Exploration of the active site of *Escherichia coli* cystathionine γ -synthase

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Abstract: Cystathionine γ -synthase (CGS) catalyzes the condensation of *O*-succinyl-L-homoserine (L-OSHS) and L-cysteine (L-Cys), to produce L-cystathionine (L-Cth) and succinate, in the first step of the bacterial transsulfuration pathway. In the absence of L-Cys, the enzyme catalyzes the futile α,γ -elimination of L-OSHS, yielding succinate, α -ketobutyrate, and ammonia. A series of 16 site-directed variants of *Escherichia coli* CGS (eCGS) was constructed to probe the roles of active-site residues D45, Y46, R48, R49, Y101, R106, N227, E325, S326, and R361. The effects of these substitutions on the catalytic efficiency of the α,γ -elimination reaction range from a reduction of only ~2-fold for R49K and the E325A,Q variants to 310- and 760-fold for R361K and R48K, respectively. A similar trend is observed for the $k_{\text{cat}}/K_m^{\text{L-OSHS}}$ of the physiological, α,γ -replacement reaction. The results of this study suggest that the arginine residues at positions 48, 106 and 361 of eCGS, conserved in bacterial CGS sequences, tether the distal and α -carboxylate moieties, respectively, of the L-OSHS substrate. In contrast, with the exception of the 13-fold increase observed for R106A, the $K_m^{\text{L-Cys}}$ is not markedly affected by the site-directed replacement of the residues investigated. The decrease in k_{cat} observed for the S326A variant reflects the role of this residue in tethering the side chain of K198, the catalytic base. Although no structures exist of eCGS bound to active-site ligands, the roles of individual residues is consistent with the structures inhibitor complexes of related enzymes. Substitution of D45, E325, or Y101 enables a minor transamination activity for the substrate L-Ala.

Keywords: reaction specificity; pyridoxal phosphate; cystathionine; mechanism; structure–function relationship

Abbreviations: APPA, DL-E-2-amino-5-phosphono-3-pentenoic acid; AVG, aminoethoxyvinylglycine; CBL, cystathionine β -lyase; CGL, cystathionine γ -lyase; CGS, cystathionine γ -synthase; CTCPO, 5-carboxymethylthio-3-(3'-chlorophenyl)-1,2,4-oxadiazol; L-Cth, L-cystathionine; L-Hcys, L-homocysteine; IPTG, Isopropyl- β -D-thiogalactopyranoside; Ni-NTA, Ni-nitrilo triacetic acid; L-OPHS, *o*-phospho-L-homoserine; L-OSHS, *o*-succinyl-L-homoserine; PLP, pyridoxal 5'-phosphate; PPCA, 3-(phosphonomethyl)pyridine-2-carboxylic acid.

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Introduction

Cystathionine γ -synthase (CGS) is the first enzyme of the transsulfuration pathway, which converts L-cysteine (L-Cys) to L-Hcys, the immediate precursor of L-methionine (Fig. 1). The branch-point between L-methionine (L-Met) and L-threonine biosynthesis is L-homoserine in bacteria and *O*-phospho-L-homoserine (L-OPHS) in plants. Therefore, the bacterial and plant CGS enzymes condense distinct forms of activated L-homoserine, *O*-succinyl-L-homoserine (L-OSHS), and L-OPHS, respectively, with L-Cys to produce L-cystathionine (L-Cth).¹ Structures are available for *Escherichia coli* CGS (eCGS) and *Nicotiana tabacum* CGS (nCGS), as well as for the latter in complex with 3-(phosphonomethyl)pyridine-2-carboxylic acid (PPCA), 5-carboxymethylthio-3-(3'-chlorophenyl)-1,2,4-oxadiazol (CTCPO) and DL-E-2-amino-5-

Figure 1. (A) The bacterial transsulfuration pathway and (B) the structures of the nCGS inhibitors for which structures of the corresponding nCGS complexes are available.^{2,3}

phosphono-3-pentenoic acid (APPA) (Fig. 1).^{2,3} The structural similarity of the eCGS (1CS1) and nCGS (1I41) is demonstrated by the r.m.s. deviation of only 2.04 Å for ~350 C α atoms, the majority of the peptide backbone.^{3,4} Additionally, the r.m.s. deviation is only 0.3 Å between the native and inhibitor-bound forms of nCGS.² The bacterial and plant CGS enzymes are attractive targets for the development of novel anti-microbial compounds and herbicides because they are not present in mammals.

The enzymes of the γ -subfamily of fold-type I of pyridoxal 5'-phosphate (PLP)-dependent enzymes display remarkable structural similarity, as exemplified by the ~1.5 Å r.m.s. deviation between ~350 C α atoms observed on superposition of the structures of eCGS (1CS1), *Trichomonas vaginalis* methionine γ -lyase (tMGL; 1E5F), yeast (*Saccharomyces cerevisiae*) cystathionine γ -lyase (yCGL; 1N8P) and *E. coli* CBL (eCBL; 1CL1), the second enzyme of the bacterial transsulfuration pathway.⁵ These enzymes catalyze α,β and α,γ -elimination and replacement reactions on similar amino acid substrates and share common active-site features including the residues corresponding to Y46, R48, Y101, S326, and R361 of eCGS, as well as K198, the catalytic base. In contrast, D45, R106, N227, and E325 of eCGS are shared by CGL sequences, but not by CBL and eCGS-R49 is common to bacterial CGS and CBL enzymes, but is replaced by a serine in yeast CGL and a tyrosine in the plant enzyme nCGS. Interestingly, an arginine at position 106 of eCGS is con-

served in bacterial and plant CGS enzymes, but while this residue is proposed to bind the distal carboxylate of L-OSHS, it does not interact with the phosphonate moiety of APPA, an L-OPHS analog, in the structure of the nCGS complex.^{2,4}

The crystal structures of the plant nCGS enzyme in complex with inhibitors (Figs. 1 and 2) provides valuable insight into active site interactions.² However, the difference in the activated L-homoserine substrates and the active sites of the plant and bacterial enzymes complicates the use of these structures as a model for eCGS.²⁻⁴ Additionally, no information is available concerning the binding site of L-Cys. The side chains of R48, R106, Y101, S326, and R361 and those of E45, R49, and E325 were proposed to bind the L-OSHS and L-Cys substrates, respectively, of eCGS.⁴ Residues R58 and R372 of eCBL, which correspond to eCGS-R48 and R361, bind the distal and α -carboxylate moieties of the L-Cth substrate, the product of CGS.^{6,7} A key determinant of β -elimination reaction specificity in the context of the eCBL active site is S339, which is not involved in substrate binding but tethers the catalytic base following its release from the PLP cofactor upon formation of the external aldimine.⁶ A series of 16 site-directed variants of eCGS residues D45, Y46, R48, R49, Y101, R106, N227, E325, S326, and R361 was constructed to probe the specific roles of these residues in the γ -elimination and replacement reactions catalyzed by this enzyme. With the exception of R106A, the K_m^{L-Cys} of all variants is within 3-fold of the wild-type enzyme, indicating that the other nine targeted residues do not interact directly with the L-Cys substrate. In contrast, the observed increases in the K_m^{L-OSHS} values of the R48K, R106A,K, and R361K variants identify these residues as interacting with L-OSHS. The 40 and 360-fold decreases in the k_{cat} of the α,γ -elimination and replacement activities of the alanine replacement variant of S326 distinguish this residue, which guides the ϵ -amino group of K198, the catalytic base. A novel role for residues D45, Y101, and E325 as determinants of reaction promiscuity and specificity is described.

Results

Site-directed variants of *E. coli* CGS (eCGS) were constructed and characterized to probe the roles of residues D45, Y46, R48, R49, Y101, R106, N227, E325, S326, and R361 in the active site. The effects of the substitutions on the catalytic efficiency of the physiological α,γ -replacement and minor α,γ -elimination reactions, as well as the reaction with L-Ala, were investigated. The wild-type and 16 site-directed variants of eCGS were purified to homogeneity. All enzymes are soluble and, with the exceptions of R48A and R361A, possess detectable levels of the physiological α,γ -replacement, and minor α,γ -

Figure 2. (A) Observed contacts of APPA in the active-site of nCGS.² The dotted lines represent putative hydrogen bond distances of ≤ 3.3 Å between heteroatoms and the corresponding residue labels in eCGS are in given in brackets. Residues nCGS-Y111 $\approx$ eCGS-R49, nCGS-H289 $\approx$ eCGS-N227, and nCGS-P387 $\approx$ eCGS-E325 are shown in grey to indicate that they are not conserved between the plant and bacterial CGS enzymes. Other targeted active-site residues, including nCGS-E107, which is conservatively replaced by aspartate in eCGS, are shown in black. The image was constructed using ChemDraw and PDB entry 1I41.

elimination, activities and are substrate inhibited by L-Cys but not L-OSHS. The hydrolysis of *O*-acetylserine (OAS) is undetectable for the wild-type enzyme and the variants investigated, demonstrating their lack of α,β -elimination activity, in contrast with the

closely related eCBL.⁶ The L-Cth hydrolysis activity of the variants was also assayed (data not shown) and follows a similar trend to the α,γ -elimination of L-OSHS (Table II). The low catalytic efficiency of the wild-type enzyme for hydrolysis of L-Cth ($k_{\text{cat}}/K_{\text{m}}^{\text{L-Cth}}$)

Table I. Kinetic Parameters for the Condensation of L-OSHS and L-Cys by Wild-Type Site-Directed Variants of eCGS^a

Enzyme	k_{catR} (s ⁻¹)	$K_{\text{mR}}^{\text{L-OSHS}}$ (mM)	$K_{\text{mR}}^{\text{L-Cys}}$ (mM)	$K_{\text{iR}}^{\text{L-Cys}}$ (mM)	$k_{\text{catR}}/K_{\text{mR}}^{\text{L-OSHS}}$ (M ⁻¹ s ⁻¹)	$k_{\text{catR}}/K_{\text{mR}}^{\text{L-Cys}}$ (M ⁻¹ s ⁻¹)
eCGS ^b	121 $\pm$ 5	2.5 $\pm$ 0.5	0.11 $\pm$ 0.01	0.33 $\pm$ 0.09	(4.9 $\pm$ 0.9) $\times 10^4$	(1.06 $\pm$ 0.07) $\times 10^6$
eCGS	112 $\pm$ 5	4.4 $\pm$ 0.6	0.24 $\pm$ 0.02	1.2 $\pm$ 0.2	(2.5 $\pm$ 0.3) $\times 10^4$	(4.7 $\pm$ 0.4) $\times 10^5$
D45A	79 $\pm$ 9	19 $\pm$ 3	0.8 $\pm$ 0.1	1.1 $\pm$ 0.1	(4.2 $\pm$ 0.3) $\times 10^3$	(9.9 $\pm$ 0.6) $\times 10^4$
D45N	100 $\pm$ 20	39 $\pm$ 7	0.9 $\pm$ 0.2	1.8 $\pm$ 0.1	(2.6 $\pm$ 0.2) $\times 10^3$	(1.13 $\pm$ 0.09) $\times 10^5$
Y46F	6 $\pm$ 1	90 $\pm$ 20	0.6 $\pm$ 0.1	1.8 $\pm$ 0.2	71 $\pm$ 5	(1.13 $\pm$ 0.08) $\times 10^4$
R48K ^c	0.15 $\pm$ 0.01	n.s. ^c	0.22 $\pm$ 0.03	2.5 $\pm$ 0.5	2.1 $\pm$ 0.1	(6.8 $\pm$ 0.6) $\times 10^2$
R49A	5.6 $\pm$ 0.4	1.8 $\pm$ 0.5	0.12 $\pm$ 0.04	0.5 $\pm$ 0.2	(3.1 $\pm$ 0.9) $\times 10^3$	(5 $\pm$ 1) $\times 10^4$
R49K	130 $\pm$ 20	8 $\pm$ 2	0.36 $\pm$ 0.09	1.2 $\pm$ 0.3	(1.6 $\pm$ 0.3) $\times 10^4$	(3.6 $\pm$ 0.6) $\times 10^5$
Y101F	9.6 $\pm$ 0.2	1.9 $\pm$ 0.2	0.15 $\pm$ 0.01	0.34 $\pm$ 0.05	(5.1 $\pm$ 0.7) $\times 10^3$	(6.4 $\pm$ 0.3) $\times 10^4$
R106A	1.4 $\pm$ 0.5	11 $\pm$ 7	3 $\pm$ 1	0.6 $\pm$ 0.3	(1.2 $\pm$ 0.6) $\times 10^2$	(4.1 $\pm$ 0.5) $\times 10^2$
R106K	5.3 $\pm$ 0.5	39 $\pm$ 5	0.65 $\pm$ 0.08	2.9 $\pm$ 0.3	(1.4 $\pm$ 0.1) $\times 10^2$	(8.2 $\pm$ 0.5) $\times 10^3$
N227A	33 $\pm$ 3	8 $\pm$ 2	0.30 $\pm$ 0.06	1.8 $\pm$ 0.5	(4.2 $\pm$ 0.8) $\times 10^3$	(1.1 $\pm$ 0.2) $\times 10^5$
E325A	59 $\pm$ 3	0.7 $\pm$ 0.2	0.17 $\pm$ 0.02	0.24 $\pm$ 0.07	(9 $\pm$ 2) $\times 10^4$	(3.5 $\pm$ 0.3) $\times 10^5$
E325Q	57 $\pm$ 3	1.1 $\pm$ 0.3	0.29 $\pm$ 0.04	0.26 $\pm$ 0.08	(5 $\pm$ 1) $\times 10^4$	(2.0 $\pm$ 0.2) $\times 10^5$
S326A	0.31 $\pm$ 0.02	21 $\pm$ 3	0.20 $\pm$ 0.04	2.3 $\pm$ 0.3	15 $\pm$ 1	(1.6 $\pm$ 0.2) $\times 10^3$
R361K	0.50 $\pm$ 0.04	n.s. ^c	0.22 $\pm$ 0.04	0.25 $\pm$ 0.4	5.4 $\pm$ 0.2	(2.3 $\pm$ 0.2) $\times 10^3$

^a Kinetic parameters reported are for condensation of L-OSHS and L-Cys. Reaction conditions: 0.1–50 mM L-OSHS, 0.025–20 mM L-Cys, 1.3 mM NADH, 1.0 μ M eCBL, 1.0 μ M LDH, and 0.094–21.25 μ M wild-type or mutant eCGS, depending on the activity of the enzyme, in assay buffer at 25°C. The data were fit to the Eqs. (1)–(3).

^b Kinetic parameters of eCGS reported by Aitken *et al.*¹⁷

^c n.s. indicates that $K_{\text{m}}^{\text{L-OSHS}}$ exceeds the solubility limit of the L-OSHS, such that $k_{\text{catR}}/K_{\text{m}}^{\text{L-OSHS}}$ was determined via linear regression, at the L-Cys concentration for which maximal activity was observed, and k_{catR} , $K_{\text{m}}^{\text{L-Cys}}$ and $K_{\text{i}}^{\text{L-Cys}}$ were determined from the fit of velocity versus L-Cys, at the highest concentration of L-OSHS used, to Eq. (4).

Table II. Kinetic Parameters of the α,γ -Elimination of OSHS by Wild-Type Site-Directed Variants of eCGS^a

Enzyme	k_{catE} (s ⁻¹)	$K_{\text{mE}}^{\text{L-OSHS}}$ (mM)	$k_{\text{catE}}/K_{\text{mE}}^{\text{L-OSHS}}$ (M ⁻¹ s ⁻¹)
eCGS ^b	1.80 ± 0.05	1.3 ± 0.1	(1.35 ± 0.09) × 10 ³
eCGS	3.65 ± 0.09	1.9 ± 0.2	(2.0 ± 0.2) × 10 ³
D45A	1.61 ± 0.04	3.7 ± 0.3	(4.3 ± 0.2) × 10 ²
D45N	2.64 ± 0.05	4.6 ± 0.3	(5.8 ± 0.2) × 10 ²
Y46F	1.8 ± 0.1	23 ± 2	78 ± 3
R48K	0.7 ± 0.2	230 ± 80	2.63 ± 0.03 ^c
R49A	1.60 ± 0.04	8.2 ± 0.4	(1.96 ± 0.06) × 10 ²
R49K	2.99 ± 0.09	1.5 ± 0.2	(2.0 ± 0.2) × 10 ³
Y103F	1.74 ± 0.04	6.3 ± 0.4	(2.8 ± 0.1) × 10 ²
R106A	0.50 ± 0.02	25 ± 2	19.9 ± 0.8
R106K	0.99 ± 0.05	20 ± 2	51 ± 3
N227A	5.76 ± 0.07	7.3 ± 0.3	(7.9 ± 0.2) × 10 ²
E325A	2.4 ± 0.1	1.5 ± 0.3	(1.6 ± 0.2) × 10 ³
E325Q	2.5 ± 0.1	2.0 ± 0.3	(1.2 ± 0.1) × 10 ³
S326A	0.093 ± 0.002	9.3 ± 0.6	10.0 ± 0.4
R361K	1.6 ± 0.4	240 ± 60	6.5 ± 0.2

^a Kinetic parameters reported are for hydrolysis of L-OSHS. Reaction conditions: 0.1–50 mM L-OSHS and 1–26.25 μ M wild-type or mutant eCGS, depending on the activity of the enzyme, in assay buffer at 25°C. The data were fit to the Michaelis–Menten equation to obtain k_{cat} and $K_{\text{m}}^{\text{L-OSHS}}$ and Eq. (6) to obtain $k_{\text{cat}}/K_{\text{m}}^{\text{L-OSHS}}$.

^b Values for wild-type eCGS are from Aitken *et al.*¹⁷

^c The value of $k_{\text{catE}}/K_{\text{mE}}^{\text{L-OSHS}}$ of R48K was determined via linear regression based on the assumption that $K_{\text{mE}}^{\text{L-OSHS}} \gg [\text{L-Cth}]$, because this variant showed no evidence of saturation within the solubility limit of the substrate. Therefore, the estimates of k_{catE} and $K_{\text{mE}}^{\text{L-OSHS}}$ are provided only as estimates to illustrate the degree to which substrate binding is altered by the conservative substitution of R48 by lysine.

= 23.3 ± 0.8 M⁻¹s⁻¹), two orders of magnitude lower than the α,γ -elimination of L-OSHS ($k_{\text{catE}}/K_{\text{m}}^{\text{L-OSHS}}$ = 2000 ± 200 M⁻¹s⁻¹), precludes meaningful comparison for variants with decreased k_{cat} or increased $K_{\text{m}}^{\text{L-Cth}}$.

The D45A,N, R49A,K, and E325A,Q variants

The value of $K_{\text{mR}}^{\text{L-Cys}}$ is unchanged by site-directed substitutions targeting R49 and E325 and is increased only ~3-fold by replacement of D45 with alanine or asparagine (Table I). Although the k_{catR} of the α,γ -replacement activity is reduced 20-fold by the R49A substitution (Table I), the k_{catE} for the α,γ -elimination of L-OSHS is unchanged (Table II) and the kinetic parameters of R49K are identical to those of wild-type eCGS, within experimental error (Tables I and II). The 5- to 10-fold reductions in the $k_{\text{catR}}/K_{\text{mR}}^{\text{L-OSHS}}$ and $k_{\text{catR}}/K_{\text{mR}}^{\text{L-Cys}}$ of the alanine and asparagine replacement variants of D45 are the result of 3- to 12-fold increases in $K_{\text{mR}}^{\text{L-Cys}}$ and $K_{\text{mR}}^{\text{L-OSHS}}$ as k_{catR} is decreased ≤ 2 -fold by these substitutions (Table I). Interestingly, although the kinetic parameters of the α,γ -elimination of L-OSHS are unaffected by the alanine and glutamine substitutions of E325,

the $K_{\text{mR}}^{\text{L-OSHS}}$, and $K_{\text{mR}}^{\text{L-Cys}}$ values, for the α,γ -replacement activity, of these variants are decreased ~5-fold (Tables I and II, Fig. 3).

Upon reaction of wild-type eCGS with 200 mM L-alanine, the 421-nm absorbance, corresponding to the ketoenamine tautomer of the internal aldimine of the PLP cofactor, is decreased with the concomitant increase in a 320–330 nm shoulder [Fig. 4(A)], corresponding to the enolimine tautomer of the external aldimine of L-Ala or to a ketimine intermediate formed upon subsequent deprotonation of C α and protonation of C4'. The behavior of the R49A, R49K, and E325Q enzymes is identical to that of native eCGS [Fig. 4(A)]. In contrast, upon reaction of the D45A, D45N, and E325A substitution variants with L-Ala, the 421-nm peak of the internal aldimine decreases in intensity, with a concomitant formation of a 320-nm peak, which is distinct from the 320–330 nm shoulder observed for the wild-type enzyme (Fig. 4).

The R48K, R106A,K, R361K, and N227A variants

Residue eCGS-N227 corresponds to Y238 of eCBL, which binds the distal carboxylate of the substrate.⁶ However, in the context of the eCGS active site, replacement of N227 with alanine results in modest, <4-fold changes in kinetic parameters, compared with the wild-type enzyme (Tables I and II). The 100, 40, 760, and 310-fold decreases in the catalytic efficiency of the α,γ -elimination activity of the R106A, R106K, R48K, and R361K variants are dominated by the 20- to 25-fold and ~230-fold increases in the $K_{\text{mE}}^{\text{L-OSHS}}$ of the R106 variants and the R48 and R361 lysine-substitution variants, respectively, as the k_{catE} is decreased only 2- to 7-fold (Table II). The $K_{\text{mR}}^{\text{L-OSHS}}$ and $K_{\text{mR}}^{\text{L-Cys}}$ values, for the physiological, α,γ -replacement activity, of the R106 variants are both increased, by 9- and 3-fold, respectively (Fig. 3), and the 80- and 20-fold decreases in the k_{catR} of R106A and R106K are of similar magnitude (Table I). The data for the R48K and R361K variants could not be fit to Eqs. (1)–(3) as saturation was not observed within the solubility limit of the L-OSHS substrate. Therefore, with the assumption that $K_{\text{mR}}^{\text{L-OSHS}} \gg [\text{L-OSHS}]$, the Michaelis–Menten equation was modified to obtain $k_{\text{catR}}/K_{\text{mR}}^{\text{L-OSHS}}$, at the concentration of L-Cys at which each enzyme displays maximal activity (Table I). Values of k_{catR} , $K_{\text{mR}}^{\text{L-Cys}}$, $K_{\text{mR}}^{\text{L-OSHS}}$, and $k_{\text{catR}}/K_{\text{mR}}^{\text{L-Cys}}$ were determined for the R48K and R361K variants from the fit of velocity versus L-Cys, at the highest concentration of L-OSHS used, to Eqs. (4) and (5). Therefore, the values of k_{catR} , $K_{\text{mR}}^{\text{L-OSHS}}$, and $K_{\text{mR}}^{\text{L-Cys}}$ for these two enzymes should be considered as lower limits for the values of these kinetic parameters as the L-OSHS concentration was limited by its solubility under assay conditions. The $K_{\text{m}}^{\text{L-Cys}}$ and $K_{\text{m}}^{\text{L-OSHS}}$ values of these enzymes are within 5-fold of wild-type eCGS.

Figure 3. The dependence of the activity of wild-type eCGS (●) and the R106A (○), R106K (◇), E325A (▲), and S326A (◆) site-directed variants on substrate concentration. (A) The α,γ -elimination activity is plotted versus L-OSHS concentration. Reaction conditions: 0.1–50 mM L-OSHS and 1–26.25 μ M wild-type or mutant eCGS, depending on the activity of the enzyme, in assay buffer at 25°C. The α,γ -replacement activity is plotted versus (B) L-OSHS and (C) L-Cys concentration. Reaction conditions: 0.1–50 mM L-OSHS (at 1 mM L-Cys), 0.025–20 mM L-Cys (at 10 mM L-OSHS), 1.3 mM NADH, 1.0 μ M eCBL, 1.0 μ M LDH, and 0.05–21.25 μ M wild-type or mutant eCGS, depending on the activity of the enzyme, in assay buffer at 25°C.

Although the 750- and 220-fold decreases in k_{catR} dominate the 12,000 and 4600-fold reductions in the apparent $k_{\text{catR}}/K_{\text{mR}}^{\text{L-OSHS}}$ values of R48K and R361K (Table I), the degree to which $K_{\text{mR}}^{\text{L-OSHS}}$ is increased cannot be accurately determined. The behavior of the R48, R106, and R361 targeted substitution variants in the presence of 200 mM L-Ala is identical to that of wild-type eCGS.

The Y46F, Y101F, and S326A mutants

The 350-fold decrease in the $k_{\text{catR}}/K_{\text{mR}}^{\text{L-OSHS}}$ of the α,γ -replacement activity of eCGS-Y46F is comprised of equal, $\sim$ 20-fold changes in k_{catR} and $K_{\text{mR}}^{\text{L-OSHS}}$, whereas the $K_{\text{mR}}^{\text{L-Cys}}$ is increased by only 3-fold. Although the k_{catE} of the minor α,γ -elimination activity of Y46F is unchanged, compared to the wild-type enzyme, a 12-fold increase is observed for $K_{\text{mE}}^{\text{L-OSHS}}$, similar to that of $K_{\text{mR}}^{\text{L-OSHS}}$. The k_{catE} and k_{catR} of eCGS-Y101F are reduced 2- and 12-fold, respectively, and $K_{\text{m}}^{\text{L-OSHS}}$ values of both the γ -elimination and replacement reactions are within 5-fold of the wild-type enzyme. The 40- and 360-fold decreases in k_{catE} and k_{catR} of eCGS-S326A are unique among the site-directed variants investigated and the $K_{\text{mE}}^{\text{L-OSHS}}$ and $K_{\text{mR}}^{\text{L-OSHS}}$ values of this variant are increased by only 5-fold (Tables I and II, Fig. 3). Although the behavior of S326A in the presence of 200 mM L-Ala is identical to that of eCGS, the Y46F enzyme displays a minor 320-nm peak following a 4-h incubation. In contrast, complete conversion of the 421-nm species to one with a distinct 320-nm absorbance,

Figure 4. The effect of incubation with 200 mM L-Ala on the PLP spectrum of the wild-type eCGS enzyme and site-specific variants of residues D45 and E325. (A) The spectrum of wild-type eCGS (solid line) and following 4.2 h incubation with 200 mM L-Ala (mixed dotted/dashed line). (B) The spectra of wild-type eCGS (mixed dotted/dashed line) and the D45A (solid line), E325A (dashed line), and E325Q (dotted line) variants following 4.2 h incubation with 200 mM L-Ala.

Figure 5. The effect of consecutive incubation with L-Ala and pyruvate on the PLP spectrum of the Y101F variant of eCGS. (A) Time course of incubation of 20 μ M Y101F with 200 mM L-Ala for 4.4 h demonstrates the concomitant decrease in intensity at 421 nm and increase at 320 nm. The absorbance spectra of the PLP cofactor of 20 μ M (B) wild-type eCGS and (C) eCGS-Y101F were recorded before the addition of substrate (solid line), following a 4.4 h incubation with 200 mM L-Ala (dashed line) and following a 4.4 h incubation of the L-Ala-treated enzyme with 50 mM pyruvate (dotted line). The enzymes were dialyzed for 1 h following both incubations to remove excess L-Ala and pyruvate. As the effect of incubation of the wild-type enzyme with L-Ala is shown in Figure 4A, the spectra in panel B focus on the 420-nm region.

identical to D45A, D45N, and E325A, is observed for Y101F [Figs. 4(B) and 5]. To investigate the nature of the 320-nm species, the Y101F variant was incubated with 200 mM L-Ala for 4 hours followed by dialysis, to remove the excess substrate, and a subsequent, 4-h incubation with 50 mM pyruvate to return any pyridoxamine (PMP), produced on reaction with L-Ala, to the internal aldimine of PLP. Incubation with pyruvate resulted in a 3.8-fold increase in the intensity of the 421-nm band of Y101F, suggesting that PMP was formed upon reaction with L-Ala and that it reacted with the α -ketoacid pyruvate to produce alanine and regenerate the PLP form of the cofactor, thereby completing a transamination catalytic cycle (Fig. 5).

Discussion

The enzymes of the γ -subfamily of fold-type I, including those of the transsulfuration pathways, comprise an ideal model system for investigation of the structure-function relationships underlying substrate and reaction specificity in PLP-dependent enzymes. The structural similarity of CGS, CBL, and CGL extends to the active site as five of the residues proposed to participate in substrate binding and catalysis are conserved in these three enzymes, from

both prokaryotic and eukaryotic species.^{5–7} These conserved positions include a pair of arginines (eCGS-R48 and R361), two tyrosines (eCGS-Y46 and Y101), and a serine residue (eCGS-S326). An additional five active-site residues demonstrate conservation with either CGL (eCGS-D45, R106, N227, and E325) or CBL (eCGS-R49). The conformation and orientation of substrate(s) within the active site as well as the degree of freedom of rotation about the C α -C β bond, of the substrate covalently bound to the cofactor, have been proposed to be determinants of reaction specificity among the enzymes of the γ -subfamily of fold-type I of PLP-dependent enzymes.^{7,8} However, these factors are subtle and not evident from comparison of the available crystal structures of the unliganded or inhibitor-bound complexes of these enzymes, from a mixture of bacterial and plant species, as a conserved residue may play different roles dependent on the context of the specific active site. Additionally, interconversion of the two residues most distinct between the active sites of the α,γ -elimination catalyzing eCGS (D45 and E325) and yCGL (E48 and E333) and eCBL (F55 and Y338), which catalyzes an α,β -elimination reaction, does not result in a corresponding increase in α,γ or α,β -elimination activity.⁹ Therefore, exploration of the roles of active-site residues in these enzymes, dependent on the versatile PLP cofactor, is necessary to decipher the subtle and complex structure-function relationships of these structurally similar but mechanistically distinct enzymes. The characterization of a series of 16 site-directed variants of 10 eCGS active-site residues (D45, Y46, R48, R49, Y101, R106, N227, E325, S326, and R361) is the focus of this study.

The D45A,N, R49A,K, and E325A,Q variants

Clausen *et al.* proposed that the acidic residues D45 and E325 and basic R49 interact with the α -amino and α -carboxylate groups, respectively, of the L-Cys substrate.⁴ The near-native K_{mR}^{1-Cys} values of the alanine substitution variants of these residues, which remove their hydrogen-bonding capacity, do not substantiate this mode of L-Cys binding (Table I). The observation that, with the exception of R106A, K_{mR}^{1-Cys} was increased, ≤ 3 -fold, by only D45A,N and Y46F, of the 16 site-directed variants of eCGS active-site residues investigated, suggests that L-Cys may bind via backbone or water-mediated interactions or that the succinate leaving group of L-OSHS may be involved in the positioning of this substrate. The 20-fold reduction in the k_{catR} and the 4-fold increase in the K_{mE}^{1-OSHS} , of the α,γ -replacement and elimination reactions, respectively, suggest that replacement of R49 with alanine subtly alters the architecture of the active site. The wild-type kinetic parameters of the R49K variant, unaltered despite the ~ 1 Å shorter side-chain of lysine, confirm that this residue does not play a direct role in substrate binding or

catalysis. In contrast with the corresponding R59 of eCBL, which forms a salt bridge with residue E235 of the neighboring subunit of the catalytic dimer, the side chain of eCGS-R49 does not interact with other amino acids.^{4,10} The 4- to 9-fold reductions in the $k_{\text{catE}}/K_{\text{mE}}^{\text{L-OSHS}}$ and $k_{\text{catR}}/K_{\text{mR}}^{\text{L-OSHS}}$ of the D45A and D45N variants reflect minor, 2- to 4-fold changes in k_{cat} and $K_{\text{m}}^{\text{L-OSHS}}$ resulting from subtle alterations in active-site architecture or the positioning of water molecules. In contrast, replacement of residue D45 with phenylalanine, to mimic the corresponding F55 of eCBL, decreases k_{cat} by 16-fold and causes a 6-fold increase in $K_{\text{m}}^{\text{L-Cys}}$.⁹ These results suggest that, although this residue does not participate directly in substrate binding, it is situated in proximity to the L-Cys binding site. Similarly, the unique 4- to 6-fold decreases in the $K_{\text{mR}}^{\text{L-OSHS}}$ and $K_{\text{iR}}^{\text{L-Cys}}$ values of the alanine and glutamine substitutions of E325 (Table I, Fig. 3) indicate that this residue is located in the L-OSHS binding site and that its negative charge reduces the binding of both substrates to this site but does not contribute to the ability of the enzyme to discriminate between them. Residue E325 of eCGS is conserved as a glutamate in CGL but replaced by tyrosine in bacterial CBL (eCBL-Y338). Messerschmidt et al. proposed a role for the corresponding E333 of yCGL as a determinant of α,γ versus α,β -elimination specificity via interaction with the sulfur atom of the L-Cth substrate, common to CGL and CBL.⁵ Replacement of eCBL-Y338 with phenylalanine has negligible effect on the kinetic parameters of eCBL and no discernible effect on reaction specificity.⁶ In the context of the eCGS active site, residue E325 acts as a determinant of reaction promiscuity as the ratio of the γ -replacement/ γ -elimination catalytic efficiencies, for L-OSHS, is increased ~ 5 -fold by both the alanine and glutamine substitution variants. A possible evolutionary role for the minor γ -elimination activity is the production of propanoyl-CoA, the cellular precursor of C5 branched dibasic acids. Alternatively, the minor γ -elimination activity of eCGS may prevent alternative substrates from reacting with the ketimine intermediate in the situation that the cellular pool of the L-Cys substrate is diminished. A role for this residue as a determinant of reaction specificity is also implied by the observation that the E325A variant displays the 320-nm PMP absorption, suggestive of transamination, upon reaction with L-Ala.

The R48K, R106A,K, R361K, and N227A variants

Residue eCGS-N227 was targeted in this study because the corresponding Y238 of eCBL has been shown to interact with the distal carboxylate of the substrate.⁶ The N δ 1 and O δ 1 atoms of N227 are 3.3 and 2.8 Å from the guanidinium groups of the side chains of R106 and R48, respectively, suggesting that this residue may play a role in positioning of

one or both of these arginine residues.⁴ However, the negligible impact of removal of the hydrogen-bonding capacity of this residue on the kinetic parameters of eCGS (Tables I and II), resulting from its replacement with alanine, demonstrate that N227 does not interact with either substrate or play an important role in the positioning of other active-site residues in the context of the eCGS active site.

Docking studies with eCGS predicted roles for three arginine residues, R48/R106 and R361, in binding the distal and α -carboxylate moieties, respectively, of the L-OSHS substrate.⁴ This model is supported by the 130, ~ 10 , and 120-fold increases in $K_{\text{mE}}^{\text{L-OSHS}}$ for the α,γ -elimination activity of the R48K, R106A,K and R361K variants, respectively (Table II). Similarly, the $K_{\text{mR}}^{\text{L-OSHS}}$ value for the α,γ -replacement activity is increased 3- and 9-fold (Fig. 3), respectively, by substitution of R106 with alanine or lysine, whereas the R48K and R361K variants are not saturated within the solubility limit of L-OSHS (Table I). Residue R106 of eCGS is conserved as an arginine in prokaryotic and eukaryotic CGS and CGL sequences and replaced by an aspartate in bacterial CBL (eCBL-D116). Substitution of the side chain of D116, located 8.3 Å from the distal amino group of AVG, in the structure of the eCBL complex with this inhibitor, does not modify the kinetic parameters of eCBL.^{7,8} Similarly, the side-chain of R167 of the plant nCGS enzyme, which corresponds to eCGS-R106 and eCBL-D116, is oriented away from the phosphonate moiety of the inhibitor in the nCGS-APPA complex (Fig. 2), suggesting that although this residue likely does not bind the distal phosphate group of the L-OPHS substrate, it may interact with the α -carboxylate of L-Cys.² However, the 3- to 9- and ~ 10 -fold increases in the $K_{\text{mR}}^{\text{L-OSHS}}$ and $K_{\text{mE}}^{\text{L-OSHS}}$ values of the R106A,K variants, suggest that this residue interacts, directly or in a water-mediated manner, with the distal portion of the L-OSHS substrate of eCGS (Tables I and II, Fig. 1). The 13-fold increase in $K_{\text{mR}}^{\text{L-Cys}}$ of the alanine substitution variant of R106 is unique among the 16 active-site variants investigated. Residue R106 may be situated such that it bridges the carboxylate groups of the succinate moiety of L-OSHS and of L-Cys.

Although residue nCGS-R110, which corresponds to eCGS-R48, does not interact with the phosphonate moiety of the inhibitor in the structure of the nCGS-APPA complex, the distal carboxylate of L-OSHS was proposed to form a salt bridge with the corresponding eCGS-R48.^{2,4} This is supported by the 120-fold increase in $K_{\text{mE}}^{\text{L-OSHS}}$ and by comparison of the effect of alanine and lysine substitution of the corresponding eCBL-R58 on the K_{i} for inhibition by AVG, lacking the distal carboxylate moiety of L-Cth, and the $K_{\text{m}}^{\text{L-Cth}}$, which demonstrates the interaction between the distal carboxylate of the substrate and

R58. A pair of hydrogen bonds between the α -carboxylate group of the inhibitor and the side chain of R423, which corresponds to eCGS-R361, are apparent in the structure of the nCGS-APPA complex (Fig. 2).² The 130-fold increase in the K_{mE}^{L-OSHS} and lack of L-OSHS saturation observed for the α,γ -replacement activity of R361K (Tables I and II), support the proposed role of R361 in L-OSHS binding. A similar 80-fold increase in K_m^{L-Cth} has been reported for the corresponding R372K variant of eCBL, which also interacts with the α -carboxylate moiety of the inhibitor in the eCBL-AVG complex.^{7,8} The ≥ 220 -fold decrease in the k_{cat} of the α,γ -replacement activity of eCGS-R361K is in keeping with the 55-fold decrease in this parameter reported for the corresponding AATase-R386K but contrasts with the 4-fold decrease in the k_{cat} of CBL-R372K.^{7,11,12} The observed reductions in the activity of the eCGS and eAAT variants reflects differences in the binding orientation and conformation of the substrates, such that they are not optimally positioned for catalysis.¹² In contrast, the substantially smaller decrease in the k_{cat} of eCBL-R372K likely reflects the facile nature of the α,β -elimination of L-Cth, which, in contrast with the transamination of eAATase and α,γ -replacement reaction of eCGS, does not require the generation of a ketimine intermediate or the binding of a second substrate. The observed 80- and 750-fold decreases in the k_{catR} of the R106A and R48K variants are likely also the result of suboptimal positioning and conformation of L-OSHS within the active site of eCGS. Additionally, as R48 is one of the primary ligands of the phosphate moiety of PLP, its substitution by lysine, the side chain of which is approximately 1 Å shorter, may result in a change in the positioning of the cofactor and corresponding alteration of active-site architecture.⁴

The Y46F, Y101F, and S326A variants

Based on the structure of eCGS, a role for residues Y101 and S326 in binding the distal and α -carboxylate groups, respectively, of L-OSHS was proposed; a model which is not supported by the minor, 2- and 5-fold changes in K_{mR}^{L-OSHS} of the Y101F and S326A variants, respectively (Table I).⁴ The tyrosine at position 46 of eCGS was targeted for investigation in this study because it is conserved in many members of fold-type I and phenylalanine substitution variants of the corresponding residue in diverse enzymes, including Y56 of eCBL, Y70 of *E. coli* aspartate aminotransferase, Y121 of murine erythroid aminolevulinate synthase (meALAS), Y71 of *Citrobacter freundii* tyrosine phenol lyase and Y64 of *Treponema denticola* cystalysin, have been reported to impact the kinetic parameters of the target enzyme.^{6,13–16} The 20-fold decrease in k_{catR} and 20 and 12-fold increases in K_{mR}^{L-OSHS} and K_{mE}^{L-OSHS} of

eCGS-Y46F (Table I) are of common magnitude with the 6-fold decrease in k_{cat} and 20-fold increase in K_m^{L-Cth} of the corresponding Y56F variant of eCBL. Based on the similar, 15-fold increase in the K_m for glycine of the corresponding meALAS-Y121F variant, Tan *et al.* proposed that the observed change in K_m is due to the increase in K_d^{PLP} of the cofactor, rather than a direct interaction with the substrate, as glycine does not possess a side chain.¹⁵ Given that the hydrogen bond between the phosphate moiety of the cofactor and residue Y121 of meALAS, is conserved in eCBL and eCGS, the observed similar increases in the K_m values of the corresponding Y56F and Y46F variants, may also reflect the absence of this link rather than a direct interaction with the substrate.

The 2- to 12-fold changes in the k_{cat} and K_m values of eCGS-Y101F demonstrate that Y101 is not directly involved in catalysis, demonstrating that the role proposed for this residue in proton transfer between the α -amino group of L-OSHS and the succinate leaving group is not supported.⁴ However, the transamination of L-Ala, not observed for the wild-type enzyme, is most apparent for Y101F (Figs. 4 and 5), among the 16 variants investigated, demonstrating that the hydroxyl moiety of this residue acts as a minor determinant of reaction specificity. This contrasts with eCBL for which the corresponding Y111F variant does not demonstrate transamination activity, but is uniquely inhibited by DTNB, illustrating that conserved residues play distinct roles in the active sites of the three γ -subfamily enzymes of the transsulfuration pathways. Among the corresponding substitution variants of eCBL active-site residues that have been investigated, a novel transaminase activity was observed for S339A. Interestingly, no 320-nm absorbance, indicative of PMP was observed upon reaction of the corresponding eCGS-S326A.

The 40- and 360-fold decreases in k_{catE} and k_{catR} , respectively, of eCGS-S326A are unique among the site-directed variants investigated and in agreement with the effect of the corresponding S339A variant of the closely related eCBL (Tables I and II, Fig. 3). Therefore, a role in guiding and tethering the ϵ -amino group of K198, the catalytic base, proposed for eCBL-S339, is also predicted for eCGS-S326. The presence of a serine residue at this position is unique to the enzymes of the γ -subfamily of fold-type I. A 5600-fold decrease in k_{cat} for the hydrolysis of L-Cth has been observed for the corresponding S339A variant of eCBL.⁶ The 140-fold greater effect of the S339A substitution on the k_{cat} of α,β -elimination activity of eCBL, compared to the α,γ -elimination activity of eCGS is insightful. The ϵ -amino group of eCBL-K210 must be more strictly restrained than the corresponding eCGS-K198 because protonation of C4' of the cofactor is required

in the α,γ -elimination reaction of eCGS but must be prevented in eCBL.

CGS is an attractive target for the development of novel antimicrobial compounds because it catalyzes the first reaction in the transsulfuration pathway, which is unique to plants and bacteria. The design of effective inhibitors of the bacterial CGS enzyme requires an in-depth characterization of the active-site residues participating in substrate binding. This study has identified R48/R106 and R361 as residues interacting with the distal and α -carboxylate groups, respectively, of the L-OSHS substrate, interactions which can be targeted for the design of inhibitors. However, with the exception of the 13-fold increase observed for R106A, the negligible effect of the 16 site-directed variants on K_m^{L-Cys} demonstrates that the residues investigated do not directly participate in L-Cys binding. A role for Y101, D45, and E325 as determinants of reaction specificity is proposed as substitution of these residues resulted in a minor L-Ala transaminase activity not observed for the wild-type enzyme. Given the ~ 9 and 8 Å separation between each of these acidic residues and Y101, a direct interaction is not anticipated as the underlying structural feature preventing the transamination side reaction in the wild-type enzyme.

Materials and Methods

Reagents

L-Cth, L-Cys, OSHS, and L-lactate dehydrogenase (LDH) were purchased from Sigma. Ni-NTA resin was obtained from Qiagen. Oligonucleotide primers were synthesized by Integrated DNA Technologies and mutants were sequenced by BioBasic before expression and purification. The 6-His-tagged eCBL and D-2-Hydroxyisocaproate dehydrogenase (HO-HxoDH) coupling enzymes used in the eCGS γ -replacement and γ -elimination assays, respectively, were expressed and purified as described previously.^{17–19}

Construction, expression, and purification of site-directed mutants

Site-directed mutants of eCGS, constructed *via* the overlap-extension polymerase chain reaction method, were inserted into the pTrc-99aAF plasmid. The amino-terminal, 6-His tag, and linker encoded by this vector enables affinity purification of the expressed enzymes but, as demonstrated by Farsi *et al.*, it does not alter the kinetic parameters of eCGS.⁹ Wild-type and site-directed variants of eCGS were expressed in the *E. coli* ER1821 *metB::aadA* strain, in which the gene encoding eCGS is replaced by *aadA*, encoding resistance to streptomycin, to prevent contamination with the wild-type *E. coli* enzyme. The wild-type and site-directed mutants of eCGS were expressed and purified, *via* Ni-nitrilotriacetic acid affinity chromatography, as described by Farsi *et al.*⁹

Determination of steady-state kinetic parameters

Enzyme activity was measured in a total volume of $100\ \mu\text{L}$ at 25°C on a Spectramax 340 microtiter plate spectrophotometer (Molecular Devices). The assay buffer was comprised of $50\ \text{mM}$ Tris, pH 7.8, with $20\ \mu\text{M}$ PLP. The formation of L-Cth, *via* the condensation of L-OSHS and L-Cys, was detected using the CBL-LDH coupled assay.¹⁷ A background reading was recorded, before initiation of the reaction by the addition of the experimental enzyme, in all assays. The data for the γ -replacement activity of eCGS were fitted to Eqs. (1)–(3) for the modified ping pong mechanism described by Aitken *et al.*, in which the E and R subscripts denote the γ -elimination and γ -replacement activities, respectively, to obtain k_{catR} , K_{mR}^{L-Cys} , K_{mR}^{L-OSHS} , K_{iR}^{L-Cys} , $k_{\text{catR}}/K_{\text{mR}}^{L-Cys}$, and $k_{\text{catR}}/K_{\text{mR}}^{L-OSHS}$. The independently determined values of k_{catE} and K_{mE}^{L-OSHS} , for the γ -elimination activity, were substituted into Eqs. (1)–(3) to reduce the number of kinetic parameters to be determined.¹⁷ Data were fit by nonlinear regression with the SAS software package (SAS Institute, Cary, NC).

$$\frac{v}{[E]} = \frac{k_{\text{catE}}K_{\text{mR}}^{L-Cys}[\text{L-OSHS}] + k_{\text{catR}}[\text{L-OSHS}][\text{L-Cys}]}{K_{\text{mE}}^{L-OSHS}K_{\text{mR}}^{L-Cys} + K_{\text{mR}}^{L-Cys}[\text{L-OSHS}] + K_{\text{mR}}^{L-OSHS}\left(1 + \frac{[\text{L-Cys}]}{K_{\text{iR}}^{L-Cys}}\right)[\text{L-Cys}] + [\text{L-OSHS}][\text{L-Cys}]} \quad (1)$$

$$\frac{v}{[E]} = \frac{k_{\text{catE}}K_{\text{mR}}^{L-Cys}/K_{\text{mR}}^{L-OSHS}[\text{L-OSHS}] + k_{\text{catR}}/K_{\text{mR}}^{L-OSHS}[\text{L-OSHS}][\text{L-Cys}]}{K_{\text{mE}}^{L-OSHS}K_{\text{mR}}^{L-Cys}/K_{\text{mR}}^{L-OSHS} + K_{\text{mR}}^{L-Cys}/K_{\text{mR}}^{L-OSHS}[\text{L-OSHS}] + \left(1 + \frac{[\text{L-Cys}]}{K_{\text{iR}}^{L-Cys}}\right)[\text{L-Cys}] + 1/K_{\text{mR}}^{L-OSHS}[\text{L-OSHS}][\text{L-Cys}]} \quad (2)$$

$$\frac{v}{[E]} = \frac{k_{\text{catE}}[\text{L-OSHS}] + k_{\text{catR}}/K_{\text{mR}}^{L-Cys}[\text{L-OSHS}][\text{L-Cys}]}{K_{\text{mE}}^{L-OSHS} + [\text{L-OSHS}] + K_{\text{mR}}^{L-OSHS}/K_{\text{mR}}^{L-Cys}\left(1 + \frac{[\text{L-Cys}]}{K_{\text{iR}}^{L-Cys}}\right)[\text{L-Cys}] + 1/K_{\text{mR}}^{L-Cys}[\text{L-OSHS}][\text{L-Cys}]} \quad (3)$$

Values of k_{catR} , K_{mR}^{L-Cys} , and K_{iR}^{L-Cys} for the R48K and R361K variants, for which K_{mR}^{L-OSHS} exceeds the solubility of L-OSHS, were determined from the fit of

velocity versus L-Cys, at the highest concentration of L-OSHS used, to Eq. (4), which incorporates the K_{iR}^{L-Cys} term for substrate inhibition by L-Cys. The

parameter $k_{\text{catR}}/K_{\text{mR}}^{\text{L-Cys}}$ was obtained independently from Eq. (5).

$$\frac{v}{[E]} = \frac{k_{\text{catR}} \times [S]}{K_{\text{mR}}^{\text{L-Cys}} + [S] \left(1 + [S]/K_{\text{IR}}^{\text{L-Cys}}\right)} \quad (4)$$

$$\frac{v}{[E]} = \frac{k_{\text{catR}}/K_{\text{mR}} \times [S]}{1 + [S]/K_{\text{mR}} \left(1 + [S]/K_{\text{IR}}^{\text{L-Cys}}\right)} \quad (5)$$

The hydrolysis of L-OSHS was detected via the continuous γ -elimination assay, in which α -ketobutyrate is reduced, with concomitant oxidation of NADH ($\epsilon_{340} = 6,200 \text{ M}^{-1}\text{s}^{-1}$), by HO-HxoDH.¹⁷ Values of k_{catE} and $K_{\text{mE}}^{\text{L-OSHS}}$ were obtained by fitting of the data to the Michaelis-Menten equation and $k_{\text{catE}}/K_{\text{mE}}^{\text{L-OSHS}}$ was obtained independently from Eq. (6)

$$\frac{v}{[E]} = \frac{k_{\text{catE}}/K_{\text{mE}}^{\text{OSHS}} \times [S]}{1 + [S]/K_{\text{mE}}^{\text{OSHS}}} \quad (6)$$

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