDTP* cluster encodes an NADP⁺-dependent succinic acid semialdehyde dehydrogenase (Cozzani, Fazio, Felici, & Barletta, 1980), a pyridoxal phosphate-dependent γ -aminobutyrate (GABA) transaminase (Dover & Halpern, 1974), and a GABA-specific membrane-bound permease (Niegemann, Schulz, & Bartsch, 1993), together allowing the cells to metabolize extracellular GABA to generate intracellular succinic acid. These studies did not address the cellular role of CsiD.

2. FUNCTIONAL ASSIGNMENT OF CSID

2.1 Structural Assignment of CsiD as an Fe(II)/2OG-Dependent Oxygenase

Crystal structures were reported for CsiD from *E. coli* strain K-12 that was truncated by 14-residues at its *N*-terminus and from *E. coli* strain B that was full-length, but with the first 14 residues disordered (Table 1; PDB ID: 1JR7 and 2R6S, respectively) (Chance et al., 2002; Lohkamp & Dobritzsch, 2008). The sequences of these proteins differ at six positions (Ala68/Gly68, Val102/Ile102, Lys106/Ala106, Asn201/His201, Ser318/Thr318, and Asn319/His319 for strains K-12/B, respectively), possibly accounting for the observed tetrameric versus octomeric crystal structures (although differences in pH alternatively may have impacted the quaternary structure). In solution, both proteins behave as tetramers of a subunit that possesses a double-stranded β -helix fold containing a ferrous ion bound to a 2-histidine-1-carboxylate motif involving His160, Asp162, and His292 (Fig. 3). This protein fold and metallocenter arrangement is a hallmark of Fe(II)/2OG-dependent oxygenases (Aik et al., 2015), a family of enzymes that catalyzes hydroxylation, halogenation, ring formation, desaturation, epimerization, and other reactions (Hausinger, 2004; Herr & Hausinger, 2018; Martinez & Hausinger, 2015). The diversity of potential reactions and the known breadth of substrates utilized by Fe(II)/2OG-dependent oxygenase family members, including proteins, oligonucleotides, lipids, amino acids, bases, terpenoids, and more (Hausinger, 2015; Herr & Hausinger, 2018; Islam, Leissing, Chowdhury, Hopkinson, & Schofield, 2018; Wu, Meng, &

Tang, 2016), initially hindered assignment of the primary substrate of CsiD and its cellular function.

2.2 Biochemical Confirmation that CsiD is an Fe(II)/2OG oxygenase

Because of our long-standing interest in Fe(II)/2OG-dependent oxygenases (Hausinger, 2004, 2015; Herr & Hausinger, 2018; Martinez & Hausinger, 2015) and as an extension of our earlier characterization of LhgO from *E. coli* (Kalliri & Hausinger, 2008), we initiated investigations of CsiD from this microorganism. We found that CsiD catalyzes an uncoupled reaction in which oxygen and 2OG are consumed in the absence of the enzyme's primary substrate if an external reductant such as ascorbic acid is provided, as previously noted for several other members of the Fe(II)/2OG dioxygenase family (Hausinger, 2004). For example, incubation of CsiD (2 μ M final concentration) in 25 mM imidazole buffer, pH 6.75, containing 100 μ M ferrous ions, 200 μ M ascorbate, and 1 mM 2OG led to oxygen consumption at an initial rate of 700–900 nmoles min^{-1} (mg protein) $^{-1}$ when measured with a Clark-type oxygen electrode. Furthermore, a similar enzyme preparation (2.5 μ M CsiD in 25 mM imidazole buffer, pH 6.75, containing 50 μ M ferrous ions, 0.4 mM ascorbate, and 1 mM 2OG) resulted in 2OG consumption and succinate production at initial rates of 738 nmol min^{-1} (mg protein) $^{-1}$ and 612 nmol min^{-1} (mg protein) $^{-1}$, respectively, when measured by HPLC. These results support the assignment of CsiD as a 2OG-dependent oxygenase.

Another diagnostic property of members within the Fe(II)/2OG-dependent oxygenase family is the development of a pink or purple color when anaerobic apoprotein is incubated with Fe(II) and 2OG (Proshlyakov, McCracken, & Hausinger, 2017). This chromophore has been attributed to metal-to-ligand charge-transfer (MLCT) transitions associated with the bidentate coordination of the metal by the C1 carboxylate and C2 keto group of 2OG (Pavel et al., 1998). CsiD was treated with EDTA to remove any contaminating nickel or other metal ions that might be bound to the purified protein. After dialysis against EDTA-free buffer containing 25 mM Tris and 1 mM dithiothreitol, an Fe(II)/ascorbate mixture was added, followed by addition of 2OG to yield the difference spectrum in Fig. 4. The position and intensity of the λ_{max} (510 nm with $\epsilon_{510} = 158 \text{ M}^{-1}\cdot\text{cm}^{-1}$) is similar to the situation for many other proteins of this family such as the sulfonate-degrading enzyme TauD, the herbicide-metabolizing representative TfdA, and the biosynthetic enzyme clavamate synthase (λ_{max} 500–530 nm and ϵ_{530} in the range of 140–375 $\text{M}^{-1}\cdot\text{cm}^{-1}$) (Hegg et al., 1999; Pavel et al., 1998; Ryle, Padmakumar, & Hausinger, 1999). These results confirm that CsiD binds ferrous ion plus 2OG in a bidentate coordination complex.

2.3 Identification of CsiD as a Glutarate L-2 Hydroxylase

Because *E. coli csiD* lies adjacent to *lhgO* encoding an L-2HG oxidase (Kalliri & Hausinger, 2008) or L-2HG dehydrogenase (Knorr et al., 2018), we reasoned that CsiD may be a 2OG-dependent glutarate L-2-hydroxylase. To test this hypothesis, we incubated CsiD with equal amounts of 2OG and glutaric acid while monitoring the substrate mixture and products by ^1H NMR spectroscopy. We observed the complete consumption of both 2OG and glutarate with conversion of the substrates to 2HG and succinate (Fig. 5). Essentially identical NMR results were published by others (Knorr et al., 2018). Confirmation of this reaction was

obtained by mass spectrometric analyses with the *E. coli* protein (Knorr et al., 2018) and by HPLC quantifications using an organic acids column with the *P. putida* enzyme (Zhang et al., 2018).

To assess the enantiospecificity of the 2HG produced by CsiD, we examined the effect of mixing the product of the CsiD reaction with LhgO that is known to be specific for L-2HG (Kalliri & Hausinger, 2008). A CsiD reaction mixture was incubated at 37 °C and timed aliquots were examined for the remaining concentrations of 2OG by colorimetric quantification using *ortho*-phenylenediamine (OPD), a reagent that reacts with ketones and aldehydes (Hayashi, Tsuchiya, Todoriki, & Naruse, 1982). After the 2OG was consumed at 2 h, LhgO was added to convert L-2HG to 2OG that was quantified by the OPD assay. The levels of 2OG generated were stoichiometric with the amount originally provided (Fig. 6), thus demonstrating that CsiD specifically forms the L-isomer of 2HG. Formation of the L-isomer product also was demonstrated by derivatization with diacetyl-L-tartaric anhydride and analysis by liquid chromatography-mass spectrometry (Knorr et al., 2018). In addition, L-specificity was inferred for the product obtained by *P. putida* CsiD on the basis of its inability to be utilized by a D-specific 2HG dehydrogenase (Zhang et al., 2018).

3. CHARACTERIZATION OF GLUTARATE L-2-HYDROXYLASE

3.1 Enzyme Kinetics of Glutarate L-2-Hydroxylase

Selected kinetic parameters have been obtained for CsiD isolated from *E. coli*, *P. putida*, *Klebsiella pneumoniae*, and *Salmonella typhimurium* (Knorr et al., 2018; Zhang et al., 2018) (Table 2). The two specific activity values reported in studies of the *E. coli* protein were more than two-fold different, perhaps due to the distinct assay conditions used. Although both measurements utilized an oxygen electrode and included 400 μ M ascorbate and 1 mM 2OG, one was carried out in 100 mM MOPS buffer (pH 7.2) containing 5 mM glutarate, 70 mM KCl, and 4 μ M $\text{Fe}(\text{NH}_4)_2\text{SO}_4$ at 30 °C (Knorr et al., 2018), whereas the other was performed in 20 mM imidazole (pH 6.7) containing 1 mM glutarate, no added salt, and 50 μ M ferrous ions at an unstated temperature (Zhang et al., 2018). The specific activity values for CsiD from all sources are the same order of magnitude, and for *E. coli* the glutarate 2-hydroxylase activity corresponds to at least 100-fold the uncoupled rate for oxidative decarboxylation of 2OG in the presence of ascorbate. The K_m parameters were determined only for the *E. coli* and *P. putida* proteins; values for glutarate were more than 4-fold different, whereas those for 2OG were identical. In the case of *P. putida* CsiD, the K_m of oxygen (266 ± 14 μ M) also was determined (Zhang et al., 2018). Although k_{cat} and k_{cat}/K_m values for glutarate, 2OG, and oxygen were provided in the *P. putida* CsiD study, these numbers are inconsistent with the reported specific activity and V_{max} values (Zhang et al., 2018) and should not be used when comparing to such values for enzymes from other sources.

CsiD appears to be highly specific for glutaric acid as the primary substrate, with no activity observed when this compound was substituted by 5-aminovalerate, 2-aminoadipate, 2-oxoadipate (2OA), succinate, adipate, L-lysine, or glutaryl-CoA using the *P. putida* enzyme (Zhang et al., 2018). Studies with the *E. coli* CsiD found no activity when substituting glutarate with other dicarboxylic acids including oxalate, malonate, succinate, adipate, and

pimelate. Furthermore, CsiD proteins from *Halobacillus* sp. BAB-2008, *P. putida*, and *E. coli* were confirmed to lack activity using malonate, succinate, adipate, propionate, butyrate, pentanoate, and hexanoate while retaining the capacity to hydroxylate glutarate (Thompson et al., 2019). While 2OA was not used as the primary substrate, *E. coli* CsiD is active when 2OA replaces 2OG in the reaction (unpublished observations and Thompson et al., 2019). Several other family members can substitute 2OG with 2OA, a 2-oxo acid containing an extra methylene carbon, for catalyzing their reactions (e.g., (Eichhorn, van der Ploeg, Kertesz, & Leisinger, 1997; Fukumori & Hausinger, 1993; Kahnert & Kertesz, 2000; Martinez & Hausinger, 2016)).

Inhibitors of CsiD include a variety of metal chelators, which remove the essential ferrous ion from the active site (unpublished observations), and *N*-oxalylglycine (NOG), a 2OG mimic that is incapable of supporting catalysis (Knorr et al., 2018). These compounds are known to inhibit a multitude of other Fe(II)/2OG-dependent oxygenase (Aik et al., 2015).

3.2 Structures of Glutarate L-2-Hydroxylase:Ligand Complexes

Knorr *et al.* describe four crystal structures of CsiD (Fig. 7) that provide keen insights into how the protein interacts with its substrates (Knorr et al., 2018). In agreement with the previously described structures of this protein (Chance et al., 2002; Lohkamp & Dobritzsch, 2008), the catalytic metal ion is bound within the double-stranded β -helix fold by the His160, Asp162, and His292 sidechains, with the octahedral coordination geometry completed by three water molecules (Fig. 7A). Several positively-charged Arg residues located in the active site pocket are potentially poised to interact with the carboxylate-containing substrates (2OG and glutarate).

The structure of the 2OG-bound enzyme was approximated by using the 2OG analog NOG (Fig. 7B). Structures have been solved for many other Fe(II)/2OG oxygenase family members in the presence of NOG or 2OG, and they typically exhibit bidentate metal coordination of the ligand with the C2 carbonyl group opposite the carboxylate side chain and the C1 carboxyl group located opposite one of the His ligands or at an intermediate position. Furthermore, most other family representatives possess two arginine residues at the active site; one Arg forms a hydrogen bond with the proximal carboxylate of 2OG/NOG and a second Arg stabilizes the distal carboxylate of 2OG/NOG in a salt bridge (Aik et al., 2015). For CsiD, NOG coordinates the metal in a bidentate manner with its C2 carbonyl located opposite Asp162 and the C1 carboxylate positioned approximately mid-way opposite the two His residues. A hydrogen bond from Arg309 stabilizes the metal-bound carboxyl group, and very long hydrogen bonds are potentially provided by Arg294 and Arg305 to the distal carboxylate.

The structure of CsiD with the bound product succinate (Fig. 7C) is notable in two respects. First, the metal coordination geometry involving His160, Asp162, and the two carboxylate oxygen atoms of the product is nearly planar, leading to an open coordination site opposite Asp292. This open site indicates the most likely position for the L-2HG hydroxyl oxygen atom if that product were present. Second, the distal carboxylate of succinate is hydrogen bonded to one of two conformations of Arg305 (whereas the distance of this carboxylate of glutarate to Arg294 has expanded to 5.2 Å). The flexibility of this side chain suggests that it

also may be capable of adopting a conformation that could form a salt bridge with this product or with NOG/2OG.

The glutarate-bound structure (Fig. 7D) depicts a severely bent substrate with one carboxylate forming a long and asymmetric salt bridge (3.2 and 3.8 Å in length) with a highly twisted Arg311 side chain (and the backbone amide of Gly163, not shown). The second carboxyl group of glutarate binds to the metal ion from which two water molecules have been displaced. This aspect of the binding mode is unlikely to be relevant to the situation that occurs when both glutarate and 2OG are coordinated to the protein during catalysis. In particular, 2OG is nearly certain to occupy two metal coordination sites and one site must be available to bind oxygen, so the glutarate-Fe(II) bond is not catalytically significant. Furthermore, the C2 carbon atoms of glutarate are inappropriately positioned for hydride abstraction by an Fe(IV)-oxo intermediate in order to yield the L-2HG product.

We generated a hypothetical model of the 2OG- and glutarate-bound structure of CsiD (Fig. 8). This view was created from the structure of NOG-bound CsiD by substituting the N atom of NOG with a carbon atom and rotating the position of the C1 carboxylate to lie closer to the plane formed by His160, Asp162, and the Fe(II) atom. A salt bridge between the 2OG C5 carboxylate and Arg305 was formed by rotating bonds of the arginine side chain. Small bond rotations in Arg309 allowed for hydrogen bonding to the 2OG C1 carboxylate and to glutaric acid. The substrate glutarate was shifted in position from that found in the glutarate-only structure and made less bent. Furthermore, bond rotations of Arg311 and Arg294 allowed for salt bridge stabilization of each carboxylate. This pose of the substrate is notable because the methylene carbon adjacent to the Arg311-interacting carboxylate is well positioned for hydrogen abstraction followed by hydroxyl radical rebound to yield the correct enantiomer: L-2HG. We stress that this figure presents only a theoretical view and a crystal structure of the authentic complex is needed for verification.

3.3 Reaction Mechanism of Glutarate L-2-Hydroxylase

On the basis of the known properties of glutarate L-2-hydroxylase described above along with the wealth of information available for other family members (Schofield & Hausinger, 2015), we propose the enzymatic reaction mechanism shown in Fig. 9. The resting state of the enzyme (boxed) possesses Fe(II) bound to three protein side chains (His160, Asp162, and His292) and with three coordinating water molecules. Bidentate binding of 2OG via a C1 carboxylate oxygen (opposite His160) and C2 keto oxygen (opposite Asp262) results in the release of two water molecules, generating the MLCT transitions that are diagnostic of this enzyme family (Fig. 4). The binding of 2OG is stabilized by Arg305 forming a salt bridge with the C5 carboxylate and by Arg309 hydrogen bonding to the C1 carboxylate (not depicted in the figure). The primary substrate, glutarate, binds near the metal center, but without direct coordination to the metal and possibly forms salt bridges with Arg311 and Arg294 (Fig. 8). The binding of the primary substrate results in perturbation of the MLCT features (data not shown), displacement of the final water molecule, and creation of an oxygen binding site. Binding of oxygen likely results in an Fe(III)-superoxo state that has not been directly observed in any representative of this group of enzymes. The reaction scheme next shows a plausible series of sequential steps involving a cyclic peroxo-Fe(IV)

species (also not directly observed for any family member), a persuccinate bound Fe(II) (crystallographically observed in VioC (Mitchell et al., 2017), and an Fe(IV)-oxo (ferryl) species; the pathway to reach the ferryl species remains uncertain, but direct evidence for the existence of the ferryl state has been obtained for several other members of this enzyme family (Chang et al., 2014; Chang, Li, Lee, Cronican, & Guo, 2016; Dunham et al., 2018; Galoni, Barr, Walsh, Bollinger, & Krebs, 2007; Hoffart, Barr, Guyer, Bollinger, & Krebs, 2006; Matthews et al., 2009; Mitchell et al., 2017; Price, Barr, Glass, Krebs, & Bollinger, 2003; Price, Barr, Tirupati, Bollinger, & Krebs, 2003; Proshlyakov, Henshaw, Monterosso, Ryle, & Hausinger, 2004). Accompanying formation of the ferryl species is the production of succinate and carbon dioxide.

The ferryl intermediate is positioned appropriately for in-line (Hausinger, 2004) abstraction of the hydrogen atom from the L prochiral position of the glutarate C2 atom, yielding Fe(III)-hydroxide and a substrate radical. Hydroxyl radical rebound would then yield L-2HG and restore the Fe(II) state of the enzyme. Alternatively, deprotonation of Fe(III)-hydroxide may lead to an Fe(III)-oxo state that reacts with the substrate radical to produce glutarate alkoxide bound to Fe(II) (not shown in Fig. 9), as suggested for other enzymes in this family on the basis of spectroscopy (Grzyska, Appelman, Hausinger, & Proshlyakov, 2010), crystallographic evidence (Mitchell et al., 2017), or computational methods (Fang & Cisneros, 2013). In either case, product dissociation and rebinding of three water molecules completes the cycle.

4 DISTRIBUTION OF GLUTARATE L-2-HYDROXYLASE

Zhang et al. identified homologs of *csiD* in 454 *Proteobacteria* and homologs of *lhgO* in 608 *Proteobacteria* and 4 *Actinobacteria* (Zhang et al., 2018). The distribution of each gene was suggested to be narrow and sporadic. Whereas genes encoding CsiD appear to be produced only in bacteria, those encoding LhgO also occur in eukaryotic microbes, animals, and plants. While 164 genomes included only *lhgO* (perhaps used for synthesis of a repair enzyme to remove this harmful metabolite), 448 genomes had both *csiD* and *lhgO*, and only 6 genomes had *csiD* without *lhgO*. The phylogenetic distribution of *csiD* also was assessed using the refseq database from May 2018 (Knorr et al., 2018); homologs are found in α -, β -, γ -, and ϵ -proteobacteria and bacilli.

5 CELLULAR ROLE OF GLUTARATE L-2-HYDROXYLASE

Deletion mutants of *csiD* (*csiD*) exhibit significant growth phenotypes compared to wild-type cells. For example, *E. coli* BL21(DE3) cells in the late stages of growth benefited by the inclusion of glutarate in minimal glucose medium, whereas a corresponding *csiD* culture reached stationary phase regardless of the presence of this supplemental nutrient (Fig. 10). Furthermore, the *csiD* strain of *E. coli* BW25113 leads to a growth defect after reaching an OD₆₀₀ of about 2 compared to that wild-type strain when grown in lysogeny broth (LB) medium (Knorr et al., 2018). In addition, deletion of *csiD* in *P. putida* results in delayed growth on glutarate medium (Zhang et al., 2018). These results are consistent with the notion that the *csiD* strains fail to utilize glutaric acid during stationary phase growth and suggest that CsiD is utilized for metabolizing glutaric acid. In addition, a transposon

insertion into *csiD* of *P. putida* results in decreased fitness when the cells are grown with 5-aminovaleate, an upstrate metabolite in L-lysine degradation (Thompson et al., 2019).

Glutarate has been detected in mid-exponential phase *E. coli* and other cells growing on mineral salts medium with glucose provided as the carbon source (van der Werf, Overkamp, Muilwijk, Coulier, & Hankemeier, 2007). Furthermore, using gas chromatography-mass spectrometric analysis of cell extracts, we have shown that glutarate is present in stationary phase *E. coli* cells grown in TB medium (data not shown). When glutarate is generated in sufficient quantities within the *E. coli* cell, its metabolism via CsiD may be an important metabolic strategy—especially during stationary phase. In particular, the combined action of CsiD and LhgO allow cells to convert glutarate to CO₂ and succinic acid that can be further metabolized (Fig. 11).

The amino acid L-lysine appears to be the primary source of glutaric acid in both *E. coli* (Knorr et al., 2018) and *P. putida* (Zhang et al., 2018)(Thompson et al. 2019). The *E. coli* pathway for lysine utilization is of special interest because of its ties to the GABA metabolism genes (Fig. 12). *E. coli* decarboxylates L-lysine to generate cadavarine by using two pyridoxal phosphate-dependent enzymes: LdcC and CadA (Lemonnier & Lane, 1998; Sabo, Boeker, Byers, Waron, & Fischer, 1974). The cadavarine is used to transaminate 2OG, forming glutamate and 5-aminopentanal, by the enzyme PatA, named for its analogous role in putrescine metabolism (Samsonova, Smirnov, Altman, & Ptitsyn, 2003). Similarly, PatD uses NAD⁺ to oxidize 5-aminopentanal thus generating 5-aminovaleate in addition to its better known function of converting 4-aminobutyraldehyde to GABA (Jorge, Perez-Garcia, & Wendisch, 2017; Knorr et al., 2018). Purified GabT and GabD were shown capable of converting 5-aminovaleate to glutarate with efficiencies comparable to those for conversion of GABA to succinate (Knorr et al., 2018). In *P. putida*, glutarate also is derived from L-lysine via 5-aminovaleate, but in this case the pathway uses the *davBA*, *davT*, and *davD* gene products (Liu et al., 2014; Revelles, Espinosa-Urgel, Molin, & Ramos, 2004; Zhang et al., 2018)(Thompson et al., 2019). In both microorganisms, CsiD and LhgO complete the lysine degradation pathways. Glutarate also is available from several sources in the environment; this molecule is formed by various microbial species via hydrolysis of glutaryl-CoA, during benzoate degradation, via decomposition of L-tryptophan (or its metabolites including L-kynurenine, anthranilate, 2-aminomuconate, and 2-oxoadipate), and other pathways (Kanehisa et al., 2014).

6 CONCLUSIONS

CsiD is a 2OG-dependent glutarate L-2-hydroxylase that allows *E. coli* cells to obtain energy from L-lysine metabolism during stationary phase. Given this function, Knorr *et al.* suggest the corresponding gene name be replaced by *glaH* (for glutarate hydroxylase) (Knorr et al., 2018). The period of carbon starvation is an important, but still understudied, phase of bacterial growth (Finkel, 2006; Kolter, Siegele, & Tormo, 1993; Navarro Lloren, Tormo, & Martinez-Garcia, 2010; Nyström, 2004). It has been reported that 2.3% of *E. coli* genes are associated with this life period according to the GenBank annotation (Tani, Khodursky, Blumenthal, Brown, & Matthews, 2002). CsiD/GlaH is regulated by RpoS, a sigma factor affecting nearly 500 genes or ~10% of the *E. coli* genome (Lacour & Landini,

2004; Patten, Kirchhof, Schertzberg, Morton, & Schellhorn, 2004; Weber, Polen, Heuveling, Wendisch, & Hengge, 2005), and Lrp, a transcription factor modulating more than 400 genes (Tani et al., 2002), with many of the functions for these genes remaining unidentified. As described here, the stationary phase-dependent regulation of *csiD/glaH* and its neighboring genes leads to the production of a complex pathway allowing the organism to metabolize L-lysine as a carbon source.

7 EXPERIMENTAL SECTION

7.1 Strain Construction

The *csiD* strain of *E. coli* BL21(DE3) was generated by P1 transduction (Thomason, Constantino, & Court, 2007) using the *csiD* strain of *E. coli* BW25113 of the Keio collection obtained from the Coli Genetic Stock Center (Baba et al., 2006).

For overproduction of the CsiD protein for biochemical studies, full-length *csiD* was amplified from *E. coli* MG1655 DNA using 5'-CATAAGAGGATCGCTTCATATGAATGCACTGACCG-3' and 5'-AAGCTTTTACTGATGCGTCTGGTA-3' primers that introduced NdeI and HindIII sites at the 5' and 3' end of the gene, respectively. Ligation of the endonuclease treated PCR product into identically digested pET42b (Novagen) provided an in-frame region encoding CsiD with an *N*-terminal His₆ tag. The resulting plasmid, pET42b-*csiD*, was transformed into DH5α competent cells, and the amplified plasmid was transformed into *E. coli* BL21(DE3) or the *csiD* cells that were plated on LB agar containing 50 µg/ml of kanamycin.

7.2 Purification of CsiD

A colony of *E. coli* BL21(DE3) pET42b-*csiD* was used to inoculate a culture of 50 ml LB medium containing 50 µg/ml of kanamycin and this was grown overnight at 37 °C. An aliquot (15 ml) was used to inoculate 1 L of terrific broth (DOT scientific) containing 50 µg/ml kanamycin, and this culture was grown at 37 °C until reaching OD₆₀₀ of 0.2. The culture was shifted to 25 °C and shaken vigorously until reaching OD₆₀₀ of 0.5 at which point the cells were induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside and the growth continued overnight at 25 °C. The cells were harvested by centrifugation at 7,500 *g* for 10 min at 4 °C. Approximately 3 g of harvested *E. coli* BL21(DE3) pET42b-*csiD* cells was suspended in 10 ml of lysis buffer containing 20 mM imidazole, 300 mM NaCl, 50 mM Na₂HPO₄, and 1 mM DTT, pH 8. The suspension was sonicated using a Sonifier 450 (Branson) for 1 min at 30 W output power, and 50 % pulse duration. The sonication was repeated four more times with 1 min intervals of cooling in ice. The broken cells were centrifuged at 100,000 *g* for 1 h at 4 °C. The soluble cell-free extracts (~9 ml) were loaded onto an 8 ml Ni-Sepharose 6-Fast Flow column (GE Healthcare), which had been equilibrated with buffer A (20 mM imidazole, 300 mM NaCl, 50 mM Na₂HPO₄, pH 8). The column was washed with buffer B (100 mM imidazole, 300 mM NaCl, 50 mM Na₂HPO₄, pH 8) in order to remove any protein weakly bound to the resin. CsiD was eluted with buffer C (500 mM imidazole, 300 mM NaCl, 50 mM Na₂HPO₄, pH 8). The fractions containing the purified protein were concentrated then passed through a PD-10 desalting column (GE Healthcare) for buffer exchange into 25 mM Tris, 1 mM DTT, pH 8. In some cases (where

apoprotein was desired) the CsiD solution was incubated with 1 mM EDTA prior to buffer exchange against the same Tris buffer. Enzyme samples were stored frozen at -80°C until use. Protein concentrations were determined by using the Bradford assay (Bradford, 1976).

7.3 Product Analyses and Enzyme Assays

In addition to the NMR analysis to monitor glutarate, 2OG, 2HG, and succinate (Fig. 5) and the OPD assay to monitor 2OG (Fig. 6), we measured the consumption of 2OG or glutarate and production of succinate or 2HG by HPLC (HPX-87H organic acids column, Bio-Rad). Reaction mixtures (containing enzyme and reactants in the buffer specified) were incubated for various times at 30°C , and quenched by adding $5\text{ }\mu\text{l}$ of 6 M sulfuric acid to the $300\text{ }\mu\text{l}$ aliquots. After centrifugation at $10,000\text{ }g$ for 5 min the supernatants were centrifuged in spin columns (Amicon ultrafree-MC from Millipore) at $10,000\text{ }g$ for 1 min, and $200\text{ }\mu\text{l}$ aliquots were injected onto the organic acids HPLC column that had been previously equilibrated with 13 mM sulfuric acid. The refractive index and absorbance at 214 nm were monitored and the integrated peak intensities were compared to those of authentic standards.

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REFERENCES

- Aik WS, Chowdhury R, Clifton IJ, Hopkinson RJ, Leissing T, McDonough MA, et al. (2015). Introduction to structural studies on 2-oxoglutarate-dependent oxygenases and related enzymes In Schofield CJ & Hausinger RP (Eds.), 2-Oxoglutarate-Dependent Oxygenases. Cambridge, U.K.: Royal Society of Chemistry.
- Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. Molecular and Systems Biology, 2, 2006.0008.
- Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248–254. [PubMed: 942051]
- Chance MR, Bresnick AR, Burley SK, Jiang J-S, Lima CD, Sali A, et al. (2002). Structural genomics: A pipeline for providing structures for the biochemist. Protein Science, 11, 723–738. [PubMed: 11910018]
- Chang W. c., Guo Y, Wang C, Butch SE, Rosenzweig AC, Boal AK, et al. (2014). Mechanism of the C5 stereoinversion reaction in the biosynthesis of carbapenem antibiotics. Science, 343, 1140–1144. [PubMed: 24604200]
- Chang W. c., Li J, Lee JL, Cronican AA, & Guo Y (2016). Mechanistic investigation of a non-heme iron enzyme catalyzed epoxidation in (–)-4'-methoxycyclophenin biosynthesis. Journal of the American Chemical Society, 138, 10390–10393. [PubMed: 27442345]
- Cozzani I, Fazio AM, Felici E, & Barletta G (1980). Separation and characterization of NAD- and NADP-specific succinate-semialdehyde dehydrogenase from *Escherichia coli* K-12 3300. Biochimica Et Biophysica Acta, 613, 309–317. [PubMed: 7004491]
- Dover S, & Halpern YS (1974). Genetic analysis of the γ -aminobutyrate utilization pathway in *Escherichia coli* K-12. Journal of Bacteriology, 117, 494–501. [PubMed: 4590473]
- Dunham NP, Chang W. c., Mitchell AJ, Martinie RJ, Zhang B, Bergman JA, et al. (2018). Two distinct mechanisms for C-C desaturation by iron(II)- and 2-(oxo)glutarate-dependent oxygenases:

- Importance of α -heteroatom assistance. *Journal of the American Chemical Society*, 140, 7116–7126. [PubMed: 29708749]
- Eichhorn E, van der Ploeg JR, Kertesz MA, & Leisinger T (1997). Characterization of α -ketoglutarate-dependent taurine dioxygenase from *Escherichia coli*. *Journal of Biological Chemistry*, 272, 23031–23036. [PubMed: 9287300]
- Fang D, & Cisneros GA (2013). Alternative pathway for the reaction catalyzed by DNA dealkylase AlkB from ab initio QM/MM calculations. *Journal of Chemical Theory and Computation*, 10, 5136–5148.
- Finkel SE (2006). Long-term survival during stationary phase: evolution and the GASP phenotype. *Nature Reviews in Microbiology*, 4, 113–120. [PubMed: 16415927]
- Fukumori F, & Hausinger RP (1993). Purification and characterization of 2,4-dichlorophenoxyacetate/ α -ketoglutarate dioxygenase. *Journal of Biological Chemistry*, 268, 24311–24317. [PubMed: 8226980]
- Galoni DP, Barr EW, Walsh CT, Bollinger JM Jr., & Krebs C (2007). Two interconverting Fe(IV) intermediates in aliphatic chlorination by the halogenase CytC3. *Nature Chemical Biology*, 3, 113–116. [PubMed: 17220900]
- Grzyska PK, Appelman EH, Hausinger RP, & Proshlyakov DA (2010). Insight into the mechanism of an iron dioxygenase by resolution of steps following the Fe(IV)=O species. *Proceedings of the National Academy of Sciences*, 107, 3982–3987.
- Hausinger RP (2004). Fe(II)/ α -ketoglutarate-dependent hydroxylases and related enzymes. *Critical Reviews in Biochemistry and Molecular Biology*, 39, 21–68. [PubMed: 15121720]
- Hausinger RP (2015). Biochemical diversity of 2-oxoglutarate-dependent oxygenases In Schofield CJ & Hausinger RP (Eds.), *2-Oxoglutarate-Dependent Oxygenases* (pp. 1–58). Cambridge, U.K.: Royal Society of Chemistry.
- Hayashi T, Tsuchiya H, Todoriki H, & Naruse H (1982). High-performance liquid chromatographic determination of alpha-keto acids in human urine and plasma. *Analytical Biochemistry*, 122, 173–179. [PubMed: 6808860]
- Hegg EL, Whiting AK, Saari RE, McCracken J, Hausinger RP, & Que L Jr. (1999). Herbicide-degrading α -keto acid-dependent enzyme TfdA: Metal coordination environment and mechanistic insights. *Biochemistry*, 38, 16714–16726. [PubMed: 10600135]
- Hengge R (2008). The two-component network and the general stress sigma factor Rpo(σ s) in *Escherichia coli*. *Advances in Experimental Medicine and Biology*, 631, 40–53. [PubMed: 18792681]
- Herr CQ, & Hausinger RP (2018). Amazing diversity in biochemical roles of Fe(II)/2-oxoglutarate oxygenases. *Trends in Biochemical Sciences*, 43, 517–532. [PubMed: 29709390]
- Hoffart LM, Barr EW, Guyer RB, Bollinger JM Jr., & Krebs C (2006). Direct spectroscopic detection of a C-H-cleaving high-spin Fe(IV) complex in a prolyl-4-hydroxylase. *Proceedings of the National Academy of Sciences*, 103, 14738–14743.
- Islam MS, Leissing TM, Chowdhury R, Hopkinson RJ, & Schofield CJ (2018). 2-Oxoglutarate-dependent oxygenases. *Annual Review of Biochemistry*, 87, 585–620.
- Jorge JMP, Perez-Garcia F, & Wendisch VF (2017). A new metabolic route of the fermentative production of 5-aminovalerate from glucose and alternative carbon sources. *Bioresource Technology*, 245, 1701–1709. [PubMed: 28522202]
- Kahnert A, & Kertesz MA (2000). Characterization of a sulfur-regulated oxygenative alkylsulfatase from *Pseudomonas putida* S-313. *Journal of Biological Chemistry*, 275, 31661–31667. [PubMed: 10913158]
- Kalliri E, & Hausinger RP (2008). Identification of *Escherichia coli* YgaF as an L-2-hydroxyglutarate oxidase. *Journal of Bacteriology*, 190, 3793–3798. [PubMed: 18390652]
- Kanehisa M, Goto S, Sato Y, Kawashima M, Furumichi M, & Tanabe M (2014). Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Research*, 42, D199–D205. [PubMed: 24214961]
- Knorr S, Sinn M, Galetskiy D, Williams RM, Wang C, Müller N, et al. (2018). Widespread bacterial lysine degradation proceeding via glutarate and L-2-hydroxyglutarate. *Nature Communications*, 9, 5071.

- Kolter R, Siegele DA, & Tormo A (1993). The stationary phase of the bacterial life cycle. *Annual Review of Microbiology*, 47, 855–874.
- Lacour S, & Landini P (2004). σ^S -Dependent gene expression at the onset of stationary phase in *Escherichia coli*: Function of σ^S -dependent genes and identification of their promoter sequences. *Journal of Bacteriology*, 186, 7186–7195. [PubMed: 15489429]
- Lemonnier M, & Lane D (1998). Expression of the second lysine decarboxylase gene of *Escherichia coli*. *Microbiology*, 144, 751–760. [PubMed: 9534244]
- Liu P, Zhang H, Lv M, Hu M, Li Z, Gao C, et al. (2014). Enzymatic production of 5-aminovalerate from L-lysine using L-lysine monooxygenase and 5-aminovaleramide amidohydrolase. *Scientific Reports*, 4(5657).
- Lohkamp B, & Dobritsch D (2008). A mixture of fortunes: The curious determination of the structure of *Escherichia coli* BL21 Gab protein. *Acta Crystallographica*, D64, 407–415.
- Marschall C, Labrousse V, Kreimer M, Wichart D, Kolb A, & Hengge-Aronis R (1998). Molecular analysis of the regulation of *csiD*, a carbon starvation-inducible gene in *Escherichia coli* that is exclusively dependent on σ^S and requires activation by cAMP-CRP. *Journal of Molecular Biology*, 276, 339–353. [PubMed: 9512707]
- Martinez S, & Hausinger RP (2015). Catalytic mechanisms of Fe(II)- and 2-oxoglutarate-dependent oxygenases. *Journal of Biological Chemistry*, 290, 20702–20711. [PubMed: 26152721]
- Martinez S, & Hausinger RP (2016). Biochemical and spectroscopic characterization of the non-heme Fe(II)- and 2-oxoglutarate-dependent ethylene-forming enzyme from *Pseudomonas syringae* pv. *phaseolicola* PK2. *Biochemistry*, 55, 5989–5999. [PubMed: 27749027]
- Matthews MM, Krest C, Barr EW, Vaillancourt F, Walsh CT, Green M, et al. (2009). Substrate-triggered formation and remarkable stability of the C-H-cleaving chloroferryl intermediate in the aliphatic halogenase, SyrB2. *Biochemistry*, 48, 4331–4343. [PubMed: 19245217]
- Metzner M, Germer J, & Hengge R (2004). Multiple stress signal integration in the regulation of the complex σ^S -dependent *csiD-ygaF-gabDTP* operon in *Escherichia coli*. *Molecular Microbiology*, 51, 799–811. [PubMed: 14731280]
- Mitchell AJ, Dunham NP, Martinie RJ, Bergman JA, Pollock CJ, Hu K, et al. (2017). Visualizing the reaction cycle in an iron(II)- and 2-(oxo)-glutarate-dependent hydroxylase. *Journal of the American Chemical Society*, 139, 13830–13836. [PubMed: 28823155]
- Navarro Lloren JM, Tormo A, & Martinez-Garcia E (2010). Stationary phase in gram-negative bacteria. *FEMS Microbiology Reviews*, 34, 476–495. [PubMed: 20236330]
- Niegemann E, Schulz A, & Bartsch K (1993). Molecular organization of the *Escherichia coli* *gab* cluster: nucleotide sequence of the structural genes *gabD* and *gabP* and expression of the GABA permease gene. *Archives of Microbiology*, 160, 454–460. [PubMed: 8297211]
- Nyström T (2004). Stationary-phase physiology. *Annual Review of Microbiology*, 58, 161–181.
- Patten CL, Kirchhof MG, Schertzberg MR, Morton RA, & Schellhorn HE (2004). Microarray analysis of RpoS-mediated expression in *Escherichia coli* K-12. *Molecular and General Genetics*, 272, 580–591.
- Pavel EG, Zhou J, Busby RW, Gunsior M, Townsend CA, & Solomon EI (1998). Circular dichroism and magnetic circular dichroism spectroscopic studies of the non-heme ferrous active site in clavamate synthase and its interaction with α -ketoglutarate cosubstrate. *Journal of the American Chemical Society*, 120, 743–753.
- Price JC, Barr EW, Glass TE, Krebs C, & Bollinger JM Jr. (2003). Evidence for hydrogen abstraction from C1 of taurine by the high-spin Fe(IV) intermediate detected during oxygen activation by taurine: α -ketoglutarate dioxygenase (TauD). *Journal of the American Chemical Society*, 125, 13008–13009. [PubMed: 14570457]
- Price JC, Barr EW, Tirupati B, Bollinger JM Jr., & Krebs C (2003). The first direct characterization of a high-valent iron intermediate in the reaction of an α -ketoglutarate-dependent dioxygenase: A high-spin Fe(IV) complex in taurine/ α -ketoglutarate dioxygenase (TauD) from *Escherichia coli*. *Biochemistry*, 42, 7497–7508. [PubMed: 12809506]
- Proshlyakov DA, Henshaw TF, Monterosso GR, Ryle MJ, & Hausinger RP (2004). Direct detection of oxygen intermediates in the non-heme Fe enzyme taurine/ α -ketoglutarate dioxygenase. *Journal of the American Chemical Society*, 126, 1022–1023. [PubMed: 14746461]

- Proshlyakov DA, McCracken J, & Hausinger RP (2017). Spectroscopic analyses of 2-oxoglutarate-dependent oxygenases: TauD as a case study. *Journal of Biological Inorganic Chemistry*, 22, 367–379. [PubMed: 27812832]
- Revelles O, Espinosa-Urgel M, Molin S, & Ramos JL (2004). The *davDT* operon of *Pseudomonas putida*, involved in lysine catabolism, is induced in response to the pathway intermediate δ -aminovaleric acid. *Journal of Bacteriology*, 186, 3439–3446. [PubMed: 15150230]
- Ryle MJ, Padmakumar R, & Hausinger RP (1999). Stopped-flow kinetic analysis of *Escherichia coli* taurine/ α -ketoglutarate dioxygenase: Interactions with α -ketoglutarate, taurine, and oxygen. *Biochemistry*, 38, 15278–15286. [PubMed: 10563813]
- Sabo DL, Boeker EA, Byers B, Waron H, & Fischer EH (1974). Purification and physical properties of inducible *Escherichia coli* lysine decarboxylase. *Biochemistry*, 13, 662–670. [PubMed: 4590109]
- Samsonova NN, Smirnov SV, Altman IB, & Ptitsyn LR (2003). Molecular cloning and characterization of *Escherichia coli* K12 *ygiG* gene. *BMC Microbiology*, 3, 2. [PubMed: 12617754]
- Schneider BL, Ruback S, Kiupakis AK, Kasbarian H, Pybus C, & Reitzer L (2002). The *Escherichia coli* *gabDTPC* operon: Specific γ -aminobutyrate catabolism and nonspecific induction. *Journal of Bacteriology*, 184, 6976–6986. [PubMed: 12446648]
- Schofield CJ, & Hausinger RP (Eds.). (2015). *2-Oxoglutarate-Dependent Oxygenases*. Cambridge, U.K.: Royal Society of Chemistry.
- Tani TH, Khodursky A, Blumenthal RM, Brown PO, & Matthews RG (2002). Adaptation to famine: A family of stationary-phase genes revealed by microarray analysis. *Proceedings of the National Academy of Sciences*, 99, 13471–13476.
- Thomason LC, Constantino N, & Court DL (2007). *E. coli* genome manipulation by P1 transduction. *Current Protocols in Molecular Biology*, 79, 1.17.11–11.17.18.
- Thompson MG, Blake-Hedges JM, Cruz-Morales P, Barajas JF, Curran SC, Eiben CB, Harris NC, Benites VT, Gin JW, Sharpless WA, Twigg FF, Skyrud W, Krishna RN, Pereira JH, Baidoo EEK, Petzold CJ, Adams PD, Arkin AP, Deutschbauer AM, & Keasling JD (2019). Massively parallel fitness profiling reveals multiple novel enzymes in *Pseudomonas putida* lysine metabolism. *mBio*, 10, e02577e18. [PubMed: 31064836]
- van der Werf MJ, Overkamp KM, Muilwijk B, Coulier L, & Hankemeier T (2007). Microbial metabolomics: Toward a platform with full metabolome coverage. *Analytical Biochemistry*, 370, 17–25. [PubMed: 17765195]
- Weber H, Polen T, Heuveling J, Wendisch VF, & Hengge R (2005). Genome-wide analysis of the general stress response network in *Escherichia coli*: σ^S -Dependent genes, promoters, and sigma factor selectivity. *Journal of Bacteriology*, 187, 1591–1603. [PubMed: 15716429]
- Weichart D, Lange R, Henneberg N, & Hengge-Aronis R (1993). Identification and characterization of stationary phase-inducible genes in *Escherichia coli*. *Molecular Microbiology*, 10, 407–420.
- Wu L-F, Meng S, & Tang G-L (2016). Ferrous iron and α -ketoglutarate dependent dioxygenases in the biosynthesis of microbial natural products. *Biochimica Et Biophysica Acta*, 1864, 453–470. [PubMed: 26845569]
- Zhang M, Gao C, Guo X, Guo S, Kang Z, Xiao D, et al. (2018). Increased glutarate production by blocking the glutaryl-CoA dehydrogenation pathway and a catabolic pathway involving L-2-hydroxylglutarate. *Nature Communications*, 9, 2114.

Fig. 1.
Genomic location of *csuD* in *E. coli* and regulation of the *csuD-lhgO-gabDTP-csiR* gene cluster.

Fig. 2.

Functions of LhgO and GabDTP. LhgO catalyzes the O_2 -dependent oxidation (as illustrated) or the menaquinone-linked dehydrogenation (not shown) of L-2HG to form hydrogen peroxide (or reduced menaquinone) and 2OG. The GabP permease allows GABA to traverse the cell membrane (indicated by the stippled lines). The GabT transaminase converts intracellular GABA into succinic acid semialdehyde while transforming 2OG into glutamic acid (Glu). GabD then catalyzes the $NADP^+$ -dependent oxidation of succinic acid semialdehyde to generate succinate.

Fig. 3. Overall structure of CsiD. The CsiD (A) octamer, (B) tetramer, and (C) monomer are depicted in cartoon mode with different subunits in the oligomers shown in distinct colors and the single subunit shown with β -strands in yellow, α -helices in red, and loops in green. Fe atoms are shown as brown spheres and the protein ligands to the metal are shown as orange sticks. (D) An expanded view of the active site depicts several positively-charged arginine side chains near the active site that could interact with 2OG and the primary substrate.

Fig. 4.

CsiD exhibits a visible absorbance spectrum in the presence of Fe(II) and 2OG. Anaerobic solutions of CsiD were prepared in sealed serum vials by alternative cycles of vacuum treatment and argon purging. Stock solutions of 2OG (100 mM) were prepared in 100 mM HEPES buffer, pH 8.0, and made anaerobic by 5 rounds of degassing and flushing with argon. Mixtures of ferrous ammonium sulfate (18 mM) and sodium dithionite (100 mM) were prepared by three rounds of degassing and argon flushing of the solids, followed by addition of the desired volume of anoxic water. The protein (25 μ M) was mixed with 125 μ M $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ and 1 mM 2OG in 25 mM Tris buffer, pH 8.0, at room temperature and the difference spectrum was obtained after subtracting the spectrum of the same concentrations of enzyme and iron.

Fig. 5.

CsiD converts 2OG and glutarate into succinate and 2HG as shown by ^1H -NMR spectroscopy. CsiD ($2\ \mu\text{M}$) was incubated with $1\ \text{mM}$ 2OG, $1\ \text{mM}$ glutarate, and $0.1\ \text{mM}$ ferrous ions in $0.7\ \text{mL}$ of $1\ \text{mM}$ Tris buffer ($\text{pH}\ 8.0$) at 37°C . Samples were boiled for $3\ \text{min}$ at 100°C and centrifuged at $10,000\ g$ for $10\ \text{min}$, then the supernatants were subjected to vacuum in a Speed-Vac until the solvent had evaporated. The remaining solids were dissolved in deuterated water and $300\ \text{MHz}$ proton ^1H NMR spectra were obtained. The upper panel depicts the spectrum obtained at zero time with peaks corresponding to 2OG and glutarate. The lower panel shows the spectrum after $2\ \text{h}$ of incubation revealing the disappearance of substrates and formation of succinate and 2HG.

Fig. 6.

CsiD generates the L-isomer of 2HG. CsiD (2 μ M) was incubated with 1 mM glutarate, 1 mM 2OG, and 0.1 mM ferrous ions in 100 mM HEPES buffer (pH 7.0). Aliquots (250 μ L) were removed at the times indicated and the OPD assay was used to monitor the remaining 2OG. LhgO (2 μ M, red squares) was added and the recovery of 2OG was assessed using the OPD assay.

Fig. 7.

Ligand-bound structures of glutarate L-2-hydroxylase: (A) CsiD holoprotein (PDB code: 6GPE), (B) CsiD:NOG (PDB code: 6GPN), (C) CsiD:succinate (PDB code: 6HL9), and (D) CsiD:glutarate (PDB code: 6HL8). Fe(II) atoms are shown as brown spheres, coordinated waters are red spheres, and metal-binding residues are shown as sticks with orange carbon atoms. Selected Arg side chains (green carbon atoms) near the active site are depicted, with panel C showing two conformations for Arg305. Selected distances are indicated.

Fig. 8.
Hypothetical view of the CsiD active site with bound 2OG (yellow) and glutarate (red).

Fig. 9.

Proposed enzymatic reaction mechanism of glutarate L-2-hydroxylase. Potential electron transfer reactions are shown by red arrows and the oxygen atoms derived from dioxxygen are indicated in bold red. Two species that have not yet been directly detected in any 2OG-dependent oxygenase are shown in brackets.

FIG. 10. CsiD increases cellular fitness in response to supplemental glutarate. (A) BL21(DE3) wild-type cells were grown in minimal medium A with 0.02% glucose and the presence of varying amount of glutarate (0 mM, circles; 1 mM, squares; and 5 mM triangles). (B) BL21(DE3) *csiD* cells were grown in identical conditions. The optical density at 600 nm (OD₆₀₀) was monitored to assess cell growth.

Fig. 11.

CsiD and LhgO combine to allow the metabolism of glutaric acid to form succinic acid plus carbon dioxide.

Fig. 12.

Lysine degradation in stationary-phase *E. coli* cells occurs by a pathway that includes CsiD and LhgO. The functions shown for GabT and GabD are expanded beyond their well-known roles. The oxidase activity shown for LhgO may alternatively utilize a menaquinone-dependent dehydrogenase activity.

Table 1

Summary of CsiD crystal structures

PDB ID	Metal	Ligand(s)	pH and Precipitates	Space Group	Cell Constants a, b, c (Å) α, β, γ (°)	Resolution (Å)
1JR7	Fe	None	pH 5.6; (NH ₄) ₂ SO ₄	I 4 2 2	120, 120, 136 90, 90, 90	2.0
2R6S	Fe	None	pH 9, 1.6 M (NH ₄) ₂ SO ₄	I 4 2 2	120, 120, 137 90, 90, 90	2.1
6GPE	Fe	None	pH 6, 30% 2-methylpentanediol	P 4 2 ₁ 2	121, 121, 137 90, 90, 90	2.2
6HL8	Fe	Glutarate	pH 6, 30% 2-methylpentanediol	P 4 2 ₁ 2	120, 120, 136 90, 90, 90	2.4
6GPN	Fe	NOG ^a	pH 7, 3.5 M sodium formate	P 4 2 ₁ 2	121, 121, 137 90, 90, 90	2.2
6HL9	Fe	Succinate	pH 7, 1 M succinate	P 4 2 ₁ 2	123, 123, 138 90, 90, 90	2.3

^aNOG, N-oxalylglycine

Table 2

Kinetic parameters of CsiD from different sources

Source of CsiD	Specific Activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	Glutarate K_m (μM)	2OG K_m (μM)	Reference
<i>E. coli</i>	53 ± 3	650 ± 20	100 ± 7	(Knorr et al., 2018)
<i>E. coli</i>	126.8 ± 0.4	NR ^a	NR	(Zhang et al., 2018)
<i>P. putida</i>	177 ± 3	145.7 ± 1.5	95.3 ± 1.0	(Zhang et al., 2018)
<i>K. pneumoniae</i>	118.1 ± 0.4	NR	NR	(Zhang et al., 2018)
<i>S. typhimurium</i>	194.2 ± 0.3	NR	NR	(Zhang et al., 2018)

^aNR, not reported