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Mutational analysis of Sep-tRNA:Cys-tRNA synthase reveals critical residues for tRNA-dependent cysteine formation

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Abstract

In methanogenic archaea, Sep-tRNA:Cys-tRNA synthase (SepCysS) converts Sep-tRNA^{Cys} to Cys-tRNA^{Cys}. The mechanism of tRNA-dependent cysteine formation remains unclear due to the lack of functional studies. In this work, we mutated 19 conserved residues in *Methanocaldococcus jannaschii* SepCysS, and employed an *in vivo* system to determine the activity of the resulting variants. Our results show that three active-site cysteines (Cys39, Cys42 and Cys247) are essential for SepCysS activity. In addition, combined with structural modeling, our mutational and functional analyses also reveal multiple residues that are important for the binding of PLP, Sep and tRNA. Our work thus represents the first systematic functional analysis of conserved residues in archaeal SepCysSs, providing insights into the catalytic and substrate binding mechanisms of this poorly characterized enzyme.

Keywords

SepCysS; aminoacyl-tRNA; protein synthesis

1. Introduction

Aminoacyl-tRNAs (aa-tRNAs) are utilized as substrates for protein synthesis by the ribosome [1]. Proteinogenic amino acids are normally directly attached to tRNAs by aminoacyl-tRNA synthetases (aaRSs). However, four amino acids have been shown to lack corresponding aaRSs in certain organisms, and are thus synthesized on tRNAs via indirect pathways [2]. For example, no cognate aaRS has been found to attach selenocysteine (Sec) to tRNAs, and certain methanogenic archaea lack cysteinyl-tRNA synthetase [3,4]. In bacteria, Sec-tRNA^{Sec} is converted from Ser-tRNA^{Sec} (formed by seryl-tRNA synthetase) by selenocysteine synthase, while in archaea and eukaryotes, *O*-phosphoseryl-tRNA kinase (PSTK) first phosphorylates Ser-tRNA^{Sec} to form Sep-tRNA^{Sec} [5], which is then modified

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to Sec-tRNA^{Sec} by Sep-tRNA:Sec-tRNA synthase (SepSecS) [6,7]. In methanogenic archaea, Cys-tRNA^{Cys} is transformed by Sep-tRNA:Cys-tRNA synthase (SepCysS) from Sep-tRNA^{Cys}, which is formed by phosphoseryl-tRNA synthetase (SepRS) [3].

SepCysS is a pyridoxal phosphate (PLP)-dependent enzyme that uses an unknown sulfur donor to convert Sep-tRNA^{Cys} to Cys-tRNA^{Cys}. The crystal structure of *Archaeoglobus fulgidus* SepCysS reveals that PLP is deeply bound at the active site near the dimer interface, and is covalently linked to Lys209 [8]. In addition, a sulfate ion is also bound at the active site, possibly mimicking the phosphate group of Sep attached to tRNA^{Cys}. Functional analysis of SepCysS has been limited by the lack of a proper sulfur donor *in vitro*. Although it has been shown that sulfide, thiophosphate and cysteine can all be used as sulfur donors by purified SepCysS, the catalytic efficiencies using such sulfur donors are extremely low [9]. Further, *in vitro* purification and activity measurement of SepCysS requires stringent anaerobic conditions, making it inconvenient to employ enzymatic assays to study the function of SepCysS. Recently, an *in vivo* system was established to test SepCysS activity [10]. In this assay, SepCysS converts Sep-tRNA^{Sec} to Cys-tRNA^{Sec} in *E. coli*, resulting in cysteine incorporation at selenocysteine codons. This restores activity to formate dehydrogenase H (FDH_H), an enzyme that requires a selenocysteine or cysteine in the active site. In this study, we combined mutational, functional, and structural modeling approaches to identify critical SepCysS residues, which is essential for understanding the substrate recognition and catalytic mechanisms of SepCysS.

2. Materials and methods

Clones and strains

Methanocaldococcus jannaschii SepCysS and PSTK were constructed into pET15b and pACYC, respectively, as previously described [6,10]. Construction of double deletion strain Δ*SelA* Δ*SelD* (MH1) was previously described [10]. *M. jannaschii* SepCysS mutants were obtained using site-directed mutagenesis (Agilent) and verified by DNA sequencing.

Complementation assay

The benzyl viologen assay was performed as previous reported [10]. Strains carrying SepCysS variants or empty plasmids were grown overnight in Luria Broth (LB) with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. 1 mL LB agar supplemented with 0.05M sodium formate, 1 μM Na_2MoO_4, 10 μM isopropyl β-D-1-thiogalactopyranoside, 20 μg/mL ampicillin, and 5 μg/mL chloramphenicol, was allocated into each well of a 24-well plate. 5 μL of overnight culture were spotted at the center of each well and grown under anaerobic condition for 24–28 hours at 37 °C. Then 0.5 ml of 1% top agar with 1 mg/mL benzyl viologen, 0.25 M sodium formate, and 25 mM KH_2PO_4, was added on top of each well, and the purple color was developed under anaerobic condition for 1 hour. Plates were scanned and the color intensity was calculated using Adobe Photoshop (Adobe Systems Inc.).

Western blot of SepCysS variants

Strains carrying SepCysS variants or empty plasmids were grown in 5 ml LB for 16–18 hours. Cells were spun down, washed in 20 mM phosphate buffer pH 7.2 and lysed on ice

using BugBuster protein extraction reagent (Novagen). Cell lysates were run on 12% SDS-PAGE gels (Bio-Rad), transferred to PVDF membranes by semi-dry blotting. His-tagged proteins were detected using the Amersham ECLTM Western blotting system (RPN2108).

3. Results and discussion

3.1. Functional screening of conserved SepCysS residues

The crystal structure of *Af*SepCysS complexed with PLP has been solved at high resolution, yet the catalytic mechanism remains elusive due to the lack of functional studies [8]. To identify SepCysS residues that are critical for substrate binding or catalysis, we mutated 19 conserved residues located at the active site and the putative tRNA binding domain (Table 1, Fig. S1). The resulting mutants were tested in a benzyl viologen assay previously adapted to measure SepCysS activities *in vivo* [10]. Specifically, an *E. coli* Δ*selA* Δ*selD* strain, which was unable to synthesize Sec-tRNA^{Sec}, was transformed with vectors expressing *M. jannaschii* PSTK and His-tagged SepCysS. *Mj*PSTK recognizes *E. coli* tRNA^{Sec} and forms Sep-tRNA^{Sec}, which is then converted to Cys-tRNA^{Sec} by SepCysS. In the Δ*selA* Δ*selD* strain, Cys-tRNA^{Sec} restores the activity of a selenoprotein – FDH_H, turning cells to purple in the benzyl viologen assay. Although this assay also requires the activity of PSTK, the SepCysS mutations are not expected to affect the production of Sep-tRNA as no interaction is required between PSTK and SepCysS. Of the 18 SepCysS variants tested, 9 showed no activity and 2 showed significantly reduced activities compared to the wild-type (WT) SepCysS (Fig. 1 and Table 1). To confirm that the SepCysS variants are expressed as soluble proteins under experimental conditions, we performed Western Blot on the cell lysate using an anti-His antibody. The WT and mutant proteins were all well expressed and soluble (Fig. 2), suggesting that the mutations introduced to SepCysS do not significantly induce protein misfolding that leads to protein degradation or aggregation. As expected, no His_6-tagged SepCysS was detected in the negative control with an empty pET15b vector.

3.2. Critical residues involved in PLP binding

Conversion of Sep-tRNA to Cys-tRNA by SepCysS requires PLP as a cofactor. The structure of *Af*SepCysS [8] shows that PLP is covalently linked to Lys209 (equivalent to Lys234 in *Mj*SepCysS, *Af* numbering is used below) via a Schiff base with the ε-amino group (Fig. 3). A conservative mutation of Lys209 to arginine abolished the SepCys activity *in vivo* (Fig. 1 and Table 1), suggesting that arginine is unable to form a Schiff base with PLP. The phosphate group of PLP forms a hydrogen bond with His208, which interacts with the hydroxyl group of Ser206. The H208A and S206A SepCysS variants showed no activity in *E. coli*. It is likely that His208 positions PLP in an orientation favoring Schiff base formation with Lys209.

3.3. Role of Cys residues in sulfur donation

SepCysS contains three universally conserved cysteine residues at the active site: Cys39, Cys42, and Cys247. It has been proposed that one of these cysteines forms a persulfide intermediate that transfers a sulfur atom to the PLP-activated Sep-tRNA. Recent biochemical and mass spectrometric results suggest that the equivalent of Cys39 or Cys42 in *Mj*SepCysS carries a persulfide group (Liu *et al.*, manuscript submitted). Our data show that

each of the conservative mutations C39S, C42S, and C247S abolishes the SepCysS activity, suggesting that all three cysteines are critical for either catalysis or substrate binding. The region containing Cys39 and Cys 42 in the *Af*SepCysS structure is disordered, limiting us from modeling these two residues relative to the substrate. Given the proximity of Cys39 and Cys42 in the primary structure, it is likely that both Cys residues play a role in catalysis, thus affecting the k_{cat} value of tRNA-dependent Cys formation. The function of Cys247 remains unclear. One possibility is that Cys247 participates in the recruitment of an unknown sulfur donor; mutating Cys247 may thus affect the interaction between SepCysS and a protein sulfur donor and increase the K_m level for the donor.

3.4. Binding of Sep moiety

A sulfate ion is bound to the active site in the *Af*SepCysS structure, likely mimicking the binding of the phosphate group of the Sep moiety. Arg79, His103, and Tyr104 all form interactions with the sulfate ion (Fig. 3). The R79A and H103A variants of SepCysS showed no activity, while the activity of Y104A reduced significantly compared with the WT. During the catalysis, the phosphate groups of PLP and Sep are simultaneously bound to the active site, accumulating substantial negative charges. The positively charged Arg79 is thus critical for neutralizing the negative charges of Sep. An unperturbed histidine side chain has a pKa of ~6.0, and is thus uncharged at neutral pH. Interestingly, the hydroxyl group of Ser105 interacts with His103, likely to increase the pKa of the imidazole ring and stabilize binding of the Sep moiety. Although we favor that Arg79, His103, and Tyr104 are required for Sep binding, we do not exclude the possibility that these residues might be involved in the binding of protein sulfur donors.

3.5. Model for tRNA binding

SepCysS recognizes three $tRNA^{Cys}$ isoacceptors and a distinct *E. coli* $tRNA^{Sec}$, suggesting high plasticity in tRNA binding. To better understand substrate recognition, we constructed a docking model using the crystal structures of *Af*SepCysS and human SepSecS:$tRNA^{Sec}$ complex. SepCysS and SepSecS are both Fold Type I PLP-dependent enzymes, and share similar folding in tertiary structures. The docking model suggests that the 3′-CCA end, the discriminator base, and the acceptor stem of $tRNA^{Sec}$ form contacts with SepCysS, while the anticodon stem-loop and the long variable arm are distant from the enzyme. In line with this docking model, mutating Arg345 (close to the 3′-end of the tRNA) to alanine significantly decreased *in vivo* SepCysS activity (Table 1). It is thus likely that the major tRNA identity elements for SepCysS reside in the phosphate group of Sep and the acceptor stem. SepCysS has been shown to form a binary complex with SepRS, which improves its affinity for Sep-$tRNA^{Cys}$ [11]. SepRS recognizes the discriminator base and the anticodon loop of $tRNA^{Cys}$ [12], and thus likely discriminates against $tRNA^{Sec}$. Nevertheless, the *E. coli* system we utilized does not contain SepRS, allowing $tRNA^{Sec}$ to be effectively used as a substrate by SepCysS.

3.6. Concluding remarks and future prospects

Our mutational and functional analyses in this work have revealed 11 SepCysS residues critical for catalysis and substrate binding, which is an important step for understanding the

mechanism of tRNA-dependent Cys formation. Because the physiological sulfur donor remains unknown, current *in vitro* assays are not suitable for measuring the kinetics of SepCysS. Under our *in vitro* assay conditions, we are not able to detect Cys-tRNA formation by WT *Mj*SepCysS using sodium sulfide, thiophosphate or cysteine as substrates (Fig. S2). To further dissect the roles of these residues, an efficient *in vitro* approach using the correct sulfur donor needs to be developed. A co-crystal structure of SepCysS complexed with Sep-tRNA is also needed to elucidate how SepCysS recognizes the substrate. Such work is expected to provide new insights into the mechanisms of Fold Type I PLP-dependent enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Mutational and functional studies identified 11 residues critical for SepCysS activity.
- All three active site cysteines of SepCysS are essential for tRNA-dependent Cys formation.
- The phosphate group of Sep is recognized by Arg79, His103 and Tyr104.

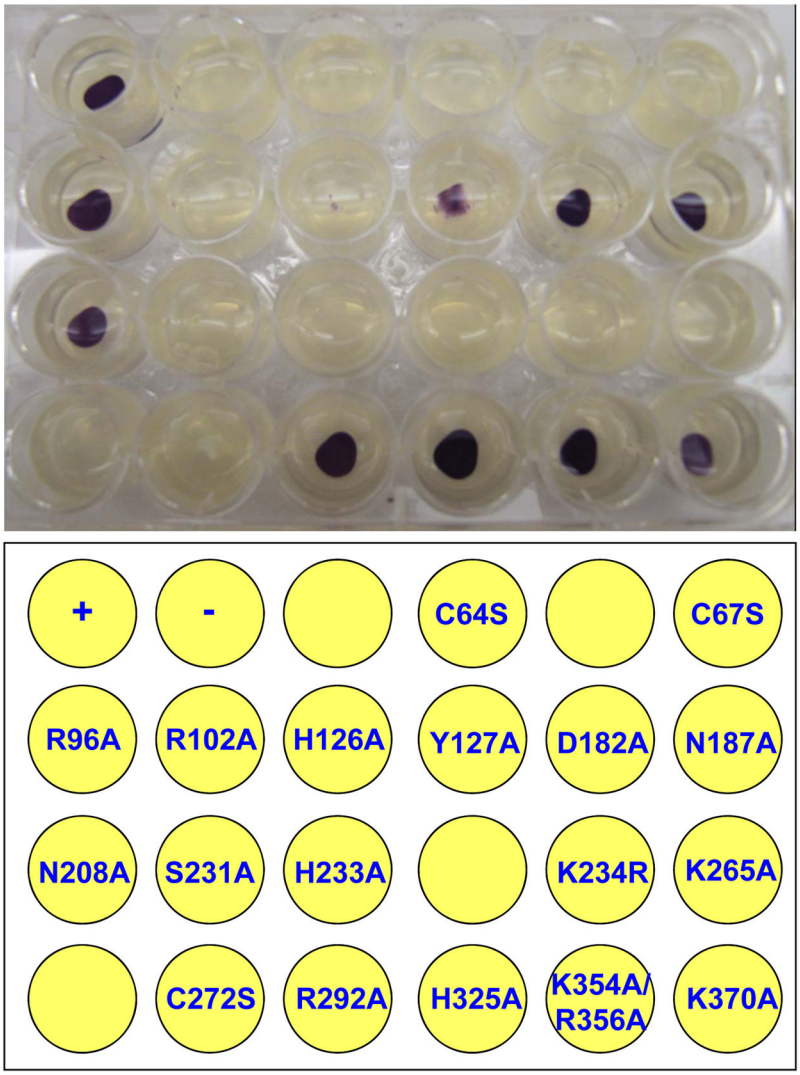

Figure 1. Benzyl viologen assay of *M. jannaschii* SepCysS variants

The purple color indicates SepCysS activity in *E. coli*. +, WT SepCysS; −, no SepCysS. The experiment was repeated 3 times with a representative figure shown here.

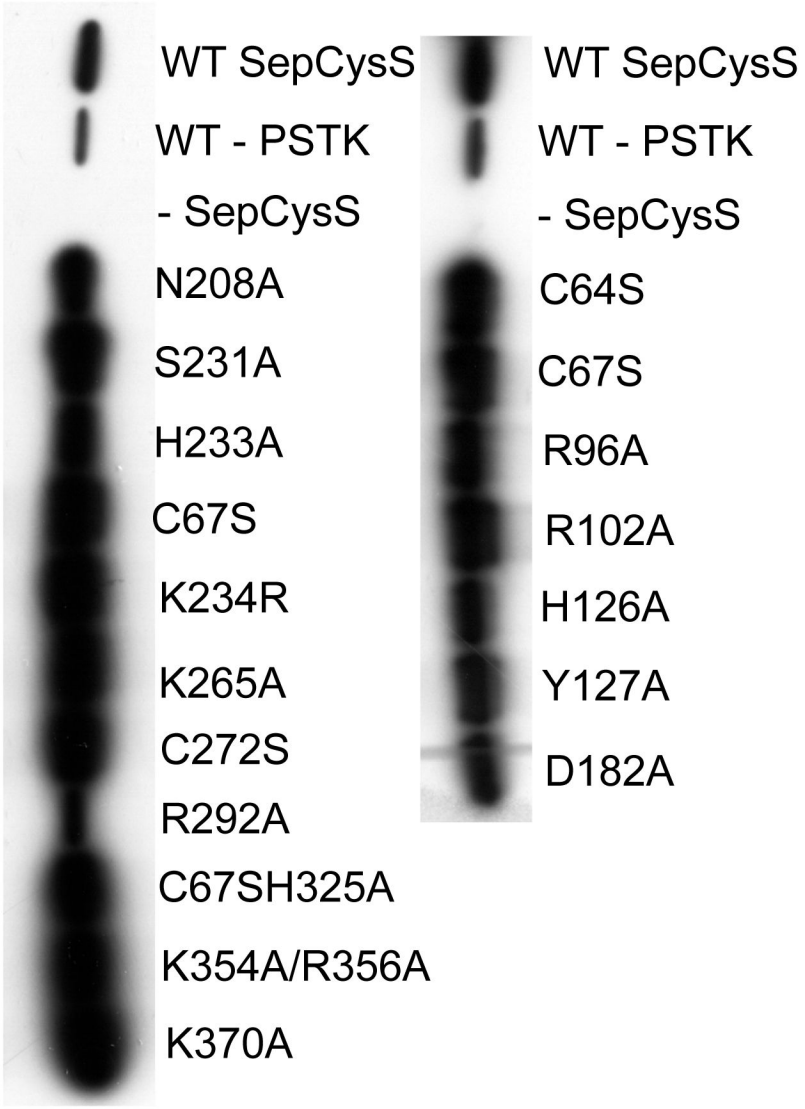

Figure 2. Western blot analysis of SepCysS variants

E. coli strain MH1 expressing His-tagged SepCysS variants were lysed, and SepCysS was detected with anti-His antibody.

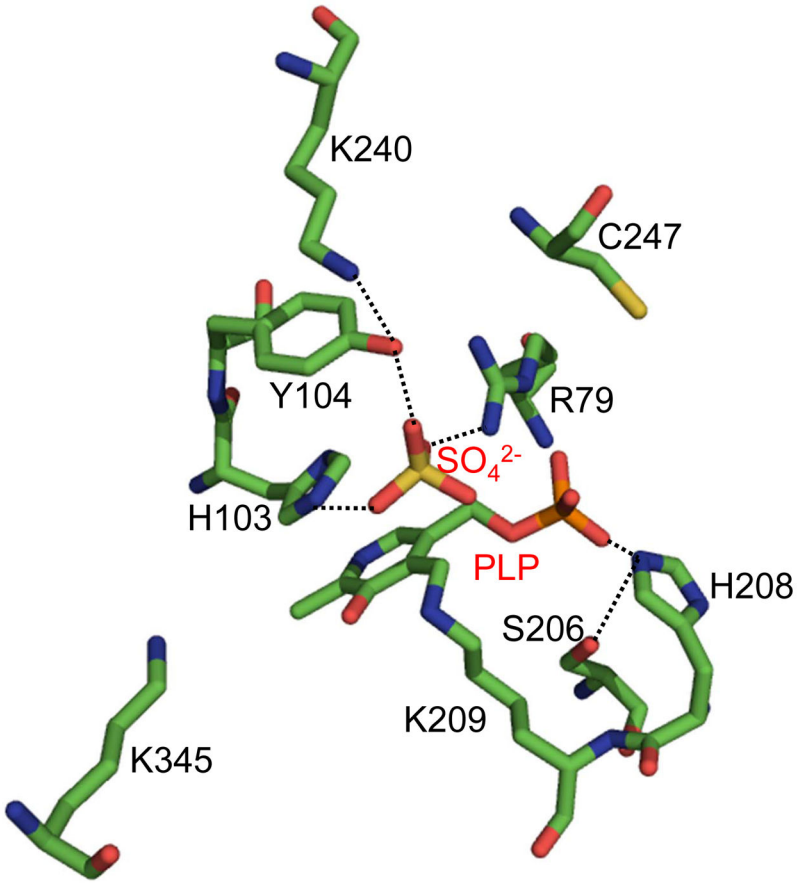

Figure 3. Residues critical for SepCysS activity identified using *in vivo* screening

The crystal structure of *Af*SepCysS (PDB: 2E7J) reveals that PLP is covalently linked to Lys209, and three residues coordinates a sulfate ion at the active site [8]. Our functional assay showed that Arg79, His103 and Tyr104 are all important for SepCysS activity, suggesting that these residues may interact with the phosphate group of Sep-tRNA, which is mimicked by the sulfate ion in the crystal structure. The region containing two critical residues Cys39 and Cys42 is disordered in the structure.

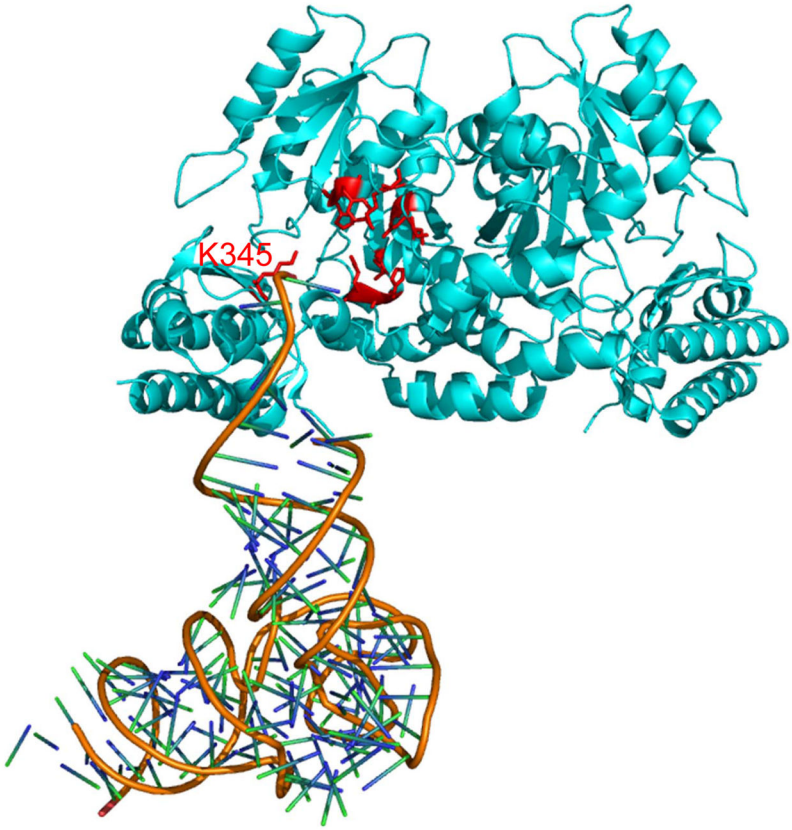

Figure 4. Binding model of tRNA to SepCysS

The crystal structure of *Af*SepCysS (PDB: 2E7J [8]) was superimposed onto the structure of human SepSecS complexed with $tRNA^{Sec}$ (PDB: 3HL2 [13]) using PylMOL (Molecular Graphics System, Version 1.0, Schrödinger, LLC). Residue Lys345 is close to the phosphate backbone of the tRNA 3′-end and is critical for the activity of SepCysS. The Sep moiety and A76 of the tRNA are missing in the structure.

Table 1

Activities of *M. jannaschii* SepCysS variants in *E. coli*

***Mj* SepCysS**	***Af* numbering**	**Relative activity**	***Mj* SepCysS**	***Af* numbering**	**Activity**
WT		100 ± 0	S231A	S206	0 ± 0
C64S	C39	0 ± 0	H233A	H208	0 ± 0
C67S	C42	0 ± 0	K234R	K209	0 ± 0
R96A	R73	89 ± 27	K265A	K240	0 ± 0
R102A	R79	0 ± 0	C272S	C247	0 ± 0
H126A	H103	0 ± 0	R292A	R267	99 ± 51
Y127A	Y104	35 ± 17	H325A	H300	124 ± 28
D182A	D157	107 ± 22	K354A/R356A	K329/R331	92 ± 47
N187A	N162	85 ± 17	K370A	K345	33 ± 22
N208A	N183	101 ± 24			

SepCysS activities were determined using a benzyl viologen assay. Relative activities were assigned to SepCysS variants based on the color intensity of three repeats with standard deviations indicated.