Examining the function of the residue C207 in the cysteine desulfurase reaction of an essential NifS-like protein from Synechocystis PCC6803

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I. Abstract

Biochemical analysis showed the NifS protein is essential to the production of many metalloenzymes, specifically iron-sulfur clusters. Slr0077 is thought to play a vital role in the shuttling of sulfur, through a two-step persulfide cleavage involving several key sites elucidated by comparative analysis of the crystal structures of the WT versus variants of the following genes: Δ 0077, Δ 0387, Δ0704. It has been shown that the essential Δ0077 mutant contains a unique site – C207. A site directed variant has been made to analyze its role in enzymatic activity. Preliminary characterization using steady state analysis showed a complex behavior of C207S toward cysteine and DTT. C207S binds L-cysteine generating Cys aldimine/Cys ketimine intermediate state and the spectrum slowly returns to that of the native enzyme. Steady state analysis of WT CD-0077 does not show substrate inhibition under the conditions tested. Preliminary data shows that C207S, on the other hand, exhibits a very different behavior, indicating that this residue might contribute to the cysteine desulfurase activity of WT CD-077. Further experiments will investigate the specific role of C207S in the formation and cleavage of the persulfide.

II. Introduction

Cysteine desulfurases were first discovered by Zheng et. al[1]. These enzymes were found to be involved in incorporating sulfur into iron-sulfur (Fe-S) clusters and other biologically important compounds (***Figure 1***). Homologs of these proteins exist both in prokaryotes and eukaryotes, some of which have been characterized.

The cyanobacterium *Synechocystis* sp. PCC 6803 encodes three NifS-like proteins: Slr0077, Slr0387, and Sll0704. Genetic experiments, performed by the Bollinger lab group and others, have shown that both *slr0387* and *sll0704* can be inactivated without altering the growth of the cell; whereas, only merodiploids can be obtained when attempting to inactivate *slr0077*, implying the essentiality of Slr0077.

Crystallographic studies, performed in conjunction with colleagues from Dennan Lab at MIT, have allowed for the identification and study of the active site of Slr0077. The active site has the cofactor pyridoxal-5'-phosphate (PLP) surrounded by the following residues: H128, K 231C207, K231, H370, and C372. Sequence alignments with characterized cysteine desulfurases suggested that C372 was the nucleophile that generates the persulfide intermediate.

This work will focus on investigating a site-directed variant protein – C207S. It should be noted that the C207 position was selected because of its' uniqueness, having only been identified in Slr0077. Over-expression, purification, and characterization have been performed and reported in this study. The preliminary results suggest C207S might indeed have, as yet, an unidentified role in cysteine desulfurase activity.

III. Materials and Methods

L-cysteine was purchased from Sigma. Tris hydrochloride, magnesium chloride, and glycerol were also obtained from Sigma. All restriction enzymes were purchased from New England Biolabs (*EcoR1* and *Nde1*). BL21 (DE3) Star cells were purchased from Invitrogen.

B. Overexpression of C207S:

C207S plasmid was transformed into BL21 (DE3) Star cells. Starter cultures were started using individual colonies that were then used to inoculate 100mL LB Rich media with appropriate antibiotic, Ampilicillin (10μL of 150mg/mL stock). Cultures were grown in LB Rich broth (3.5% tryptone, 2.0% yeast extract, and 0.5% sodium chloride) containing 150mg/L ampicillin until an OD_{600} of 0.5-0.8, and then induced by addition of 200μM IPTG overnight (20-24 hours) at 16-20^{o}C. Cells were spun down (6000rpm for 15 minutes) and stored in liquid nitrogen until further use.

C. Protein Purification of C207S:

Cells were resuspended in 5mL/g of 50mL Tris/HCl buffer (pH=7.8) with 0.005% PMSF (protease inhibitor) and allowed to thaw on ice. To lyse, we then French Press cells at 16,000psi and cellular debris was collected via centrifugation (16,000g for 15 minutes). A final centrifugation (12,000g for 10 minutes) yielded a protein rich supernatant, which was pelleted after ammonium sulfate precipitation. The pellet was resuspened in 50mL Tris/HCl buffer (pH=7.8) and transferred to a MWCO dialysis bag of 12-14 kDa and dialyzed for 4-6 hours. Final concentration of C207S protein was calculated based on final volume of solution in the dialysis bag.

MonoQ column (dimensions 10/10 1cm x 10cm, 8mL total volume) was equilibrated with 50mL Tris/HCl buffer (pH=7.8) and 4mL of protein solution was eluted at a time using FPLC. Fractions were collected and analyzed for (protein absorbance) by scanning from 240-600nm, specifically looking for absorbances at 280nm (protein peak) and 425nm (PLP cofactor peak).

D. Spectral analysis of the C207S upon addition of L-cysteine:

Varying concentrations of L-cysteine were added to known concentrations of C207S and the complexing ability was analyzed. Spectral data were collected and recorded using the HP8453 spectrophotometer as a function of absorbance versus wavelength (***Figure 2***). Using this data, the rate (v/Et) was determined and plotted as a function versus cysteine and DTT concentration (***Figure 3***).

E. Assay to Measure Cysteine Desulfurase Activity:

An assay was developed in order to quantify sulfide production by the action of cysteine desulfurase via the radioactive isotope S-35* incorporated into the cysteine substrate ([^{35}S]-cysteine).

Using 500µL Sodium Hydroxide (NaOH) in a cylindrical tube, a reaction mix (250mM cys, 100mM Hepes buffer – pH=7.8) is combined in a separate cup with varied concentrations of DTT (0.05,0.1, 0.5, 1, 2, 4, 5, 10, 50, 100mM) and the appropriate enzyme (C207S). The enzymes is allowed to react for 1 minute, after which, the reaction is quenched with 0.72N sulfuric acid (H_2SO_4) thereby halting any further enzymatic activity. The reaction chambers are left for approximately 1 hour with gentle shaking in order for the H_2S gas, produced as a result of enzyme activity, to react with the NaOH in the collection tube by the reaction. The apparatus is pictured in ***Figure 4***.

$$\mathbf{H_2S + NaOH \rightarrow NaS^* + H_2O}$$

A Scintillation counter was then used to quantify the ^{35}S present in unreacted substrate and resultant product in each of the two chambers: reaction chamber (unreacted sulfur substrate) and collection chamber (reacted sulfur product. Using these numbers, the enzyme's reaction rate is calculated. Reaction rates, variant versus wild type, are compared to determine role, if any, of C207 in cysteine desulfurase activity.

IV. Results

A. Expression and purification of C207S:

Over-expression of C207S was achieved using the BL21 (DE3) Star cells. The protein was purified by a series of three columns: Q-Sepharose, Mono Q, and finally Superose-12. The protein purification was monitored throughout, via SDS gel electrophoresis (***Figure 5***). The C207S variant protein appears as a 46.8KD protein band on SDS-PAGE gel. Approximately 3 mg of pure C207S was obtained per gram of cells, after the final column purification.

B. PLP-Binding analysis:

Pyridoxal-5'-phosphate (PLP) cofactor has a characteristic bright yellow color that allowed for easy identification of our protein throughout the purification process. Spectral analysis of C207S showed two main peaks: a protein absorption maxima at 280nm and the PLP cofactor absorption maxima at 425nm (***Figure 6***).

C. Spectral changes associated with addition of L-cysteine to C207S:

The internal aldimine of the C207S absorbs at 425nm, while the spectral peak at 342nm indicates the complex formed upon addition of the substrate, L-cysteine, which results in the formation of a cysteine ketimine intermediate. It should also be noted a dramatic decrease in aldimine is observed initially upon addition of L-cysteine but the peak slowly returns to native state. Addition of stoichiometric amounts of L-cysteine results in the formation and eventual decay of the complex noted at 342nm concomitant with reduction of subsequent increase in the 425nm peak. Owing to this complex behavior determination of Kd for cysteine was not possible.

D. Steady State behavior of C207S:

WT CD-0077 does not demonstrate typical steady-state kinetic behavior. Preliminary data with C207S shows that this variant shows a typical steady state behavior pattern with varying cysteine concentration and DTT concentration. C207S has a turnover number of $5min^{-1}$ with cysteine as substrate and $3.5min^{-1}$ with DTT as substrate.

V. Discussion

The protein analyzed within this study is a member of the NifS family of proteins that is involved in sulfur metabolism. This supports the hypothesis that the enzyme complex serves mainly as a sulfur shuttle, providing necessary sulfur to be transferred from the dietary amino acid cysteine to other essential molecules, such as: biotin, lipoic acid, molybpterin, 4-uridine, and iron-sulfur clusters (***Figure 1***).

WT CD-0077, as mentioned earlier, does not demonstrate typical steady state kinetics. We propose that the reductant performs the characteristic C-S bond cleavage (in the cys-ketimine intermediate) at higher cysteine concentrations. It appears in the C207S however, that the cys-ketimine becomes inaccessible to the reductant.

The role of C207S is still unclear. However, the results argue that the residue might have an important contribution towards the altered mechanism in WT CD-0077 setting it apart from other characterized cysteine desulfurases. Further investigations addressing the role of C207 in cysteine desulfurase activity is being presently conducted.

VI. References

1.) Zheng L, White RH, Cash VL, Jack RF, Dean DR. Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. Proc Natl Acad Sci USA. 1993 Apr 1;90(7):2754-8.

2.) Zheng L, White RH, Cash VL, Dean DR. Mechanism for the desulfurization of L-cysteine catalyzed by the nifS gene product. Biochemistry. 1994 Apr 19;33(15):4714-20.

3.) Flint DH. Escherichia coli contains a protein that is homologous in function and N-terminal sequence to the protein encoded by the nifS gene of the Azotobacter vinelandii and that can participate in the synthesis of the Fe-S cluster of dihydroxy-acid dehydratase.

4.) Jaschkowitz K, Seidler A. Role of a NifS-like protein from the cyanobacterium Synechocystis PCC 6803 in the maturation of FeS proteins. Biochemistry. 2000 Mar 28;39(12):3416-23.

5.) Mihara H, Kurihara T, Yoshimura T, Esaki N. Kinetic and maturational studies of three NifS homologs from Escherichia coli: mechanistic difference between L-cysteine desulfurase and L-selenocysteine lyase reactions. J Biochem (Tokyo). 2000 Apr;127(4):559-67.

6.) Urbina HD, Silberg JJ, Hoff KG, Vickery LE. Transfer of sulfur from IscS to IscU during Fe/S cluster assembly. J Biol Chem. 2001 Nov 30’276(48)44521-6.

Figure 1:

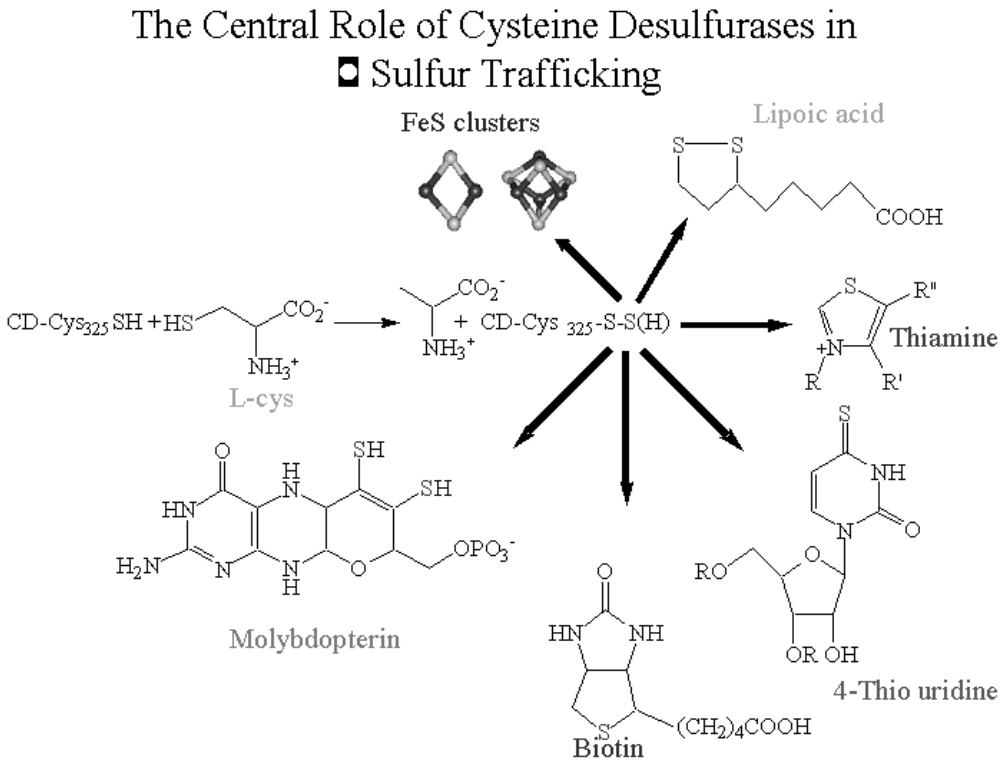


Figure 1 – This figure illustrates the sulfur trafficking in the body. This relates the possible functions of the Nif-like enzymes, defining their significance in the body.

Figure 2:

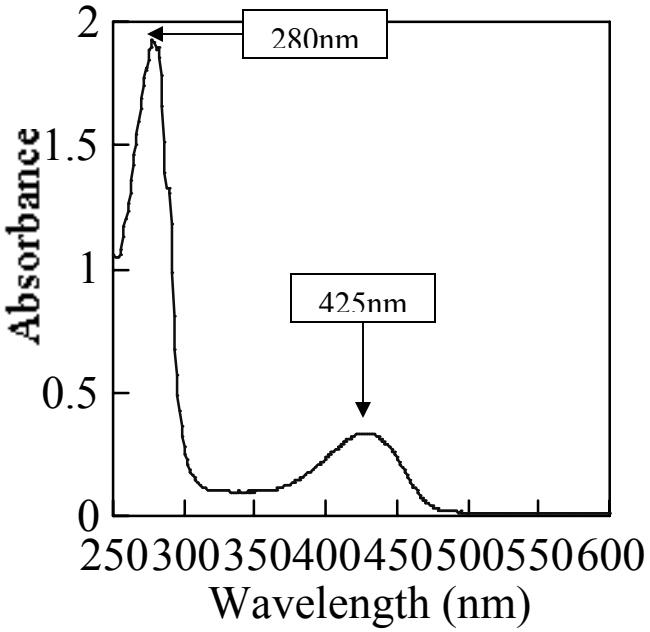
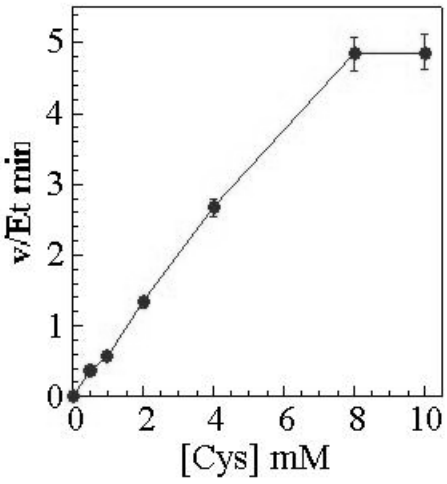


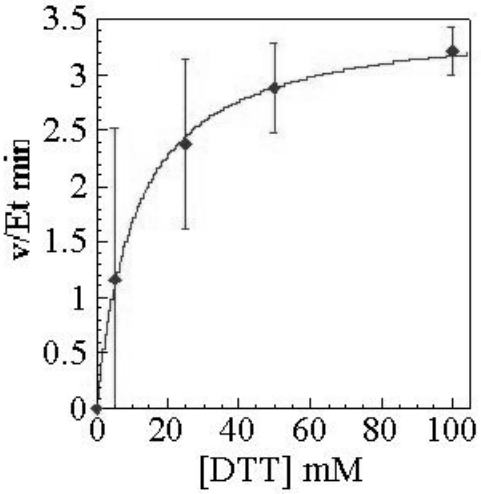
Figure 2 – This figure illustrates the purification of the C207S protein. Notice the protein peak (~280nm) and the PLP cofactor peak (~425nm).

Figure 3:

Dependence of Cysteine desulfurase Activity of C207S on Varying Concentrations of Cysteine and DTT



This figures illustrates the cysteine desulfurase activity with a maximum turnover number of 5/minute.



This figure illustrates the dependence of cysteine desulfurase activity of C207S variant on varying DTT concentrations. DTT (dithiothreitol) acts as a reductant that cleaves the persulfide.

Figure 4:

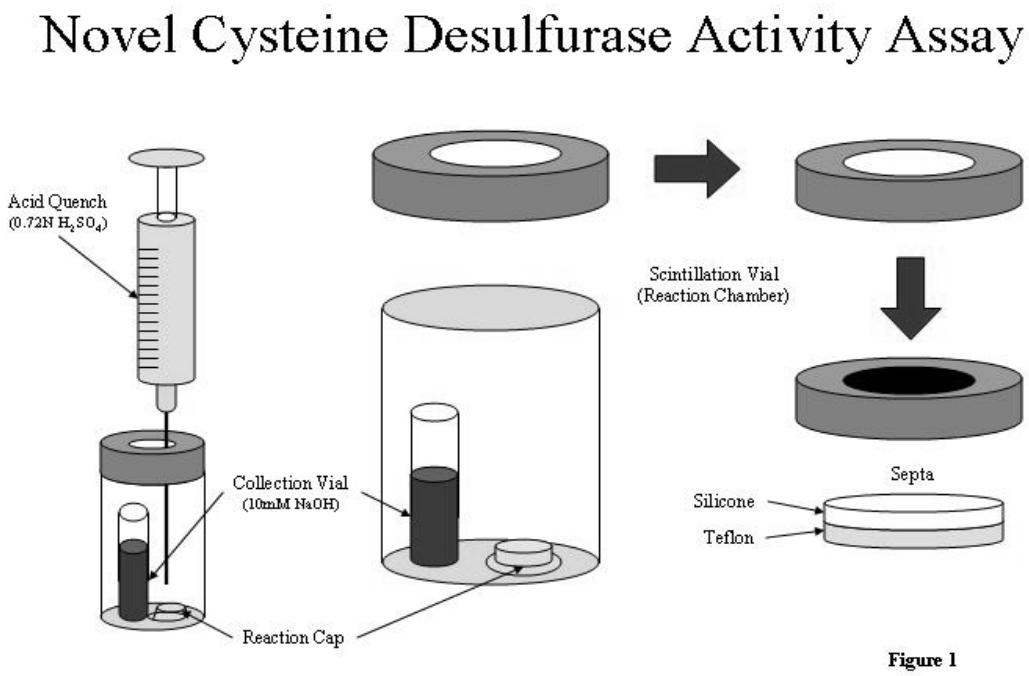


Figure 4 – This figure illustrates the novel cysteine assay used to monitor the desulfurase reaction and activity.

Figure 5:

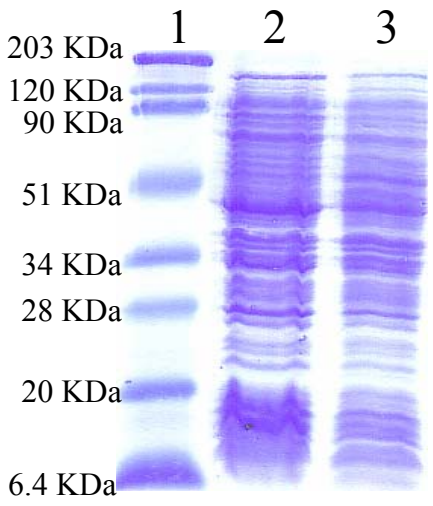
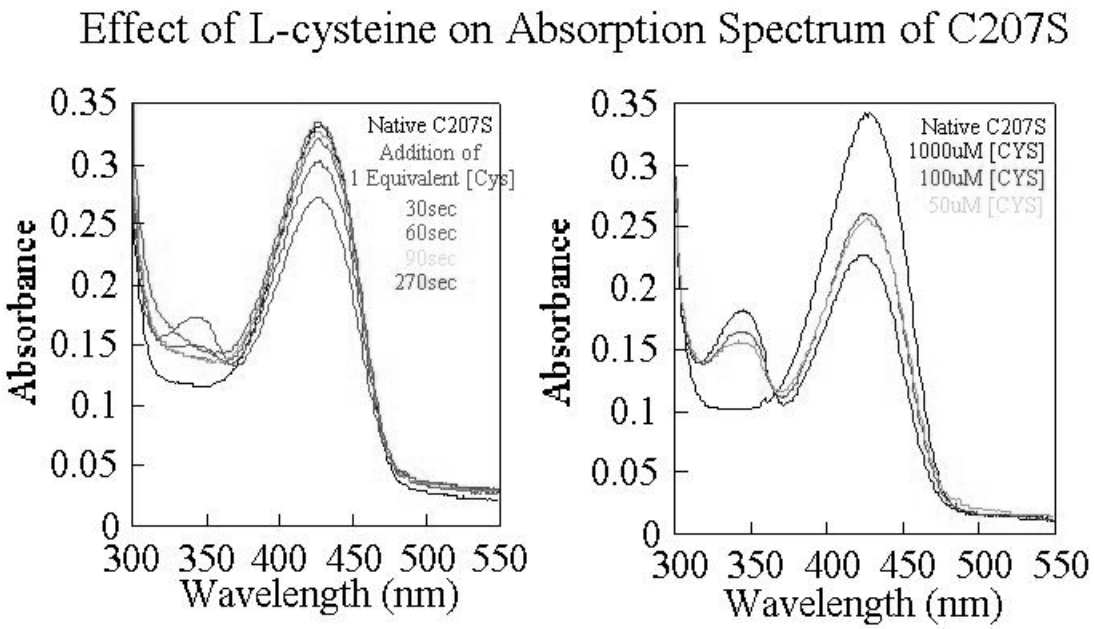


Figure 5 – SDS PAGE illustrates induction. The molecular weight marker (1), the post-induced (2), and the pre-induced (3) samples allow the comparison and identification of the C207S protein (~46.8 KDa).

Figure 6:



• Spectral changes with time upon addition of one equivalent L-cysteine to C207S.

• Spectral Changes upon addition of increasing concentrations of L-cysteine.

Figure 7:

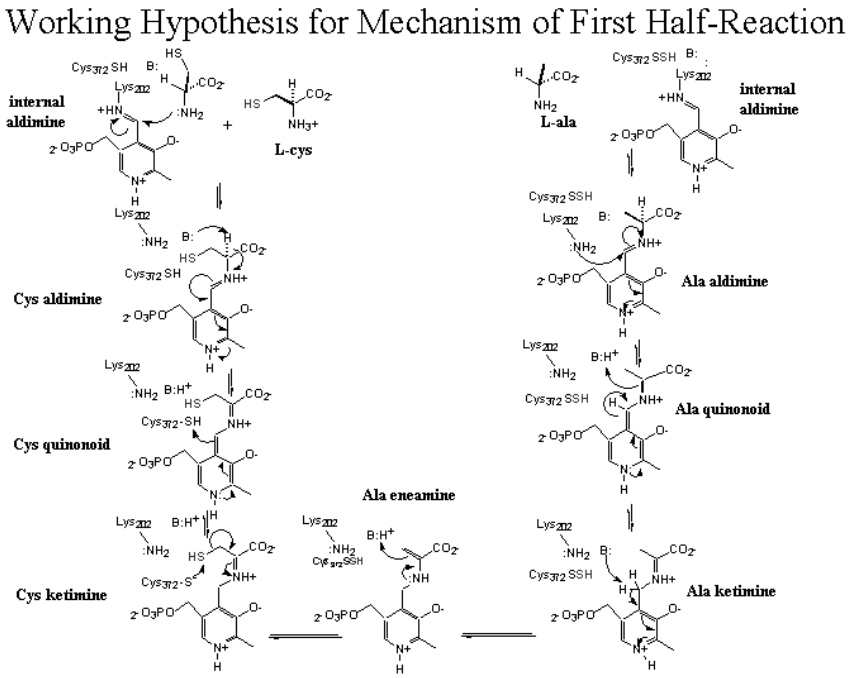


Figure 8 – This figure illustrates the proposed chemical reaction of cysteine desulfurase activity thought to be similar, or identical, to the pathway of the NifS mechanism.