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Exploration of structure–function relationships in *Escherichia coli* cystathionine γ-synthase and cystathionine β-lyase *via* chimeric constructs and site-specific substitutions

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Cystathionine γ-synthase (CGS) and cystathionine β-lyase (CBL) share a common structure and several active-site residues, but catalyze distinct side-chain rearrangements in the two-step transsulfuration pathway that converts cysteine to homocysteine, the precursor of methionine. A series of 12 chimeric variants of *Escherichia coli* CGS (eCGS) and CBL (eCBL) was constructed to probe the roles of two structurally distinct, ~25-residue segments situated in proximity to the amino and carboxy termini and located at the entrance of the active-site. *In vivo* complementation of methionine-auxotrophic *E. coli* strains, lacking the genes encoding eCGS and eCBL, demonstrated that exchange of the targeted regions impairs the activity of the resulting enzymes, but does not produce a corresponding interchange of reaction specificity. In keeping with the *in vivo* results, the catalytic efficiency of the native reactions is reduced by at least 95-fold, and α,β *versus* α,γ-elimination specificity is not modified. The midpoint of thermal denaturation monitored by circular dichroism, ranges between 59 and 80 °C, compared to 66 °C for the two wild-type enzymes, indicating that the chimeric enzymes adopt a stable folded structure and that the observed reductions in catalytic efficiency are due to reorganization of the active site. Alanine-substitution variants of residues S32 and S33, as well as K42 of eCBL, situated in proximity to and within, respectively, the targeted amino-terminal region were also investigated to explore their role as determinants of reaction specificity *via* positioning of key active-site residues. The catalytic efficiency of the S32A, S33A and the K42A site-directed variants of eCBL is reduced by less than 10-fold, demonstrating that, while these residues may participate in positioning S339, which tethers the catalytic base, their role is minor.

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1. Introduction

The transsulfuration pathway of bacteria and plants transfers the sulfhydryl moiety of cysteine (Cys) to the amino acid backbone of an activated form of homoserine, yielding homocysteine (Hcys), the immediate precursor of methionine (Met) (Fig. 1). The two enzymes comprising this pathway, cystathionine γ-synthase (CGS) and cystathionine β-lyase (CBL), belong to the γ-subfamily of the large and catalytically diverse fold-type I family of pyridoxal 5′-phosphate (PLP)-dependent enzymes [1,2]. Comparison of the structures of 14 distinct enzymes of the γ-subfamily demonstrates a high degree of conservation in the overall structures, as well as the active sites, of these enzymes [3]. For example, an r.m.s. deviation between ~340 C_α atoms of only ~1.5 Å was reported by Messerschmidt et al. for superposition of the monomers of *Escherichia coli* CGS (eCGS), *E. coli* CBL (eCBL), *Trichomonas vaginalis* methionine γ-lyase (tMGL), and yeast (*Saccharomyces cerevisiae*) cystathionine γ-lyase (yCGL) [4]. Therefore, this family of enzymes provides a useful model system for investigating the structure–function relationships underlying substrate and reaction specificity in enzymes dependent on the catalytically versatile PLP cofactor [5].

Comparison of eCGS and eCBL, which catalyze α,γ-elimination/replacement and α,β-elimination reactions, respectively, reveals that the only two structurally distinct regions are situated at the mouth of the active site, which is located at the interface between the catalytic and carboxy-terminal domains (Fig. 2A and B). The resulting difference in the architecture of the active-site entrances of the two enzymes raises the possibility that these distinct structural features may act as determinants of reaction specificity [6,7]. These regions correspond to segments of ~25 amino acids and are located near the amino (region 1: residues 36–56 of eCBL and 35–46 of eCGS) and carboxy (region 2: residues 347–372 of eCBL and 334–361 of eCGS) termini of the enzymes (Fig. 3). A series of chimeric constructs,

Abbreviations: OAS, *O*-acetylserine; AVG, aminoethoxyvinylglycine; CBL, cystathionine β-lyase; CGL, cystathionine γ-lyase; CGS, cystathionine γ-synthase; Cth, cystathionine; Hcys, homocysteine; HO-HxoDH, D-2-Hydroxyisocaproate dehydrogenase; IPTG, Isopropyl-β-D-thiogalactopyranoside; LDH, L-lactate dehydrogenase; MGL, methionine γ-lyase; MS, methionine synthase; MTHF, 5-methyltetrahydrofolate; Ni-NTA, Ni-nitrilotriacetic acid; OSHS, *O*-succinylhomoserine; PLP, pyridoxal 5′-phosphate; THF, tetrahydrofolate

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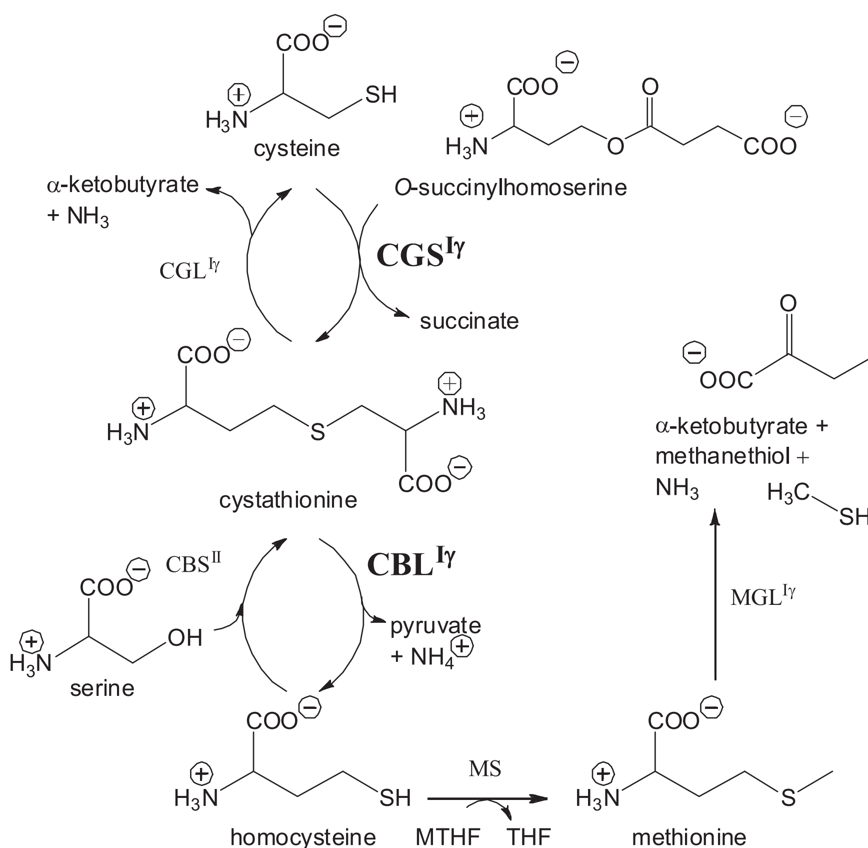

Fig. 1. The metabolic relationship and reactions catalyzed by bacterial CGS and CBL, CGL and MGL. Enzymes are identified as members of the γ-subfamily of fold-type I (Iγ) or fold-type II (II). Enzymes and metabolites are abbreviated as follows: CGS, cystathionine γ-synthase; CBL, cystathionine β-lyase; CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; MGL, methionine γ-lyase; MS, methionine synthase; MTHF, 5-methyltetrahydrofolate; OSHS, *O*-succinylhomoserine; THF, tetrahydrofolate.

interchanging either the ~50-residue amino and/or carboxy termini or the ~25-residue internal segments corresponding to regions 1 and/or 2, was constructed to investigate the hypothesis that regions 1 and 2 act as determinants of specificity.

Typical of the enzymes of the γ-subfamily of fold-type I, eCGS and eCBL share common active-site residues including Y56, R58, R59, Y101, S339 and R372 (eCBL numbering), as well as K210, the catalytic base. In contrast, eCBL residues F55, D116, Y238, Y338 and W340 differ from the corresponding D45, R106, N227, E325 and L327, respectively, of eCGS. Interestingly, several of these active-site residues, reported to play key roles in the positioning of the substrate(s), PLP cofactor or catalytic residue(s), thereby acting as determinants of substrate and/or reaction specificity, are situated adjacent to the structurally distinct regions 1 (eCBL F55, Y56, R58, R59) and 2 (eCBL Y338, S339, W340, R372) (Fig. 3) [3,5,8–10]. The challenge of identifying the underlying mechanisms that modulate reaction specificity is exemplified by eCBL–S339 (eCGS–S326), which is conserved in 12 of the 14 structures available for enzymes of the γ-subfamily and is proposed to tether the catalytic base (eCBL–K210) [3]. The degree to which the catalytic base can be allowed flexibility of movement, *versus* a requirement for constraint in this regard, is dependent on the nature of the reaction. For example, the α,γ-elimination reactions of CGL, MGL and CGS require proton transfers between C4′, Cα and Cβ, while the α,β-elimination catalyzed by CBL requires that access of the catalytic base be limited to Cα. Therefore, the positioning of S339, which tethers the catalytic base, is one factor enabling these structurally similar enzymes to catalyze distinct reactions with high specificity [3,5]. Residue S339 is positioned between Y338 and W340, in the eCBL active site, which are distinct from E325 and L327 that flank the corresponding S326 of eCGS. The side chain hydroxyl moiety of eCBL–Y338 is 2.8 Å from the ε-amino group of K42, a residue situated in the middle of the nine-residue insertion of eCBL region 1, not present in eCGS (Figs. 2C and 3). The side chain hydroxyl moieties of residues S32 and S33 of eCBL, which immediately precede region 1 and correspond to S31 and T32 of eCGS, are 3.4 and 2.7 Å from the side chain hydroxyl and backbone carbonyl groups of S339 and the adjacent Y338, respectively (Figs. 2C and 4) [6]. Therefore, residues S32, S33 and K42 of eCBL were replaced with alanine in this study to determine their impact on activity and reaction specificity.

Exchange of the ~25-residue segments corresponding to regions 1 and/or 2 does not impair the ability of the chimeric enzymes to adopt a stable, folded conformation, but could result in changes in the architecture and/or dynamics of the active site such that the catalytic efficiency of the resulting enzymes is drastically impaired. In contrast, substitution of S32, S33 or the K42 of eCBL with alanine reduced the catalytic efficiency by less than 10-fold. The results of this study illustrate the complexity of the mechanisms regulating reaction specificity in enzymes dependent on the catalytically versatile PLP cofactor.

2. Materials and methods

2.1. Reagents

Cth, *O*-acetyl-L-serine (OAS), *O*-succinylhomoserine (OSHS) and L-lactate dehydrogenase (LDH) were purchased from Sigma. Ni-nitrilotriacetic acid (Ni-NTA) resin was obtained from Qiagen. Oligonucleotide primers were synthesized by Integrated DNA Technologies and chimeric constructs and site-directed mutants were sequenced

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