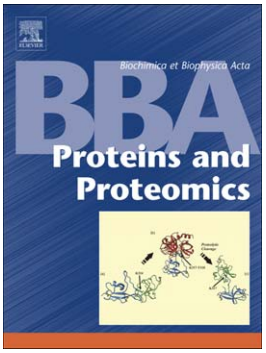

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Emily M.S. Hopwood, Duale Ahmed, Susan M. Aitken

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A Role for Glutamate-333 of *Saccharomyces cerevisiae* Cystathionine γ-Lyase as a Determinant of Specificity.

Emily M.S. Hopwood, Duale Ahmed and Susan M. Aitken*

Department of Biology, Carleton University, Ottawa, Canada, K1S 5B6.

*To whom correspondence should be addressed. Telephone: (613) 520-2600. Fax: (613) 520-3539. E-mail: susan_aitken@carleton.ca

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ABBREVIATIONS: AAT, aspartate aminotransferase; ACCS, 1-aminocyclopropane-1-carboxylate synthase; AMPSO, N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid; bicine, 2-(Bis(2-hydroxyethyl)amino)acetic acid; CAPS, 3-(Cyclohexylamino)-1-propanesulfonic acid; CBL, cystathionine β-lyase; CGL, cystathionine γ-lyase; CGS, cystathionine γ-synthase; L-Cth, L-cystathionine; DTNB, 5,5'-Dithiobis(2-nitrobenzoic acid); L-Hcys, L-homocysteine; HO-HxoDH, D-2-hydroxyisocaproate dehydrogenase; IPTG, Isopropyl-β-*D*-thiogalactopyranoside; LDH, L-lactate dehydrogenase; MGL, methionine γ-lyase; NADH (reduced form), β-nicotinamide adenine dinucleotide; Ni-NTA, Ni-nitrilo triacetic acid; L-OAS, *O*-acetyl-L-serine; L-OSHS, *O*-succinyl-L-homoserine; OASS, OAS sulfhydrylase; PLP, pyridoxal 5′-phosphate; TAPS, N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid.

1. ABSTRACT

Cystathionine γ-lyase (CGL) catalyzes the hydrolysis of L-cystathionine (L-Cth), producing L-cysteine (L-Cys), α-ketobutyrate and ammonia, in the second step of the reverse transsulfuration pathway, which converts L-homocysteine (L-Hcys) to L-Cys. Site-directed variants substituting residues E48 and E333 with alanine, aspartate and glutamine were characterized to probe the roles of these acidic residues, conserved in fungal and mammalian CGL sequences, in the active-site of CGL from *Saccharomyces cerevisiae* (yCGL). The pH optimum of variants containing the alanine or glutamine substitutions of E333 is increased by 0.4-1.2 pH units, likely due to repositioning of the cofactor and modification of the pK_a of the pyridinium nitrogen. The pH profile of yCGL-E48A/E333A resembles that of *Escherichia coli* cystathionine β-lyase. The effect of substituting E48, E333 or both residues is the 1.3-3, 26-58 and 124-568-fold reduction, respectively, in the catalytic efficiency of L-Cth hydrolysis. The $K_m^{L\text{-}Cth}$ of E333 substitution variants is increased ~17-fold, while $K_m^{L\text{-}OAS}$ is within 2.5-fold of the wild-type enzyme, indicating that residue E333 interacts with the distal amine moiety of L-Cth, which is not present in the alternative substrate *O*-acetyl-L-serine. The catalytic efficiency of yCGL for α,γ-elimination of *O*-succinyl-L-homoserine ($k_{cat}/K_m^{L\text{-}OSHS}$ = 7 ± 2), which possesses a distal carboxylate, but lacks an amino group, is 300-fold lower than that of the physiological L-Cth substrate ($k_{cat}/K_m^{L\text{-}Cth}$ = 2100 ± 100) and 260-fold higher than that of L-Hcys ($k_{cat}/K_m^{L\text{-}Hcys}$ = 0.027 ± 0.005), which lacks both distal polar moieties. The results of this study suggest that the glutamate residue at position 333 is a determinant of specificity.

2. INTRODUCTION

Natural and specialty amino acids comprise a multi-billion-dollar market with 5-7% growth annually and an expanding range of uses in the chemical, pharmaceutical, cosmetic and agri-food industries [1,2]. Unnatural amino acids also expand the scope of biocatalytic applications of enzymes [3]. Both areas rely on the engineering of amino acid biosynthetic enzymes and pathways. This will be facilitated by elucidation of the structure-function relationships that underlie substrate and reaction specificity among enzymes catalyzing transformations of amino acids, which are typically dependent on the catalytically versatile pyridoxal 5'-phosphate (PLP) cofactor. The enzymes of the microbial transsulfuration pathways, which interconvert the sulfur-containing amino acids L-cysteine (L-Cys) and L-homocysteine (L-Hcys), provide an ideal model system for this purpose as they share common structural features, but catalyze distinct side-chain rearrangements of similar amino-acid substrates (Figure 1) [4].

Cystathionine γ-lyase (CGL) is the second enzyme of the reverse transsulfuration pathway, which converts the L-Hcys product of S-adenosylmethionine metabolism to L-Cys (Figure 1). Comparison of the structures of *Escherichia coli* cystathionine γ-synthase (eCGS) and cystathionine β-lyase (eCBL), of the bacterial transsulfuration pathway, *Saccharomyces cerevisiae* CGL (yCGL) and *Trichomonas vaginalis* methionine γ-lyase (tMGL) illustrates the structural similarity among the enzymes of the γ-subfamily of the large and catalytically diverse fold-type I of PLP dependent enzymes. Although these enzymes share only ~35% amino acid sequence identity, including several common active-site residues, the r.m.s. deviation for the superposition of ~350 C_α atoms is 1.5 Å [5-8]. Typical of members of the γ-subfamily, these enzymes catalyze the α,γ-

replacement (CGS) and α,β (CBL) or α,γ-elimination (CGL and MGL) of similar sulfur-containing amino acid substrates (Figure 1).

The conformation and orientation of substrate(s) within the active site has been proposed to be a determinant of reaction specificity among the enzymes of the γ-subfamily [9,10]. However, these factors are subtle and not evident from comparison of the available crystal structures. Multiple residues and structural features contribute to the regulation of specificity and the roles of active-site residues, even those conserved between enzymes with distinct function, are dependent on the context of the specific enzyme [4,11,12]. The pseudosymmetric L-Cth substrate of eCBL and yCGL must be bound in distinct orientations in the active site to enable hydrolysis via α,β versus α,γ-elimination mechanisms. Roles for residues E48 and E333 of yCGL (Figure 2), conserved as acidic residues in eCGS (D45 and E325) and replaced by aromatic residues in eCBL (F55 and Y338), as determinants of reaction specificity were proposed based on structural comparison [5,6]. Farsi and coworkers subsequently observed that interconversion of these two residues in eCBL, eCGS and yCGL reduces wild-type activity but is not sufficient to result in a corresponding modification of specificity [13]. Similarly, the exchange of a pair of ~25-residue structurally distinct regions situated at the mouth of the active site and in proximity to residues E48 and E333, via construction of chimeric variants of eCBL and eCGS, also drastically diminishes the native reaction without an observable increase in the *in vivo* targeted activity [12]. A novel role for residues D45 and E325 of eCGS as determinants of reaction specificity was recently described by Jaworski *et al.*, as substitution with alanine and asparagine or glutamine, respectively, enables the deamination of alanine [11]. These studies demonstrate the

complexity of the system and illustrate the challenge of identifying the determinants of specificity. This study builds on our earlier work focusing on structure-function relationships among the structurally similar, but mechanistically distinct members of the γ-subfamily. We describe the characterization of a series of 9 site-directed variants of yCGL, which provide insight into the roles of active-site residues E48 and E333 [12,13].

3. MATERIALS AND METHODS

3.1. Reagents. L-Cth [*S*-(2-amino-2-carboxyethyl)-L-homocysteine], L-Cys, L-Hcys, *O*-acetyl-L-serine (L-OAS), β-nicotinamide adenine dinucleotide (β-NADH, reduced form) and L-lactate dehydrogenase (LDH) were purchased from Sigma-Aldrich (Oakville, Ontario). L-Hcys was prepared from the thiolactone as in Kashiwamata and Greenberg [14]. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Pierce and nickel-nitriloacetic acid (Ni-NTA) resin was from Qiagen (Toronto, Ontario). 2-(Bis(2-hydroxyethyl)amino)acetic acid (bicine), N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS), N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid (AMPSO) and 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) were purchased from Fisher Scientific (Ottawa, Ontario).Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, Iowa) and site-directed variants were sequenced by BioBasic (Markham, Ontario) prior to expression. The D-2-Hydroxyisocaproate dehydrogenase (HO-HxoDH) coupling enzyme was expressed and purified as described previously [15].

3.2. Construction, expression and purification of site-directed mutants. Site-directed mutants of yCGL, 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 and does not alter the kinetic parameters of yCGL [13]. Wild-type and site-directed variants of yCGL were expressed in *E. coli* strain ER1821*metC::cat*, in which the gene encoding eCBL is replaced by chloramphenicol acetyltransferase, to prevent contamination with

the β-lyase activity of the host eCBL enzyme, and purified, *via* Ni-NTA affinity chromatography [13].

3.3. Determination of steady-state kinetic parameters. Enzyme activity was measured in a total reaction volume of 100 μL at 25°C and detected on a Spectramax 340 microtiter plate spectrophotometer (Molecular Devices). The assay buffer was composed of 50 mM potassium phosphate, pH 7.2, containing 20 μM PLP. Hydrolysis of L-Cth was monitored via reaction of DTNB (ε_{412} = 13 600 M^{-1} cm^{-1}) with the free thiol group of the product [13]. The α,β-elimination of L-Cys and L-OAS and the α,γ-elimination of L-Hcys were detected via continuous, coupled assays in which the pyruvate and α-ketobutyrate products are reduced by LDH (3.5 μM) and HO-HxoDH (3.4 μM), respectively, with concomitant oxidation of NADH (ε_{340} = 6,200 $M^{-1}cm^{-1}$) [16,17]. The concentration of yCGL enzyme in the assay was between 0.45 – 19.5 μM, depending on the activity of the specific site-directed variant. A background reading was recorded in all cases before the reaction was initiated by the addition of yCGL enzyme. Data were fitted to the Michaelis-Menten equation to obtain values of k_{cat} and K_m for the hydrolysis of L-Cth and L-OAS and k_{cat}/K_m was obtained independently from equation 1. For site-directed variants for which saturation was not observed, including the hydrolysis of L-Hcys, k_{cat}/K_m values were obtained by linear regression, based on the assumption that K_m >> [substrate]. The L-Cys hydrolysis data were fitted to equation 2, which modifies the Michaelis-Menten equation to incorporate the $K_i^{L\text{-}Cys}$ term for substrate inhibition by L-Cys, and $k_{cat}/K_m^{L\text{-}Cys}$ values were obtained independently from equation 3.

$$\frac{v}{[E]} = \frac{k_{cat}/K_m \times [S]}{1 + [S]/K_m} \quad (1)$$

$$\frac{v}{[E]} = \frac{k_{cat} \times [S]}{K_m^{L-Cys} + [S]\left(1 + [S]/K_i^{L-Cys}\right)} \quad (2)$$

$$\frac{v}{[E]} = \frac{k_{cat}/K_m \times [S]}{1 + [S]/K_m\left(1 + [S]/K_i^{L-Cys}\right)} \quad (3)$$

3.4. Evaluation of the pH dependence of yCGL. The pH dependence of L-Cth hydrolysis by yCGL was determined using the continuous DTNB assay in a three-component buffer, comprised of 50 mM MOPS (pK_a = 7.2), 50 mM bicine (pK_a = 8.3) and 50 mM proline (pK_a = 10.7) [18,19]. The kinetic parameters were determined at a range of pH values between pH 6.4-10 in the presence of 20 μM PLP, 2 mM DTNB, 0.45 – 19.5 μM yCGL (depending on the activity of the site-directed variant) and 0.01-5.5 mM L-Cth. The $k_{cat}/K_m^{L\text{-}Cth}$ versus pH data were fitted to the bell-shaped curve described by equation 4, where k_{cat}/K_m^{max} is the upper limit for $k_{cat}/K_m^{L\text{-}Cth}$ at the pH optimum [16].

$$k_{cat}/K_m = \frac{k_{cat}/K_m^{\max}}{1 + 10^{pK_{a1} - pH} + 10^{pH - pK_{a2}}} \quad (4)$$

3.5. Spectrophotometric titration of the internal aldimine. The internal aldimine was titrated versus pH with 20 μM enzyme in 5 mM TAPS (pK_a = 8.4; pH 6.70) containing 0.5 M KCl as a starting point. The pH was increased by successive additions of 0.5 M AMPSO (pH 10.6; pK_a = 9.0) at pH <9.0, 0.5 M CAPS (pH 11.5; pK_a = 10.4) between pH 9.0 and 10.5, and 1.0 M NaOH at pH >10.5. The pH of the solution was determined prior to each absorbance measurement [16]. Spectra were recorded on an HP 8453 spectrophotometer. The data recorded at 390 and 421 nm were fitted to equations 5 and 6, respectively:

$$A = \frac{A_1 - A_2}{1 + 10^{pH - pK_{spec}}} + A_2 \quad (5)$$

$$A = \frac{A_1 - A_2}{1 + 10^{pK_{spec} - pH}} + A_2 \quad (6)$$

where $A1$ and $A2$ are the high and low absorbance limits at a particular wavelength, respectively.

4. RESULTS

The nine single and double site-directed substitution variants targeting residues E48 and E333 of yCGL were purified to homogeneity via Ni-NTA affinity chromatography. The proteins are soluble, with yields between 2.7-6 milligrams of protein obtained per litre of culture. Hydrolysis of the physiological L-Cth substrate as well as the α,β-elimination of L-Cys and *O*-acetylserine (OAS) is detectable for all variants (Tables 1 and 2). The α,γ-elimination of L-Hcys was also assayed. The enzymes, including wild-type yCGL, are not saturated within the solubility limit of this substrate. This, in combination with the low catalytic efficiency of the wild-type enzyme for hydrolysis of L-Hcys ($k_{cat}/K_m^{L\text{-}Hcys}$ = 0.027 ± 0.005), which is 78,000-fold lower than for the hydrolysis of L-Cth ($k_{cat}/K_m^{L\text{-}Cth}$ = 2100 ± 100; Table 1), precludes meaningful comparison of kinetic parameters for L-Hcys.

4.1. Comparison of yCGL with eCBL. Although yCGL and eCBL both catalyze the hydrolysis of L-Cth, the $k_{cat}/K_m^{L\text{-}Cth}$ of eCBL is 90-fold greater than that of yCGL (Table 1). The pH profiles of both enzymes are bell shaped, with distinct pH optima of 7.2-8.0 and 8.5-9.5 for yCGL and eCBL, respectively (Figure 3). The value of pK_{a1} (yCGL = 8.1 ± 0.4; eCBL = 8.28 ± 0.06) is within experimental error for both enzymes. In contrast, pK_{a2} of yCGL (7.1 ± 0.4) is 3 pH units lower than that of eCBL (10.20 ± 0.06). Spectral titration of eCBL and yCGL was performed to investigate a potential role for the protonation state of the aldimine as a determinant of specificity, as exemplified by 1-aminocyclopropane-1-carboxylate synthase (ACCS) and aspartate aminotransferase (AAT), which possess similarly conserved active sites but differ in the pK_a of the internal

aldimines by 2.3 pH units [20]. The increase in absorbance at 290 nm above pH 9.5 (Figure 4), and corresponding decrease at 277 nm, observed for yCGL and eCBL likely reflects tyrosine ionization (pK_a = 10.13). The 421 nm absorbance of yCGL and eCBL shifts to 390 nm at pH > 9 and 10, respectively, within one pH unit of the observed 277-to-290 nm transition. In contrast with the increase in pH optimum observed for yCGL-E48A/E333A (Figure 3), the spectral titration of this variant (Figure 4) is identical to the wild-type enzyme. The pK_a values for the loss of 421 nm absorbance are 10.04 ± 0.05 and 8.28 ± 0.03 and for the increase in absorbance at 390 nm are 11.08 ± 0.08 and 8.62 ± 0.05 for eCBL and yCGL respectively. Although the ~2 pH unit difference between the corresponding pK_a values of the two enzymes is consistent, the shift to 390 nm and the mismatch between the 390 and 421 nm pK_a values for each suggests dissociation of the cofactor rather than deprotonation of the aldimine in the context of the active site [16]. This is further supported by the lack of an isobestic point for eCBL, indicating that the observed spectral shift does not reflect a two-state system (Figure 4).

4.2. Effect of substitutions on the pH profile of yCGL. The pH optima of the $k_{cat}/K_m^{L\text{-}Cth}$ versus pH profiles of the E48 substitution variants are within 0.4 pH units of the wild-type enzyme (Table 1). Substitution of residue E333 with alanine (yCGL-E333A) or glutamine (E333Q) results in an increase of 0.4 and 0.6 pH units. In contrast, replacement with aspartate (E333D), which maintains the carboxylate moiety of the side chain but is expected to shift its position as a result of the ~1 Å shorter side chain of aspartate, does not change the pH optimum (Table 1). Substitution of both E48 and E333 with alanine or glutamine results in increases of 0.7 and 1.2 units in the pH optimum of $k_{cat}/K_m^{L\text{-}Cth}$, to

8.4-9.2 and 7.9-8.7, respectively. The pH optimum of yCGL-E48D/E333D is also increased, although by only 0.4 pH units (Table 1; Figure 3). The reduction of up to two orders of magnitude in the observed versus maximal catalytic efficiency, resulting from the reversed pK_a values (pK_{a1} > pK_{a2}) of the wild-type and site-directed variants of yCGL, precludes accurate estimations of the pK_a values for variants of these enzymes.

4.3. Hydrolysis of L-Cth and alternate substrates. The effect on yCGL activity of substituting residue E48 with alanine, aspartate and glutamine is minor as the kinetic parameters for the hydrolysis of L-Cth, L-OAS and L-Cys are within 4-fold of the wild-type enzyme (Tables 1 and 2). In contrast, the corresponding E333 substitutions result in a ~17-fold increase in $K_m^{\text{L-Cth}}$ and 2-3-fold decrease in k_{cat} (Table 1). The catalytic efficiency of the E333 replacement variants for the α,β-elimination of L-Cys is also reduced, 2-13-fold, while $K_i^{\text{L-Cys}}$ is within 2.5-fold of the wild-type (Table 2). Substitution of E333 with alanine, aspartate or glutamine alters the catalytic efficiency of L-OAS β-elimination by less than 2.5-fold, compared to the wild-type enzyme (Table 2).

The double replacement variants of residues E48/E333 are not saturated within the solubility limit of the L-Cth substrate, and $k_{cat}/K_m^{\text{L-Cth}}$ is reduced by 570, 240, and 125-fold, respectively, compared to yCGL (Table 1). In contrast, the kinetic parameters for hydrolysis of L-OAS by the E48/E333 double replacement variants are within 3-fold of the wild-type enzyme (Table 2). The value of $K_m^{\text{L-Cys}}$ is increased ~5-fold for all double replacement variants of E48/E333 and the k_{cat} of the E48D/E333D variant for L-Cys hydrolysis is 5-fold lower than the wild type (Table 2).

5. DISCUSSION

Typical of the γ-subfamily of fold-type I, the PLP-dependent enzymes of the transsulfuration pathways catalyze side chain rearrangements of similar amino acid substrates, via α,β and α,γ-elimination and replacement reactions. Sharing a common overall fold and several active-site residues, they present an effective model system for investigation of the structure-function relationships underlying substrate and reaction specificity in enzymes dependent upon the catalytically versatile PLP cofactor [4,12,13]. The current characterization of a series of 9 site-directed variants of two yCGL active-site residues (E48 and E333) builds on our earlier work deciphering the subtle and complex structure-function relationships of these structurally similar, but mechanistically distinct enzymes [12,13].

5.1. Role of E48 and E333 in substrate binding and specificity. Our analysis of a representative selection of 16 fungal and animal CGL and 27 γ-proteobacterial CBL amino acid sequences identified 65, 54 and 50 positions classified as at least 70% conserved in CGL only (CGL-conserved, variable in CBL; e.g. yCGL-E343 and T349), in CBL only (CBL-conserved, variable in CGL), and in both but as distinct residues (conserved-different; e.g. E48 and E333 of yCGL correspond to F55 and Y338 of eCBL), respectively. It is anticipated that a subset of these positions, acting in concert with a pair of ~25-residue structurally distinct regions that frame the entrance of the active site, are the primary determinants of reaction specificity. Investigation of the relative distances between C4' of the PLP cofactor and Cα of residues categorized as conserved or variable positions shows that variable positions are common remote from the active-site,

conserved-variable (CBL-conserved and CGL-conserved) and conserved-different residues are primarily situated within 15-20 Å of the active site and positions identical in both enzymes are more broadly distributed. Similarly, roles for residues outside of the primary shell of the active-site, clustering between 10-27 Å and linked via contact pathways of van der Waals and hydrogen-bonding interactions, were demonstrated by Mendona and Marana using *Spodoptera frugiperda* β-glucosidase as a model system [21]. Among the 43 sequences aligned, the residue at position 333 (yCGL numbering) is 100% conserved as glutamate among CGL sequences and 100% conserved as an aromatic residue (70% as tyrosine) in the CBL sequences. This provides indirect support for the proposal of Huang and colleagues that increased hydrophobicity at this position enables increased H_2S production from L-Cys, a substrate that allows only α,β-elimination/replacement [22]. Roles in substrate binding and α,β versus α,γ-elimination specificity have been proposed for E48 and E333 of yCGL, and the corresponding D45 and E325 of eCGS and F55 and Y338 of eCBL, as these residues are situated in the access channel of the active-site. Interaction with the distal amino group of L-Cth and the α-amino group of L-Cys was suggested for the acidic residues of yCGL and eCGS, respectively, while hydrophobic packing with the L-Cth substrate was postulated for the corresponding aromatic residues of eCBL [5-7]. Position 333 may also participate in the definition of active-site architecture as residue yCGL-E333 interacts with T349, which is situated in the ~25-residue structurally distinct, carboxy-terminal region. In contrast, the corresponding eCBL-Y338 forms a hydrogen bond with K42, located in the nine-residue insertion, unique to CBL, in the ~25-residue structurally distinct, amino-terminal region [7].

The near-wild type kinetic parameters, for the hydrolysis of L-Cth, L-Cys, and L-OAS, of the alanine, aspartate and glutamine substitution variants of E48 do not support a direct role for this residue in reaction specificity or substrate binding (Tables 1 and 2). Replacement of the corresponding D45 of eCGS with alanine or asparagine also results in only 2-9-fold changes in kinetic parameters, but enables a minor transamination activity. This observation suggests that, while this residue does not participate directly in substrate binding, it may contribute to active-site architecture or substrate positioning to subtly influence reaction specificity [11]. Substitution of the corresponding F55 of eCBL with the aspartate, mimicking eCGS-D45, results in a 78-fold increase in $K_m^{\text{L-Cth}}$, demonstrating the importance of active-site context [13].

Replacement of yCGL-E333 with alanine, aspartate or glutamine results in a ~17-fold increase in $K_m^{\text{L-Cth}}$, confirming a role for this residue in L-Cth binding (Table 1). In contrast, the kinetic parameters for L-OAS hydrolysis by the E333 substitution variants are within 3-fold of the wild type enzyme (Table 2). This implies that yCGL-E333 interacts with the distal amino moiety of L-Cth. The L-OSHS substrate of eCGS, like L-OAS, lacks the distal amino group of L-Cth and substitution variants of the corresponding eCGS-E325 also display similar 2–4-fold changes in $K_m^{\text{L-OSHS}}$ [11]. Although the corresponding Y338 of eCBL interacts with K42, the kinetic parameters of the Y338F and K42A replacement variants are similar to the wild-type eCBL enzyme, suggesting that the Y338-K42 interaction and the hydrogen-bonding moieties of these side chains are not required for efficient L-Cth hydrolysis [12,17]. As such, E333 plays unique role in substrate binding, via interaction with the distal amino group of L-Cth, in the context of yCGL. Therefore, we propose a role for this residue as a determinant of reaction

specificity via positioning of the L-Cth substrate, in a manner distinct from eCBL. The role of interactions with the distal portion of the substrate as a determinant of specificity is supported by the observation that the catalytic efficiency of L-Hcys α,γ-elimination ($k_{cat}/K_m^{L\text{-}Hcys}$ = 0.027 ± 0.005) is five orders of magnitude lower than the hydrolysis of L-Cth ($k_{cat}/K_m^{L\text{-}Cth}$ = 2100 ± 100) (Table 1). Similarly, the catalytic efficiency of yCGL for α,γ-elimination of L-OSHS ($k_{cat}/K_m^{L\text{-}OSHS}$ = 7 ± 2), which possesses a distal carboxylate, but lacks an amino group, is 300-fold lower than that of the physiological L-Cth substrate and 260-fold higher than that of L-Hcys, which lacks both distal polar moieties [13]. These observations provide support for the theory that positioning of substrate(s) within the active site is an important determinant of reaction specificity among the enzymes of the γ-subfamily [9,10].

Concomitant substitution of both E48 and E333 with alanine, aspartate, or glutamine has a synergistic effect, in terms of the magnitude of perturbation of the kinetic parameters of yCGL. The E48/E333 double replacement variants are not saturated within the solubility limit of the L-Cth substrate and $k_{cat}/K_m^{L\text{-}Cth}$ is decreased by up to 570-fold, at least an order of magnitude greater than the 1.3-3-fold and 26-58-fold reductions in catalytic efficiency observed for the single substitution variants of E48 and E333, respectively (Table 1). In contrast, the catalytic efficiency of the nine site-directed variants for L-OAS and L-Cys hydrolysis are within 4-fold and 13-fold, respectively, of the wild-type enzyme and the effect of double replacement, compared to E333 alone, is minor (Table 2). Therefore, the role of the glutamate residue at position 333 as a determinant of reaction specificity, in the context of yCGL, is enhanced by E48, as its

concomitant substitution selectively impairs L-Cth hydrolysis, compared with the α,β-elimination-specific substrates L-OAS and L-Cys.

5.2. Effect of substitutions on yCGL pH dependence and the spectral properties of the enzyme. Although eCBL, eCGS and yCGL share a common structure and several active-site residues, the pH optima for the catalytic efficiency of the eCBL-catalyzed (8.5-9.5) α,β-elimination of L-Cth is distinct from the 7.2-7.8 range for the α,γ-eliminations of L-OSHS and L-Cth catalyzed by eCGS and yCGL, respectively (Table 1, Figure 3) [16,17]. The absorption spectra of eCBL and yCGL exhibit a λ_{max} of ~421 nm, diagnostic for the protonated form of the internal aldimine of the cofactor. Loss of this proton is accompanied by a shift in the maximum of the spectrum of the PLP aldimine to 360 nm [23]. Modulation of the pK_a of the internal aldimine as a mechanism of regulating specificity is exemplified by ACCS and AAT. The pK_as of the aldimine and substrate, which correspond to the two titrating groups of the bell-shaped catalytic efficiency versus pH profile, are balanced such that that the values of 7.0 and 9.9, respectively, of AAT reversed as 9.3 and 7.8, respectively, in ACCS to enable optimal activity at physiological pH [20]. However, the shift in aldimine absorbance to 390 nm, similar to that of the free cofactor, upon titration of eCBL and yCGL is likely due to dissociation of PLP [16]. Therefore, in contrast with AAT and ACCS, the aldimine of yCGL and eCBL, like that of eCGS, cannot be assigned to either limb of the $k_{cat}/K_m^{L\text{-}Cth}$ versus pH profile [16,20].

Although the pH optima of yCGL and eCBL are distinct, differing by ~1.5 pH units, the value of pK_{a1} (yCGL = 8.1 ± 0.4; eCBL = 8.28 ± 0.06) is within experimental error for both enzymes. The titrating group of the acidic limb of the catalytic efficiency

versus pH profile may correspond to yCGL-K203/eCBL-K210 as the ε-amino group must be in the uncharged state to abstract the Cα proton of the substrate, initiating the ensuing side-chain rearrangements. The pK_a of this lysine residue, the common catalytic base of PLP-dependent enzymes catalyzing transformations of amino acids, may be reduced from 10.5 to less than 5 in the context of the active site, as exemplified by pig heart AAT [24,25]. The pK_{a1} value of ~8.2 observed for yCGL and eCBL is similar to that reported for the corresponding K41 of *Salmonella typhimurium O*-acetylserine sulfhydrylase [26,27].

In contrast with the common pK_{a1} value, the basic limb of the pH profile of yCGL (pK_{a2} = 7.1 ± 0.4) is 3 pH units lower than that of eCBL (pK_{a2} = 10.20 ± 0.06). The 90-fold lower catalytic efficiency of yCGL, compared to eCBL, may be due to the ~1 pH unit lower value of pK_{a2}, compared to pK_{a1}, as the observed catalytic efficiency of yCGL is an order of magnitude lower than the theoretical maximum (k_{cat}/K_m^{max} = 2 ± 1 x 10^4) at the pH optimum. In contrast, the observed ($k_{cat}/K_m^{L\text{-}Cth}$ = 1.3 x 10^5) value is within 1.3-fold of the theoretical maxmum for eCBL (k_{cat}/K_m^{max} = 1.63 ± 0.07 x 10^5). The catalytic efficiencies of the α,γ-elimination of L-OSHS and L-Cth by eCGS ($k_{catE}/K_m^{L\text{-}OSHS}$ = 1350 ± 90 $M^{-1}s^{-1}$) and yCGL ($k_{catE}/K_m^{L\text{-}Cth}$ = 2100 ± 10 $M^{-1}s^{-1}$), respectively, are of similar magnitude but an order of magnitude lower than the physiological α,γ-replacement of eCGS ($k_{catR}/K_m^{L\text{-}OSHS}$ = 49000 ± 9000 $M^{-1}s^{-1}$), suggesting that a step following formation of the β,γ-unsaturated ketimine intermediate, common to both mechanisms, is rate limiting for α,γ-elimination [16,28]. The reduced catalytic efficiency of yCGL, compared to eCBL, resulting from the reversed pK_a values (pK_{a1} > pK_{a2}) of the former, may represent an evolutionary trade off between catalytic efficiency and reaction specificity as

eCBL is specific for the α,β-elimination mechanism, while yCGL catalyzes the α,β- and α,γ-elimination of L-Cth [13].

The group responsable for pK_{a2} may be one of the two amino groups of the substrate, as proposed for the hydrolysis of L-Cth by cystathionine β-synthase. Aitken and Kirsch reported pK_a values of 8.54 ± 0.01 and 9.63 ± 0.01 for the amino groups of L-Cth by direct titration with NaOH [29]. It is possible that the pK_a of one or both of these groups is influenced by the active-site environment of eCBL and yCGL, particularly as residue E333 of yCGL uniquely interacts with the distal amino group of L-Cth and is replaced with the aromatic Y338 in eCBL. Alternatively, the titrating group may be the pyridinium nitrogen of the cofactor, which is influenced by proximity to the adjacent asparate (yCGL-D178, eCBL-D185) as well as interaction between O3' of the cofactor and the protein (Figure 2) [24]. A network of polar interactions connects N1 and O3' of the cofactor in eCBL but not yCGL as eCBL-W340, which forms a hydrogen bond with PLP-O3', is conserved as leucine (yCGL-L335) in fungal and animal CGL sequences. The pK_a of the pyridinium nitrogen may be influenced by yCGL-E333 and the corresponding eCBL-Y338 via modulation of the distance between N1 and the carboxylate group of the adjacent aspartate as the 1.1-Å greater distance in yCGL diminishes the ability of this residue to promote protonation of N1, thereby reducing its pK_a relative to eCBL. Residues Y338 and W340 of eCBL and E333 and L335 of yCGL also flank the serine residue (eCBL-S339/yCGL-S334) that plays an essential role in positioning of the catalytic base (Figure 2). Although the effect of substitution with aspartate on the pH optimum is minor, the catalytic efficiency of the E333D and E48D/E333D enzymes is ~5 and ~10-fold reduced, at their pH optima, compared to the

corresponding alanine and glutamine substitution variants. This suggests that the ~1 Å difference in the length of the side chain of aspartate, compared to glutamate or glutamine, results in binding of the substrate in a strained or less catalytically productive conformation. The pH optima of the E48A and E48Q, E333A and E333Q variants are increased 0.4-0.6 and 0.7 pH units, respectively. Replacement of both residues has an additive effect, as demonstrated by the increase of 0.4, 0.7 and 1.2 units in the pH optima aspartate, glutamine and alanine double substitution variants, respectively (Table 1; Figure 3). The effect of E333 substitution is likely the result of loss of the positioning interactions of this residue with T349 and the distal amino group of the substrate, thereby resulting in a shift in the relative position of L335, in van der Waals contact with the PLP ring, allowing the cofactor to shift and shortening of the (PLP)N1-D178 H-bond (Figure 2). We propose that the conserved-different residues flanking yCGL-S334 (E333 and L335) and the corresponding eCBL-S339 (Y338 and W340) contribute to the regulation of reaction specificity via interactions that optimize the relative positions of the substrate, cofactor and catalytic base to modulate the electronic properties of the PLP and allow or restrict access of the catalytic base to the C4' and Cβ positions of the cofactor and substrate, respectively.

The enzymes of the transsulfuration pathways are attractive targets for the development of novel therapeutics, including antimicrobial compounds (CGS and CBL - unique to plants and bacteria) and modulators of H_2S production (CGL). As members of the γ-subfamily of fold-type I of PLP-dependent enzymes they also comprise a useful model system for studies exploring the molecular mechanisms that underlie specificity among enzymes reliant on this catalytically versatile cofactor, with the goal of

engineering PLP-dependent enzymes for biotechnological applications. Both of these objectives require an in-depth understanding of the structure-function relationships that are the basis for inhibitor or reaction specificity. This study has identified a unique role for E333 in binding to the distal amino moiety of the physiological substrate as well influencing the pH optimum of yCGL. These findings enhance our understanding of the complex factors regulating reaction specificity in enzymes of the γ-subfamily and confirm that modification of specificity will require multiple, simultaneous changes [4,12,13]. Given the catalytic versatility of the PLP cofactor and the central role of amino acid metabolism to cellular homeostasis, it is not surprising that PLP-dependent enzymes have evolved multiple residues and structural features for the regulation of specificity. The observed immutability of restriction endonucleases, exemplified by *Bam*HI and *Bgl*II, which share structural features and a common core in their DNA recognition sequences but are not easily interconverted, demonstrates that the challenge of identifying the determinants of reaction specificity between structurally conserved enzymes is not unique to PLP-dependent enzymes [30].

6. ACKNOWLEDGEMENTS

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7. REFERENCES

[1] Leuchtenberger, W., Huthmacher, K., and Drauz, K. 2005. Biotechnological production of amino acids and derivatives: current status and prospects. Appl Microbiol Biotechnol. 69, 1-8.

[2] Esvelt, K.M. and Wang, H.H. 2013. Genome-scale engineering for systems and synthetic biology. Molecular Systems Biology 9; Article number 641.

[3] Liu CC, Schultz PG (2010) Adding new chemistries to the genetic code. Annu Rev Biochem 79: 413–444

[4] Aitken, S.M., Lodha, P.L. and Morneau, D.J.K. 2011. The enzymes of the transsulfuration pathways: active-site characterizations. Biochim. Biophys. Acta 1814:1511-1517.

[5] Messerschmidt, A., Worbs, M., Steegborn, C., Wahl, M.C., Huber, R., Laber, B. and Clausen, T. 2003. Determinants of enzymatic specificity in the Cys-Met-metabolism PLP-dependent enzymes family: crystal structure of cystathionine gamma-lyase from yeast and intrafamiliar structure comparison. Biol. Chem. 384, 373-386.

[6] Clausen, T., Huber, R., Prade, L., Wahl, M.C. and Messerschmidt, A. 1998. Crystal structure of Escherichia coli cystathionine gamma-synthase at 1.5 A resolution. EMBO J. 17, 6827-6838.

[7] T. Clausen, R. Huber, R. Laber, H.D. Pohlenz, A. Messerschmidt, Crystal structure of the pyridoxal-5'-phosphate dependent cystathionine beta-lyase from Escherichia coli at 1.83 Å, J. Mol. Biol. 262 (1996) 202-224.

[8] Goodall, G., Mottram, J. C., Coombs, G. H. and Lapthorn, A. J. (2001). Methionine γ-lyase from *Trichomonas vaginales*, PDB Entry: 1E5F.

[9] Clausen, T., Huber, R., Messerschmidt, A., Pohlenz, H.D. and Laber, B. 1997. Slow-binding inhibition of Escherichia coli cystathionine β-lyase by L-aminoethoxyvinylglycine: a kinetic and X-ray study. Biochemistry. 36, 12633-12643.

[10] Lodha, P.H., Jaworski, A.F. and Aitken, S.M. 2010. Characterization of site-directed mutants of residues R58, R59, D116, W340 and R372 in the active site of *E. coli* cystathionine β-lyase, Protein Sci. 19, 383-391.

[11] A.F. Jaworski, P.H. Lodha, A.L. Manders, S.M. Aitken, Exploration of the Active Site of *Escherichia coli* Cystathionine γ-Synthase. Protein Sci. (2012) 21:1662-1671.

[12] Manders, A.L., Jaworski, A.F., Ahmed, M., Aikten, S.M. Exploration of structure-function relationships in *Escherichia coli* cystathionine γ-synthase and cystathionine β-lyase via chimeric constructs and site-specific substitutions. Biochim. Biophys. Acta (2013) 1834: 1044-1053.

[13] Farsi, A., Lodha, P.H., Skanes, J.E., Los, H., Kalidindi, N. and Aitken, S.M. 2009. Inter-conversion of a Pair of Active-Site Residues in *E. coli* Cystathionine γ-Synthase, *E. coli* Cystathionine β-Lyase and *S. cerevisiae* Cystathionine γ-Lyase and Development of Tools for the Investigation of Mechanism and Reaction Specificity. Biochem. Cell Biol. 87, 445-457.

[14] S. Kashiwamata, D.M. Greenberg. Studies on cystathionine synthase of rat liver. Properties of the highly purified enzyme. (1970) Biochim. Biophys. Acta 212: 488-500.

[15] Morneau, D.J.K., Abouassaf, E., Skanes, J.E., and Aitken, S.M. 2012. Development of a continuous assay and steady-state characterization of Escherichia coli threonine synthase. Anal. Biochem. 423, 78-85.

[16] Aitken, S.M., Kim, D.H. and Kirsch, J.F. 2003. Escherichia coli cystathionine gamma-synthase does not obey ping-pong kinetics. Novel continuous assays for the elimination and substitution reactions. Biochemistry. 42, 11297-11306.

[17] Lodha, P.H. and Aitken, S.M. 2011. Characterization of the side-chain hydroxyl moieties of residues Y56, Y111, Y238, Y338 and S339 as determinants of specificity in E. coli cystathionine β-lyase. Biochemistry 50: 9876-9885.

[18] Peracchi, A., Bettati, S., Mozzarelli, A., Rossi, G. L., Miles, E.W., and Dunn, M. F. Allosteric regulation of tryptophan synthase: effects of pH, temperature, and alpha-

subunit ligands on the equilibrium distribution of pyridoxal 5'-phosphate-L-serine intermediate. (1996) *Biochemistry 35,* 1872-1880.

[19] Jhee, K. H., McPhie, P., and Miles, E. W. Domain architecture of the heme-independent yeast cystathionine beta-synthase provides insights into mechanisms of catalysis and regulation. (2000) *Biochemistry 39,* 10548-10556.

[20] Eliot, A.C., Kirsch, J.F. Modulation of the internal aldimine pK_as of 1-aminocyclopropane-1-carboxylate synthase and aspartate aminotransferase by specific active site residues. (2002) Biochemistry. 41: 3836-3842.

[21] Mendonça, L.M.F. and Marana, S.R. 2011. Single mutations outside the active site affect substrate specificity in β-glucosidase. Biochim. Biophys. Acta. 1814, 1616-1623.

[22] Huang, S., Chua, J.H., Yew, W.S., Sivaraman, J., Moore, P.K., Tan, C.H., Deng, L.W. Site-directed mutagenesis on human cystathionine-gamma-lyase reveals insights into the modulation of H2S production. (2010) J. Mol. Biol. 396: 708-718.

[23] Jenkins, W. T., Yphantis, D. A., and Sizer, I. W. Glutamic aspartic transaminase. I. Assay, purification, and general properties. (1959) *J. Biol. Chem. 234*, 51-57.

[24] Kiick, D.M., Cook, P.F. pH studies toward the elucidation of the auxiliary catalyst for pig heart aspartate aminotransferase. (1983) Biochemistry. 22: 375-382.

[25] Toney, M.D., Kirsch, J.F. Bronstead analysis of aspartate aminotransferase via exogenous catalysis of reactions of an inactive mutant. (1992) Protein Sci. 1: 107-119.

[26]Daum, S., Tai, C.-H., and Cook, P.F. 2003. Characterization of the S272A,D Site-Directed Mutations of O-Acetylserine Sulfhydrylase: Involvement of the Pyridine Ring in the α,β-Elimination Reaction. Biochemistry 42, 106-113

[27] Cook, P.F., Hara, S., Nalabolo, S., and Schnackerz, K.D. 1992. pH Dependence of the Absorbance and ^{31}P NMR Spectra of *O*-acetylserine sulfhydrylase in the Absence and Presence of *O*-acetylserine. Biochemistry 31, 2298-2303

[28] Brzovic, P., Holbrook, E.L., Greene, R.C., Dunn, M.F. Reaction mechanism of Escherichia coli cystathionine gamma-synthase: direct evidence for a pyridoxamine derivative of vinlglyoxylate as a key intermediate in pyridoxal phosphate dependent gamma-elimination and gamma-replacement reactions. (1990) Biochemistry 29: 442-451.

[29] Aitken SM, Kirsch JF (2003) Kinetics of the yeast cystathionine β-synthase forward and reverse reactions: continuous assays and the equilibrium constant for the reaction. Biochemistry 42:571-578.

[30] C.M. Lukacs, R. Kucera, I. Schildkraut, A.K. Aggarwal, Understanding the immutability of restriction enzymes: crystal structure of *Bgl*II and its DNA substrate at 1.5 A resolution, Nature Struct. Biol. 7 (2000) 134-140.

[31] Steegborn. C., Messerschmidt, A., Laber, B., Streber, W., Huber, R. and Clausen, T. 1999. The crystal structure of cystathionine γ-synthase from Nicotiana tabacum reveals its substrate and reaction specificity. J. Mol. Biol. 290, 983-996.

Table 1. Kinetic parameters of L-Cth hydrolysis for wild-type eCBL and yCGL and site-directed variants of yCGL.[a]

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	pH optimum
L-Cth → L-Cys + α-ketobutyrate + NH_3 (α,γ-elimination) **L-Cth → L-Hcys + pyruvate + NH_3 (α,β-elimination)**				
eCBL[b]	34.1 ± 0.6	0.18 ± 0.01	1.9 ± 0.1 x 10^5	8.5 – 9.5
yCGL	1.51 ± 0.03	0.71 ± 0.04	(2.1 ± 0.1) x 10^3	7.2 – 7.8
E48A	0.54 ± 0.01	0.76 ± 0.04	(7.1 ± 0.3) x 10^2	7.6 – 8.4
E48D	1.4 ± 0.02	0.87 ± 0.04	(1.59 ± 0.05) x 10^3	7.0 – 7.8
E48Q	1.29 ± 0.04	0.9 ± 0.1	(1.4 ± 0.1) x 10^3	7.4 – 8.2
E333A	0.78 ± 0.05	12 ± 1	65 ± 1	7.8 – 8.6
E333D	0.98 ± 0.04	12 ± 1	82 ± 1	7.2 – 8.0
E333Q	0.48 ± 0.09	13 ± 3	36 ± 2	7.6 – 8.4
E48A/E333A	n.s	n.s.	3.7 ± 0.1	8.4 – 9.2
E48D/E333D	n.s.	n.s.	8.8 ± 0.1	7.6 – 8.4
E48Q/E333Q	n.s.	n.s.	16.9 ± 0.1	7.9 – 8.7

[a]Reaction conditions: 50 mM potassium phosphate, pH 7.2, 20 μM PLP, 2 mM DTNB, 0.01-5.5 mM L-Cth and 0.45-19.5 μM yCGL, depending on the activity of the specific enzyme variant. The data were fitted to the Michaelis-Menten equation and $k_{cat}/K_m^{L\text{-}Cth}$ was determined directly via equation 1 and error values correspond to the fit of the data to the model [13]. n.s. indicates the enzyme is not saturated within the solubility limited of the L-Cth substrate. The yCGL enzymes were assayed at pH 7.2, a pH at which the activity of eCBL, which is specific for the α,β-elimination of L-Cth, is negligible (<5% of the maximal activity at pH 8.5). In contrast, yCGL catalyzes both the α,β- and α,γ-elimination of L-Cth [13]. Therefore, the lowest pH of the optimal range of yCGL, furthest from the pH optimum of eCBL, was selected to assay the L-Cth hydrolysis activity of this enzyme as the distinct pH optima of the 2 enzymes may be a determinant of α,β- versus α,γ- reaction specificity. The $k_{cat}/K_m^{L\text{-}Cth}$ of yCGL at pH 7.2 and 7.6 (in MOPS-bincine-proline buffer), the lowest point and midpoint of the optimum range of the pH versus $k_{cat}/K_m^{L\text{-}Cth}$ profile are 2180 ± 90 and 2700 ± 100 $M^{-1}s^{-1}$, respectively.
[b]values from ref [10].

Table 2. Kinetic parameters of L-OAS and L-Cys hydrolysis for wild-type and site-directed variants of yCGL.[a]

Enzyme	k_{cat} (s^{-1})	K_m (mM)	$K_i^{L\text{-}Cys}$ (mM)[b]	k_{cat}/K_m ($M^{-1}s^{-1}$)
L-Cys → pyruvate + H_2S + NH_3				
yCGL	0.15 ± 0.01	0.09 ± 0.01	2.0 ± 0.3	(1.7 ± 0.2) x 10^3
E48A	0.09 ± 0.01	0.11 ±0.02	3.1 ± 0.6	(8 ± 1) x 10^2
E48D	0.32 ± 0.04	0.4 ± 0.1	2.2 ± 0.6	(7.2 ± 0.8) x 10^2
E48Q	0.18 ± 0.01	0.18 ± 0.02	5.7 ± 0.7	(1.02 ± 0.06) x 10^3
E333A	1.3 ± 0.5	1.2 ± 0.6	0.8 ± 0.4	(8.1 ± 0.7) x 10^2
E333D	0.24 ± 0.04	1.8 ± 0.4	1.1 ± 0.3	(1.3 ± 0.1) x 10^2
E333Q	0.5 ± 0.1	0.7 ± 0.2	3 ± 1	(4.9 ± 0.3) x 10^2
E48A/E333A	0.11 ± 0.02	0.5 ± 0.1	0.4 ± 0.1	(2.1 ± 0.1) x 10^2
E48D/E333D	0.029 ± 0.004	0.5 ± 0.1	0.9 ± 0.2	60 ± 10
E48Q/E333Q	0.16 ± 0.04	0.4 ± 0.1	0.7 ± 0.2	(3.1 ± 0.2) x 10^2
L-OAS → acetate + pyruvate + NH_3				
yCGL	0.0097 ± 0.0002	2.5 ± 0.3	-	3.8 ± 0.4
E48A	0.0142 ± 0.0004	1.5 ± 0.2	-	10 ± 1
E48D	0.0053 ± 0.0001	1.4 ± 0.1	-	3.8 ± 0.3
E48Q	0.0124 ± 0.0002	4.4 ± 0.3	-	2.8 ± 0.2
E333A	0.0058 ± 0.0002	2.2 ± 0.3	-	2.6 ± 0.3
E333D	0.0045 ± 0.0002	0.9 ± 0.2	-	5 ± 1
E333Q	0.0102 ± 0.0002	5.3 ± 0.4	-	1.9 ± 0.1
E48A/E333A	0.0047 ± 0.0002	2.9 ± 0.6	-	1.6 ± 0.3
E48D/E333D	0.0035 ± 0.0002	0.7 ± 0.2	-	5 ± 1
E48Q/E333Q	0.0092 ± 0.0004	1.5 ± 0.3	-	6 ± 1

[a]Reaction conditions: 50 mM potassium phosphate, pH 7.2, 20 μM PLP, 0.75-150 mM L-OAS or 0.04-3 mM L-Cys, 3.5 μM LDH, 1.5 mM NADH (ε_{340} = 6,200 $M^{-1}cm^{-1}$) and 2.2-9.8 μM yCGL, depending on the activity of the specific enzyme variant. The L-OAS and L-Cys data were fitted to the Michaelis-Menten equation and equation 2 and k_{cat}/K_m was determined directly via equations 1 and 3, respectively.

[b]Substrate inhibition is not observed for the hydrolysis of L-OAS over the range of substrate concentrations tested (0.75-150 mM).

FIGURE LEGENDS

Figure 1. The bacterial transsulfuration (CGS and CBL) and yeast/animal reverse transsulfuration pathways (CBS and CGL) that interconvert L-Cys to L-Hcys. The superscripts Iγ and II indicate that CGL, CGS and CBL are members of the γ-subfamily of fold-type I of PLP-dependent enzymes, while CBS is structurally distinct and belongs to fold-type II [12,31].

Figure 2. Proposed polar contacts of the yCGL active site that interact with or are influenced by E48 and E333. The mode of L-Cth binding is based on the structures of yCGL (PDB accession 1NP8) and the eCBL-aminoethoxyvinylglycine complex (PDB accession 1CL2) and the data presented in table 1 [5,9]. The corresponding positions of eCBL are shown in brackets.

Figure 3. Comparison of the pH dependence of $k_{cat}/K_m^{L\text{-}Cth}$ for L-Cth hydrolysis by wild-type yCGL (○) and (**A**) eCBL (●), the (**B**) E48A (∇), E333A (Δ), and E48A/E333A (◊), (**C**) E48D/E333D (+) and (**D**) E48A/E333A (◊) and E48Q/E333Q (×) variants of yCGL. Reaction conditions: MBP buffer (50 mM MOPS, 50 mM Bicine and 50 mM proline), 20 μM PLP, 2 mM DTNB, 0.01-5.5 mM L-Cth and 0.11 μM eCBL (values from ref [10]) or 0.45-19.5 μM yCGL variant, depending on the activity of the specific enzyme, in assay buffer at 25 ºC. The data were fitted to equation 4.

Figure 4. Spectrophotometric titration of 20 μM (**A** and **B**) eCBL and (**C** and **D**) yCGL-E48A/E333A as a function of pH. The spectra shown were recorded at pH (**A**) 7.01, 8.02,

9.00, 10.02, 11.24 and 12.08 for eCBL and (**C**) 6.98, 8.00, 8.99, 10.02, 10.86 and 12.32 for yCGL-E48A/E333A. (**B** and **D**) Absorbance at 290 nm (○), 390 (◊) and 421 nm (Δ) versus pH.

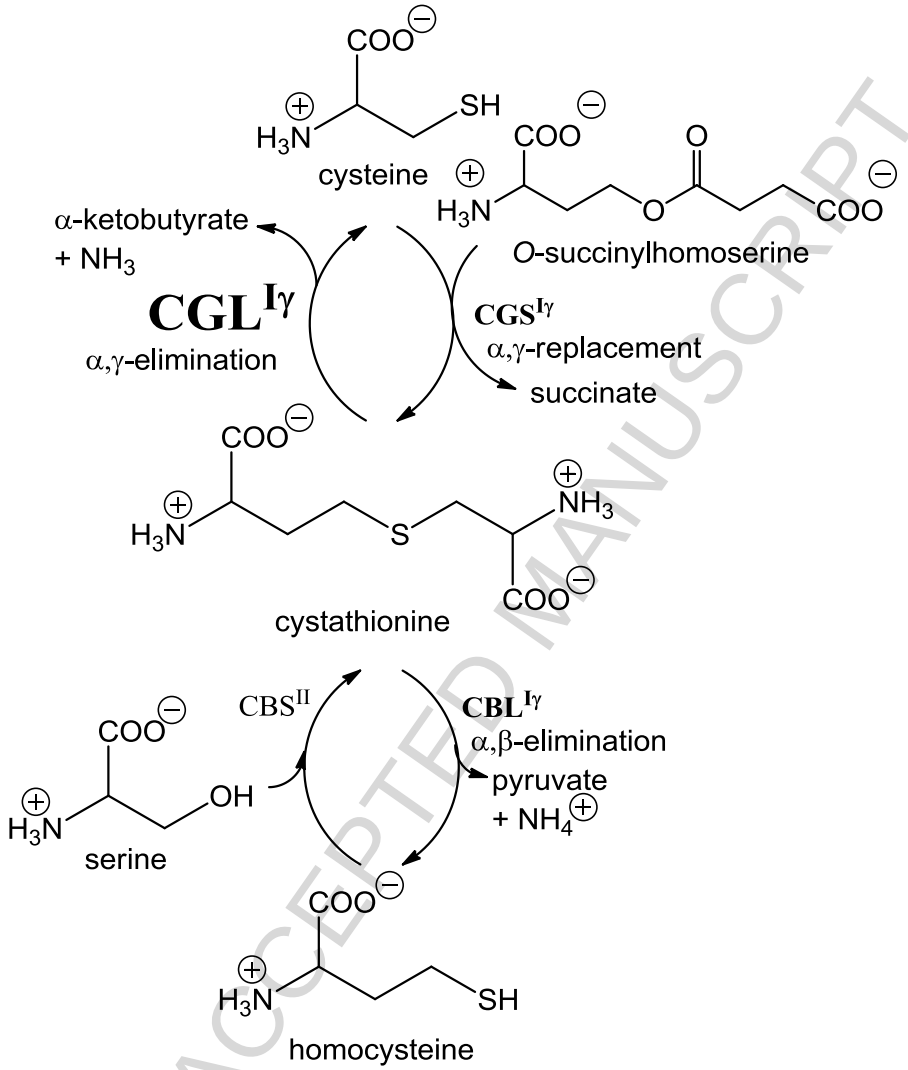

Figure 1.

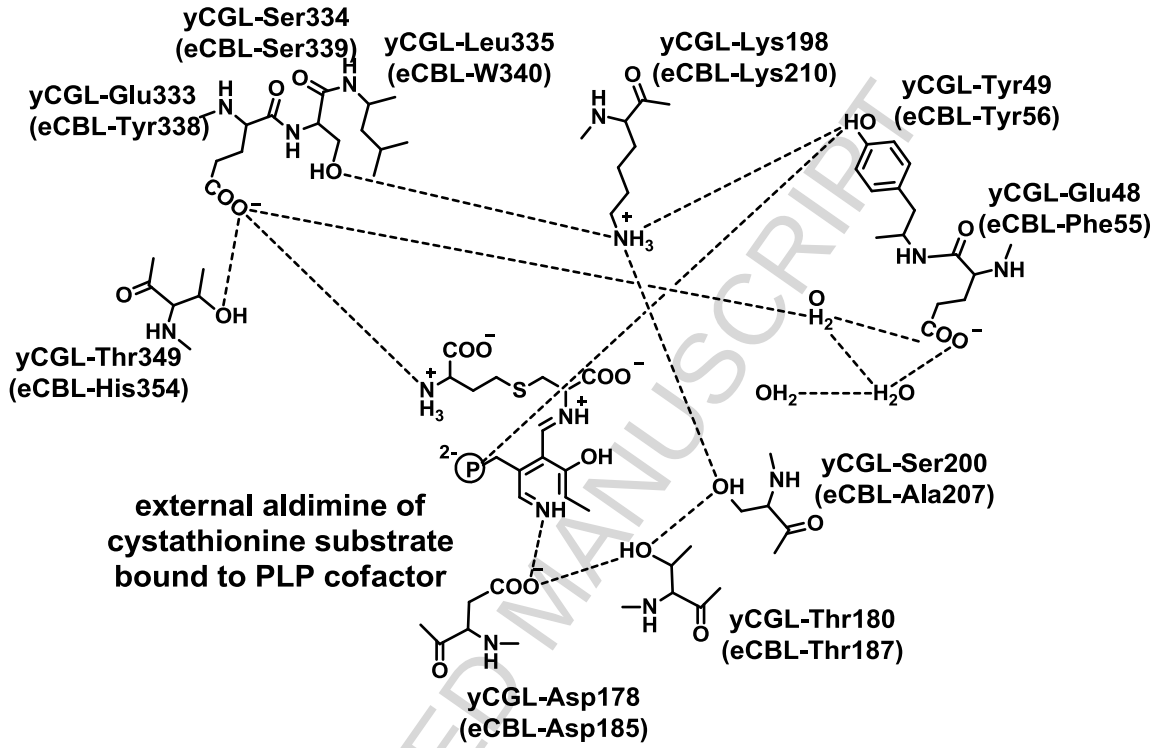

Figure 2.

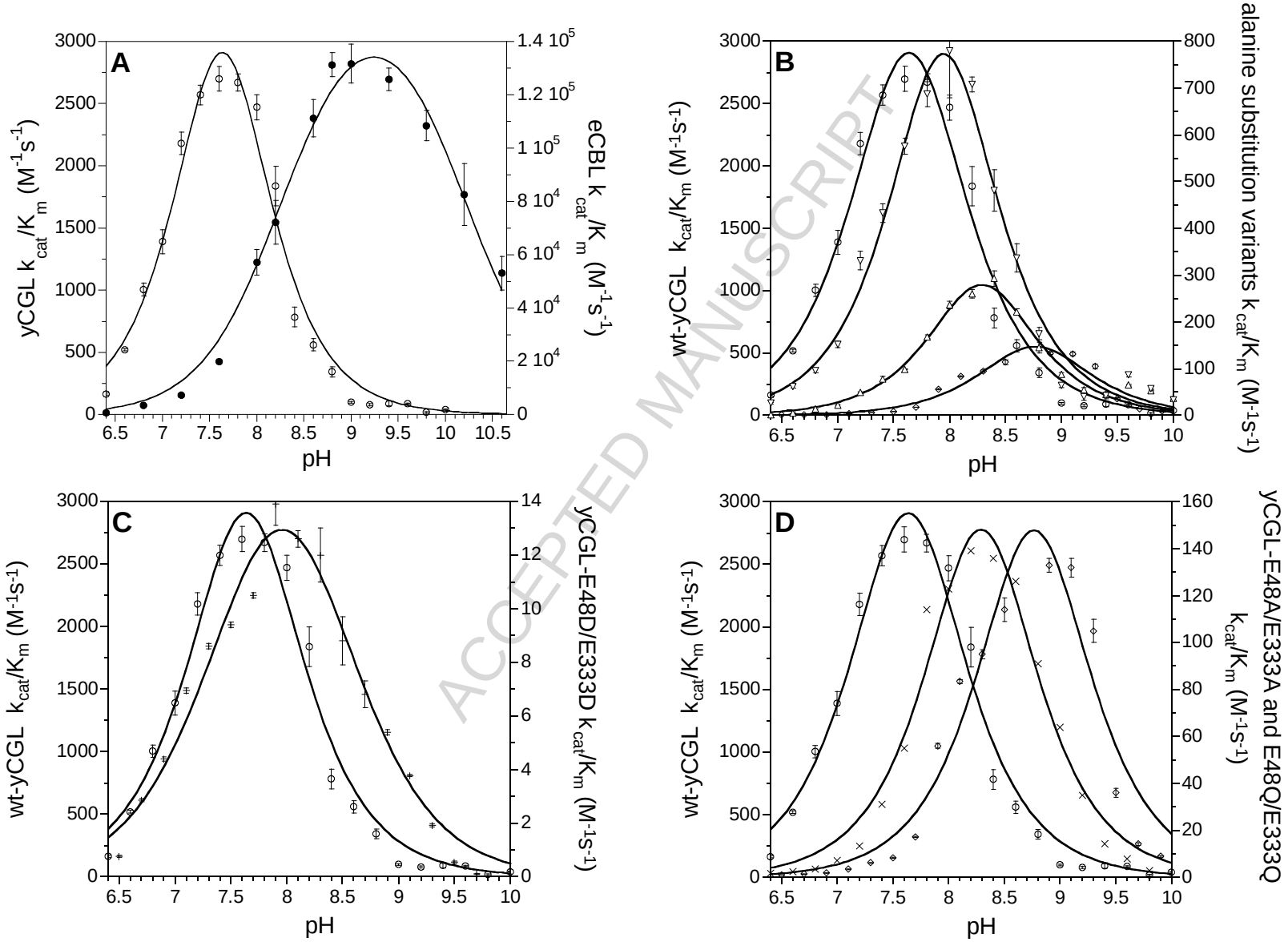

Figure 3.

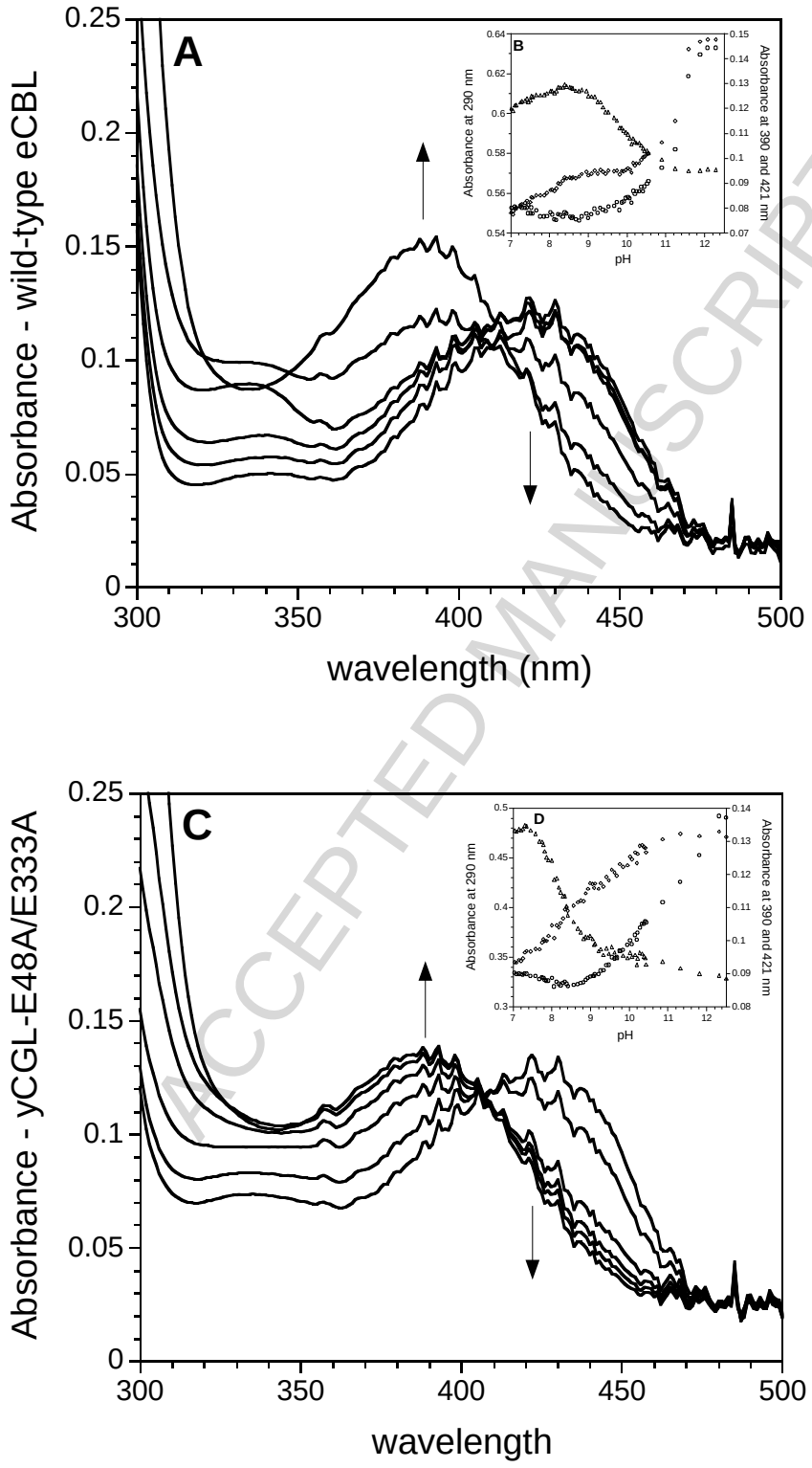

Figure 4.

Highlights of manuscript entitled "**A Role for Glutamate-333 of *Saccharomyces cerevisiae* Cystathionine γ-Lyase as a Determinant of Specificity**", submitted July 10, 2013.

- Two active-site residues probed for their roles as determinants of specificity
- 9 site-directed variants targeting E48 and E333 of yeast cystathionine gamma-lyase
- E333 interacts with distal amino group of cystathionine substrate
- Substitution of E48 and E333 shifts the pH optimum up to 1.2 pH units
- Role for E333 as a determinant of substrate and reaction specificity proposed