PURIFICATION AND CHARACTERIZATION OF A NOVEL SELENOCYSTEINE LYASE FROM *ENTEROCOCCUS FAECALIS*

by

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ABSTRACT

A previous study identified *Enterococcus faecalis* as one of two bacteria known to have the *selD* gene and other selenium related genes without having the genes necessary to make selenocysteine or selenouridine. EF2570, a gene in the cluster, was later shown to be upregulated during biofilm formation and also responsible for a selenite- and molybdate-dependent increase in biofilm formation *in vitro*. The protein encoded was identified as a selenium dependent molybdenum hydroxylase (SDMH), enzymes that contain a labile selenium atom required for activity. While the process of inserting selenocysteine into a protein is well known, the process by which a SDMH acquires a labile selenium atom has not yet been described. To begin unraveling this pathway, the *nifS*-like EF2568 from the gene cluster will be characterized. Some NifS-like proteins have been shown to have selenocysteine lyase activity, providing a source of selenium for selenophosphate synthetase, the *selD* gene product. Study of EF2568 has shown that it specifically reacts with L-selenocysteine to form selenide and alanine with L-cysteine inhibiting the reaction. Guided by homology to the well-characterized human and *E. coli* NifS-like proteins, mutants of the active site and substrate discerning residues were also characterized for activity with L-selenocysteine and L-cysteine. While mutation of the residue at position 112 thought to be responsible for substrate specificity did not affect reactivity of the enzyme with L-cysteine, it did affect reactivity with L-selenocysteine. Studying the characteristics of this novel group II selenocysteine lyase will provide a foundation for studying the remaining pathway.

I would like to dedicate this thesis to my mother, Lori Black. Her sacrifices paved the way for my success.

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INTRODUCTION

Enterococcus faecalis, a normal inhabitant of the intestine and female genital tract, is the most common cause of enterococci infections. It is a versatile opportunistic pathogen, with a proven ability to cause endocarditis, periodontal disease, and urinary tract infection. In addition, it's ability to adhere to medical devices, such as catheters, and form treatment-resistant biofilms makes it a difficult nosocomial pathogen[1,2]. While the ability of clinical isolates to form biofilms can greatly vary depending on the location of the study and infection site, the prevalence of virulence factors from *E. faecalis* involved in adherence and biofilm formation underscores the importance of these processes to the pathogenesis of *E. faecalis*[1,2]. A RIVET (recombinase *in-vivo* expression technology) screen to find genes related to biofilm formation identified EF2570, a putative xanthine dehydrogenase, as a gene expressed during that transition rather than in the planktonic state[3]. An earlier unrelated paper had also identified EF2570 as belonging to a cluster of genes that were putatively related to selenium metabolism. Interestingly, in this cluster, *E. faecalis* encoded selenophosphate synthetase, SelD, but did not encode genes necessary to incorporate selenium into selenocysteine or selenouridine, the two well-defined pathways for selenium utilization in biology[4]. The presence of *selD* and other putative selenium related genes led to the conclusion that *E. faecalis* had a third uncharacterized use for selenium[5]. Radioactive labeling of *E. faecalis* with ^{75}Se confirmed this hypothesis, with EF2570 being identified as the protein that incorporated a labile selenium atom[6]. In addition, selenium, molybdate, and uric acid supplementation caused an EF2570-dependent increase in biofilm formation. Ultimately, EF2570 was identified as a xanthine dehydrogenase and a selenium dependent molybdenum hydroxylase[6].

Molybdenum hydroxylases are molybdenum-dependent enzymes that take advantage of the multiple oxidation states of molybdenum to catalyze the exchange of oxygen between their substrate and water[7,8]. Molybdate is the most common form of molybdenum found in the environment and it must be incorporated into a pterin-type cofactor to be biologically active[9]. The only known exception is the cofactor of the nitrogenase enzyme, which incorporates molybdenum as a FeMo cofactor, coordinated by an iron-sulfur cluster[7]. The pterin-type cofactor is synthesized in four steps in which GTP is transformed into a molybdopterin molecule, sulfur atoms are exchanged for the oxygen moieties, the molecule is adenylated, and finally, the molybdenum atom is inserted[7]. The finished cofactor, known as Moco, has a molybdenum atom coordinated by the enedithiolate of the pterin molecule, a hydroxyl group, and two terminal oxygen atoms[7]. At this point, Moco can be inserted into enzymes by chaperones or, alternatively, enzymes like MobA can further process the cofactor[10]. Sulfuration of one of the terminal oxygen atoms of Moco yields the final form of the cofactor required by the xanthine oxidase family of enzymes. This family has been best studied through two the enzymes xanthine dehydrogenase and aldehyde oxidase[9,11]. The process of sulfuration has been best characterized in *Rhodobacter capsulatus*. The protein XdhC binds with and inhibits the activity of MobA, competing with it for completed Moco from MoeA[10]. XdhC is able to interact with NifS4, a cysteine desulfurase, which abstracts sulfur from L-cysteine, forming a persulfide bond which can be used to transfer a sulfur atom to Moco[12]. The sulfurated Moco is then transferred to apo-XDH, leading to a fully active enzyme[13]. Eukaryotes also contain the xanthine oxidase family of enzymes but the sulfuration process has not been as well studied. The Moco sulfurase genes were first discovered by the effects of mutant alleles such as those that caused classical type II xanthinuria in humans

or a brownish eye in drosophila, each indicating a loss of xanthine dehydrogenase activity[14–16]. The sulfuration protein in eukaryotes (HMCS in humans, ABA3 in Arabidopsis thaliana, hxB in Aspergillus nidulans, or Ma-L in Drosophila) consists of two distinct domains[14,17–19]. The N-terminal is a NifS-like cysteine desulfurase that has been shown to have the ability to react with L-cysteine, forming a persulfide bond with the abstracted sulfur atom[18,19]. The C-terminal has homology to other sulfurase genes and is able to bind Moco in the unsulfurated and sulfurated forms[15,20]. The persulfide sulfur is passed to the Moco, most likely through an intermediate cysteine residue. It was hypothesized that the sulfuration process occurred post-translationally, in contrast to co-translationally in *R. capsulatus*, due to the finding that desulfo-XDH could be purified and regenerated to an active form with sulfide and dithionite[9]. Evidence to support the co-translational or post-translational sulfuration and insertion of Moco has not yet been shown. Out of the two possibilities that the sulfurase protein can transfer the sulfur atom or the sulfurated Moco, the latter seems more likely. Still, the cofactor is deeply buried in the protein, suggesting that a chaperone would be needed for inserting the cofactor[7]. As of yet, none have been predicted. Overall, while the processes are not described as being similar, both require a cysteine desulfurase to abstract a donor sulfur atom from L-cysteine and a scaffold for sulfuration of the Moco.

In some xanthine oxidase family enzymes, the terminal sulfur atom is replaced by selenium. Compared to sulfur, selenium is the better nucleophile. While the greater reactivity of selenide, selenite, and other selenium compounds makes them toxic when present in more than trace amounts, incorporation of selenium into enzymes can dramatically increase their activity in comparison to the analogous sulfur-containing enzymes[21–23]. For example, when formate

dehydrogenase was mutated to contain a cysteine in place of a selenocysteine, only 0.3% of the wild-type activity was present[24]. While the process of incorporating a selenocysteine residue into a protein has been well characterized, the placement of the selenium in the SDMH family of enzymes has not been studied. The better-known enzymes of this family, such as nicotinic acid hydroxylase from *Eubacterium barkeri* or purine hydroxylase from *Clostridium purinolyticum*, come from organisms that have not been sequenced, negating the possibility of bioinformatic study of genes that could shed light on the pathway[25,26]. In addition, many selenium utilizing species, such as the clostridia, are able to incorporate selenium into selenocysteine or selenouridine, complicating the identification of genes solely used to insert a selenium atom in Moco. Since *E. faecalis* only has the genes for this not yet characterized third pathway of selenium utilization, it is a viable alternative to study this pathway[4].

While *E. faecalis* has been well studied compared to other SDMH-containing organisms, the only clues to what roles the putative selenium-related genes may play come from homologies to other known genes. One of the proteins predicted to have a clear function besides EF2570, the xanthine dehydrogenase, and EF2567, the selenophosphate synthase, was EF2568. The predicted protein had clear NifS homology, suggesting that it had a role as a cysteine desulfurase (CSD) or a selenocysteine lyase (SCL)[4]. Both types of enzymes form homodimers, relying on a PLP cofactor and a conserved cysteine residue for activity. They can be classified into two groups depending on the consensus sequences at the active site. Group I has a SSGSACTS sequence while Group II has RXGHHCA at the active site. Broadly, group I has a role in iron-sulfur cluster formation while group II has not been as well characterized but has more varied roles in iron homeostasis, molybdenum cofactor synthesis, and other cell functions[27]. Interestingly, even

with the high degree of homology between CSDs and SCLs, substrate specificity varies with CSDs reacting with L-cysteine and L-selenocysteine while SCLs only react with L-selenocysteine. Intriguingly, SCLs are even inhibited by L-cysteine. The similar nature of these two types of enzymes and the similarity of the substrates has led to many studies of the basis for substrate specificity. Some of the most complete work involves the three *E. coli* homologs of NifS: IscS, CsdA, and CsdB. IscS and CsdA react more efficiently with L-cysteine while CsdB reacts about 300 times more efficiently with L-selenocysteine than it does with L-cysteine[28]. The crystal structure revealed that the active site lobe of CsdB was much shorter and less mobile than in a similar NifS from *Thermotoga maritime*, suggesting that subtle variations in the protein structures may distinguish specificity[27,29]. A study of the human SCL went further and mutated a residue near the active site, D146, to lysine, resulting in the enzyme gaining cysteine desulfurase activity[30]. The study showed that a single residue could determine substrate specificity in SCLs. The group hypothesized that the aspartate residue stabilized the protonated form of the cysteine active site residue, making reaction with L-cysteine less favorable[31].

Due to the difficulty of teasing out what determines substrate specificity for CSDs and SCLs, it is impossible to predict, based on homology, which substrates EF2568 will react with. The fact that the gene is putatively present in the selenium metabolism cluster would suggest that it is more likely to be a selenocysteine lyase[4]. The NifS commonality between sulfuration pathways suggests that a selenation pathway would also require a NifS-like protein like a selenocysteine lyase to provide the selenium atom. Determining the substrate specificity and kinetics of EF2568 will provide a basis for unraveling the novel use of selenium encoded by *E. faecalis*.

METHODS

Bacterial Strains and Constructs

Bacterial strains were maintained on Luria agar (10 g/L tryptone, 5 g/L sodium chloride, 5 g/L yeast extract, 15 g/L agarose) with added 50 mg/L chloramphenicol and 100 mg/L ampicillin when needed.

Cloning and Mutagenesis

The gene EF2568 was cloned from *Enterococcus faecalis* V583 using the Champion™ pET100 Directional TOPO® Expression kit. The resulting plasmid was named pRT1 and maintained and expressed in BL21 Star™ (DE3) *E. coli* cells. Alignment of EF2568 with other *nifS* genes directed selection of C341 (active site cysteine) and E112 (similar to discerning residue in the human *nifS*) for mutagenesis. The Quikchange® II XL Site-directed Mutagenesis kit was used to change the cysteine at position 341 to alanine and the glutamate residue at 112 to lysine and alanine. The cloning and mutagenesis was confirmed by sequencing.

Protein Expression and Purification

Large-scale expression of the wild-type protein and each mutant was completed using a fermenter. Forty liters of Luria broth was sterilized and cooled to 37°C before adding 100 mg/L ampicillin, 0.1 mM pyridoxine, and 500 mL of overnight culture. The culture was stirred at 200 rpm and aerated at a rate of 15 NL/min. The expression of the EF2568 gene product was induced with 0.5 mM of IPTG once the culture reached an optical density of 0.6. The temperature of the fermenter was lowered to 25°C and allowed to continue stirring overnight. The cells were

harvested using a Sharples continuous flow centrifuge. Cells were frozen in liquid nitrogen until lysis. Cells were resolubilized in lysis buffer (50 mM Tris/HCL pH 8.0, 0.1 mM benzamidine) at the ratio of 0.5 g/mL. The resuspended cells were passed through a French Press two times to completely lyse the samples. The resulting lysate was spun down to remove cellular debris before starting the purification process.

HisPur Cobalt Resin was used to capture the histidine tagged proteins from the lysate. The column was washed with 5 volume equivalents of 50 mM Tris/HCl, pH 8.0 with 5 mM imidazole. The target protein was eluted with 2 volume equivalents 50mM Tris/HCl, pH 8.0 with 250 mM imidazole. The column was regenerated with the 5 mM imidazole solution followed by a 1 M imidazole solution to remove any leftover protein. Column was stored in 20% ethanol between uses. A final gel filtration step was used to remove any remaining contaminants. The protein concentration was determined by Bradford assay[32]. SDS-PAGE was used to determine the purity of the target protein.

Lead Acetate Assay

The selenocysteine lyase activity of EF2568 and mutant proteins was determined using a lead acetate assay to detect the selenide released from cleavage of L-selenocysteine. L-selenocysteine for the reaction was generated from selenocystine using a 2 M NaOH solution. The assay was performed in triplicate with each sample containing 1 mM pyruvate, 0.5 mM dithiothreitol (DTT), 1 mM pyridoxal-5-phosphate (PLP), 50 mM Tricine/KOH, pH 7.5, 1.25 μg of enzyme, and varying amounts of L-selenocysteine in a final volume of 100 μL. The L-selenocysteine was added last at various time points to begin the reaction. A stopping solution of

5 mM lead acetate in 0.1 M HCl was added to stop the reaction. L-selenocysteine concentrations varying from 0.05 mM to 2.0 mM were used to determine the apparent K_m of the enzymes. The time points for the reaction were chosen to reliably measure the change in product formation for the amount of L-selenocysteine added. The formation of a lead selenide complex was measured at 400 nm. Inhibition of the reaction with L-cysteine was measured using the same method with the added amount of L-cysteine ranging from 0.005 mM to 0.5 mM. An extinction coefficient of 2.36×10^4 at 400 nm was used for lead selenide as determined previously[33].

Methylene Blue Assay

Cysteine desulfurase activity of EF2568 and its mutants was measured using a methylene blue assay. The reaction mix contained 4 mM DTT, 1 mM pyruvate, 1 mM PLP, 50 mM Tricine/KOH, pH 7.5, and varying amounts of L-cysteine in a final volume of 100 µL. L-Cysteine was added last at various time points to start the reaction. The reaction was stopped by adding 10 µL of 0.02 M N,N-diethyl-p-phenylenediamine sulfate (DPDS) in 7.2 M HCl and 10 µL of 0.03 M ferric chloride in 1.2 M HCl. The reaction was incubated at room temperature for 20 minutes before detecting methylene blue formation at 650 nm. The reagents were tested for activity by creating a standard curve with sodium sulfide.

Spectroscopy

An ultraviolet-visible spectrograph was obtained for the wild-type and mutant enzymes. 60 µL of enzyme was added to a microcuvette and scanned in a UV-visible spectrophotometer. 5 µL of 100 mM L-selenocysteine or L-cysteine was added to the enzyme. The mixture was

scanned over the next five minutes to record any changes in absorbance due to reaction with the added substrates.

RESULTS

Alignment of EF2568 with NifS-like proteins

Alignment of EF2568 with the NifS-like enzymes from *E. coli* and the human selenocysteine lyase demonstrated that EF2568 contained many of the conserved residues characteristic of a CSD or SCL (Figure 1). The active site cysteine was predicted to be at position 341 with the consensus sequence at the active site categorizing EF2568 as Group II. A PLP binding site was also present, signified by a consensus sequence and conserved lysine residue. All but one of the residues known to be in the active site of CsdB from *E. coli* were conserved in EF2568.

Purification of EF2568

The purification scheme of affinity chromatography followed by gel filtration was successful at purifying the wild-type protein to homogeneity based on visible inspection of the coomassie-stained protein gel (Figure 2A). EF2568 had an approximate weight of 43 kDa on the SDS-PAGE gel, which corresponds well to the predicted weight of 43 kDa. The eluted protein had a clear yellow color and the presence of PLP as confirmed by spectroscopy (data not shown).

Substrate Specificity of EF2568

Based on homology, EF2568 is a NifS-like protein that could have cysteine desulfurase activity or selenocysteine lyase activity. As stated previously, the location of the gene suggests that it would be a selenocysteine lyase. While all mammalian SCLs studied have been shown to be specific for L-selenocysteine, CsdB from *E. coli* is able to react with both substrates[28,30,33,34].

Therefore, the first step in characterizing this novel enzyme was identifying which substrates it would react with. The methylene blue assay was used to detect cysteine desulfurase activity. When sulfide is released, it reacts with the stopping reagents (DPDS and ferric chloride) to form methylene blue, which can then be detected with a spectrophotometer. Reaction of EF2568 with L-cysteine concentrations of up to 50 mM showed no sulfide release (Figure 3B). The concentration of L-cysteine in *E. coli* is normally about 200 μM. Therefore, if there isn't any activity with cysteine even at 50 mM, then under physiological conditions, EF2568 would not have cysteine desulfurase activity. Methylene blue formation was observed with sodium sulfide, used to build a standard curve, showing that the reagents were not responsible for the lack of activity observed. The lead acetate assay was used to detect selenocysteine lyase activity. The selenide released in the reaction binds with lead to form a compound that can be detected with a spectrophotometer. Preliminary testing with 0.5 mM to 1.0 mM L-selenocysteine showed an increase in absorbance over six minutes, signifying the release of selenide. The slope increased as the concentration of selenocysteine increased, signifying that the activity of the enzyme was greater in the presence of more substrate.

Study of the three *E. coli* NifS-like enzymes showed that these catalysts can undergo abortive transamination, eliminating the amine group rather than sulfur or selenide. This abortive reaction causes inactivation of the enzyme and formation of a PMP intermediate. The change in PLP was visualized by a UV-visible scan of the enzyme with the substrate, which showed a decrease in absorbance at 420 nm and an increase at 325 nm, corresponding to the formation of PMP[28]. Addition of pyruvate could reverse the abortive transamination, increasing the rate of the enzyme. Adding PLP to the reaction also increased the rate[28]. To determine if EF2568 underwent

the same process, UV-visible scans were completed with L-cysteine and L-selenocysteine. Over the course of five minutes, the decrease in PLP and increase in PMP was observed for both substrates (data not shown). With this knowledge, the methylene blue assay was repeated with PLP and pyruvate in case the abortive transamination had inactivated the enzyme before sulfide could be detected. The presence of pyruvate and PLP did not lead to sulfide release in subsequent assays, cementing that EF2568 does not have cysteine desulfurase activity (Figure 3B). Still, it has been noted in several studies that abortive transamination can cause the enzymes to exhibit non-Michaelis-Menten kinetics[28]. Therefore, PLP and pyruvate were added to the reaction mixtures for the remainder of the study.

pH Dependence of Enzyme Activity

The activity of EF2568 with L-selenocysteine was measured over a range of pH's from 5.5 to 11.0 in order to determine the pH optimum. The activity of the enzyme followed a bell-shaped curve with the highest catalytic activity being exhibited between the pH's of 8.5 and 9.0 (Figure 4A). Generally, cysteine has a pKa of 8.4, and at these pH's, the active site cysteine would most likely be deprotonated. The bell shaped curve is characteristic of an enzyme that requires both a protonated and deprotonated amino acid for catalysis, which is in agreement with the predicted mechanism[28]. For the determination of the kinetic parameters, a pH of 7.5 was chosen as it is a more physiological relevant pH than the pH optimum of the enzyme.

Michaelis-Menten Kinetics of EF2568

The kinetic parameters of EF2568's reaction with L-selenocysteine were determined over a range of 0.05 to 3 mM L-selenocysteine. Time points were also varied in order to give a linear

response over the wide range of substrate concentrations. Sigma Plot 12 was used to determine the kinetic parameters from the initial rate data (Figure 5). The K_m^{app} was found to be 1.01 mM while the V_{max}^{app} was 0.376 μmol/min/mg (Table 1). The K_{cat} was calculated to be 17.2 min^{-1}. Overall, from this information, the enzyme is not very efficient and most likely would not turn over very often under physiological conditions. L-Selenocysteine is synthesized on the tRNA and would only be freely available from protein degradation or from the environment. Given that *E. faecalis* does not contain selenoproteins with selenocysteine, the only source of L-selenocysteine would come from the environment. Therefore, the concentration of L-selenocysteine in the cell would be far below the K_m^{app} of the enzyme.

Inhibition of Selenocysteine Lyase Activity by L-Cysteine

As mentioned previously, the selenocysteine lyases that have been previously studied were all shown to be specific for L-selenocysteine. Still, L-cysteine can react with PLP to inactivate the enzyme, as shown by scans of the protein and substrate. Studies of the selenocysteine lyase activity in the presence of L-cysteine showed that it was able to act as a competitive inhibitor of the enzyme. To determine if L-cysteine acted as an inhibitor of EF2568, the activity with L-selenocysteine was measured in the presence of L-cysteine. The concentrations ranged from 0.005 mM to 0.5 mM. The ability of L-cysteine to inhibit the reaction was apparent immediately with longer time points having to be chosen in order for a significant change in selenide production to be measured. SigmaPlot 12 was used to fit the data to inhibition models and determine the type of inhibition (Figure 6). Competitive inhibition was chosen based on knowledge from previous studies and also the high R^2 value of 0.918. The K_i

was determined to be 10.3 μM. With such a low K_i, the enzyme would be constantly inhibited by the free L-cysteine in the cell, which is normally at a concentration of 200 μM[35]. Between the low amount of L-selenocysteine and high levels of L-cysteine, EF2568 would turn over very rarely. Still, the low rate of turnover is advantageous since selenide is highly reactive and only one protein has been shown to need selenium to date in this organism.

Generation and Purification of EF2568 Mutants

The finding that the specificity of the human selenocysteine lyase can be changed by mutating a single residue led to an attempt to change the specificity of EF2568 as well. The alignment showed that the aspartate residue at 146 was not conserved in EF2568. Searching for a residue with similar character led to the glutamate residue at position 112 in EF2568. The residue is in reasonable proximity to D146 based on the alignment and has the same characteristics as an acidic side chain. For the human protein, mutagenesis to lysine produced the change in specificity[30]. For EF2568, position 112 was mutated to both alanine and lysine to see the effects of an uncharged amino acid in the same position. In addition, the active site cysteine at position 341 was mutated to an alanine to confirm that it was indeed the active site cysteine. Mutagenesis was confirmed by sequencing. The three mutants were grown and purified in the same manner as the wild-type protein. The purification procedure was successful at purifying the mutants to homogeneity based on visible inspection of a Coomassie stained gel (Figure 2B). All three proteins were able to bind PLP effectively, evidenced by the yellow color of the purified protein. E112K had a lower abundance than the other two, suggesting that the protein may not be as stable as the other two. It also came off the affinity column with a more orange color. This may

have been due to leaching of the cobalt from the affinity column since the spectrum of the protein did not differ from that of the others (data not shown). Overall, the desired mutants were successfully generated and mutated.

Reaction of EF2568 Mutants with L-Cysteine and L-Selenocysteine

First, the reactivity of C341A was tested with L-selenocysteine. For other specific selenocysteine lyases, mutation of the active site cysteine to alanine causes a loss in activity. In contrast, study of the *E. coli* cysteine desulfurases showed that mutation of the same residue did not affect selenocysteine lyase activity[28,31]. Therefore, the importance of the active site cysteine in EF2568 was determined. Compared to the wild-type enzyme, C341A showed greatly reduced activity (Table 1). Still, some background activity was left that could not be accounted for by nonspecific reactions of L-selenocysteine with other components of the reaction mix. The low reactivity could be explained by nonspecific interaction with the DTT or excess L-selenocysteine aiding in the chances that it may spontaneously leave, giving a semblance of enzyme activity that may not be present *in vivo*. Overall, the activity of the enzyme was greatly reduced by mutation of the active site cysteine, emphasizing its importance in selenocysteine lyase enzymes.

The substrate specificity of E112A and E112K was tested. This residue was mutated with the hypothesis that mutation at this site would change the substrate specificity of EF2568. Therefore, E112A and E112K were tested for cysteine desulfurase activity with 50 mM L-cysteine in the presence of PLP and pyruvate (Figure 7). No sulfide production was tested for either enzyme signifying that the substrate specificity was not affected by mutation of this residue. Further research in the literature suggested that the residue at 146 is conserved as a

histidine in bacteria and therefore the wrong residue may have been mutated. Still, each mutant was tested with L-selenocysteine to determine if the mutation affected the kinetic parameters of the enzyme. Determination of the K_m^{app} showed that E112A had a lower K_m^{app} and a lower V_{max}^{app} but the catalytic efficiency of the enzyme still increased since the K_m^{app} decreased (Table 1). Similarly, E112K had a lower K_m^{app} and lower V_{max}^{app} as well, leading to an increase in catalytic efficiency. To determine if a change in the active site was responsible for the alteration in catalytic efficiency, the pH optimum of E112A was determined. Interestingly, the pH curve for E112A was similar to the wild-type enzyme (Figure 4B). The pH optimum was also in the range of 8.5 to 9.0 with E112A just exhibiting higher levels of at activity at a majority of the pH's tested. From this experiment, it was concluded that mutation of position 112 does not affect the active site but may affect another characteristic such as access to the active site.

DISCUSSION

In this work, the novel selenocysteine lyase, EF2568, from *E. faecalis* was purified and characterized. The sequence of the protein categorized it as a group II NifS-like enzyme, making it the first specific selenocysteine lyase to be characterized from group II. Up to this point, all the selenocysteine lyases that have been studied in detail belonged to group I[30,33,34]. Still, EF2568 acted in a similar manner to other studied SCLs, reacting specifically with L-selenocysteine and being competitively inhibited by L-cysteine. It has been noted for other SCLs that the high K_m for L-selenocysteine and the low K_i for L-cysteine would lead to a very low turnover rate for the enzyme[36]. Indeed, the K_m and K_i values of 1.01 mM and 10.3 μM respectively imply that the same phenomenon would be observed for EF2568. Since L-selenocysteine is synthesized on the tRNA, it is rarely free in the cell unless it is released as a degradation product of a selenoproteins[37]. In addition, it is known that *E. faecalis* does not encode the proteins needed to make L-selenocysteine[4]. Therefore, the only source of L-selenocysteine would be from the host or environment and most likely, the intracellular concentration of L-selenocysteine would be far below the K_m. From *E. coli*, it is known that the normal concentration of L-cysteine in the cell is about 200 μM[35]. This amount is far higher than the K_i. Altogether, the enzyme would be an inefficient enzyme, rarely turning over. Indeed, even at optimal conditions such as those in the in vitro assay, the turnover was 17 per min. One of the fastest enzymes, carbonic anhydrase, is able to turn over several hundred thousand times in one second. While this enzyme is an exception, most enzymes still have rates measured in reactions per sec rather than per minute. Therefore, even under optimal conditions, EF2568 is an inefficient enzyme. Still, this is an advantage rather than a disadvantage. To date, only one protein in *E. faecalis* has been shown to require

selenium[4]. Selenium compounds are also highly reactive. If EF2568 was more active it could break down L-selenocysteine and release reactive forms into the cell faster than they could be used. Therefore, the slow turnover rate most likely provides just enough selenium to supply EF2570 without releasing toxic selenium compounds into the cell.

In all NifS-like enzymes, the active site cysteine plays a crucial role in catalyzing the beta elimination of the sulfur or selenium group. While the CSDs from *E. coli* lost cysteine desulfurase activity with the mutation of the active site cysteine, they retained their selenocysteine lyase activity[28]. This suggested that the cysteine residue may not be required for elimination of the selenium atom but mutation of specific selenocysteine lyases caused a loss of all activity with L-selenocysteine. In the case of EF2568, mutation of C341 caused the efficiency of the enzyme to be cut in half but more activity was present than expected. The greater reactivity of selenium increases the possibility of spontaneous elimination. In addition, the presence of strong reducing agents in the assay could contribute to spontaneous elimination. Whether the activity is an artifact of the in vitro assay or actual activity of the enzyme is not known. Still, spontaneous elimination is not a physiologically relevant reaction since in the normal enzyme, the cysteine is still present and formation of a perselenide is needed to specifically donate selenium to an acceptor protein[29,30].

The attempt to change the substrate specificity of EF2568 in a manner similar to that of the human SCL was not successful. Mutation of the glutamate at position of 112 changed the reactivity of the enzyme with L-selenocysteine without affecting the activity with L-cysteine. Further study of the alignments showed that another residue might be a more likely candidate for

changing substrate specificity. Alignment of EF2568 with several NifS-like proteins, including the human SCL showed that E112 is aligned with a glutamate in the human protein. Comparing the predicted structure of EF2568 with the known structure of the human SCL showed that E112 is outside the active site in a similar position as the aligned residue in the human SCL (Figure 8). This matches what was gleaned from the pH curve and reactivity with L-selenocysteine and L-cysteine. From the predicted structure, E112 might affect access to the active site. Mutation to a smaller residue or differently charged residue, such as alanine or lysine, may affect how the substrate is able to enter the active site. This might account for the changed kinetic parameters of the enzyme. In group II enzymes, the D146 from the hSCL is often conserved as a histidine[30]. Indeed, the histidine at position 100 in EF2568 is occupying a similar location to D146 from the human SCL (Figure 8). Therefore, this residue is a better candidate for changing substrate specificity of the enzyme. Work is currently ongoing to mutate H100 and determine if it changes the substrate specificity.

As stated previously, EF2568 is located in a cluster of genes putatively related to selenium metabolism and specifically selenation of Moco for EF2570, a selenium-dependent molybdenum hydroxylase. It was hypothesized that this pathway was a novel third uncharacterized pathway of selenium utilization[5]. The analogous sulfur-dependent molybdenum hydroxylases rely on a NifS-like protein to act as a sulfur donor and a scaffold to hold the Moco while it is sulfurated[10,12,13,19,20,38]. It is logical that a similar pathway is used for selenation of Moco. Indeed, *E. faecalis* has several genes that are homologs of known sulfuration genes in the same cluster as EF2568[4]. EF2571 is a homolog of XdhC, the Moco scaffold in *R. capsulatus* that interacts with a NifS. Downstream of EF2570, there is a MobA homolog. MobA interacts with

XdhC in *R. capsulatus* during sulfuration of Moco. EF2568 has been shown to be a NifS-like protein with specific selenocysteine lyase activity. Together, this information suggests that the cluster of genes is involved in the selenation of Moco for use by EF2570, the xanthine dehydrogenase. EF2568 would be the NifS-like protein acting as a selenium donor to the Moco. The fact that the enzyme is more activity with the active site cysteine residue supports the formation of a perselenide residue that would control the donation of selenium. Assuming a pathway similar to the sulfuration pathway in *R. capsulatus* would also suggest that EF2571 binds Moco, interacts with EF2568 for selenation, and then interacts with EF2570 to insert the completed cofactor. The role of MobA in *R. capsulatus* is further processing of Moco for use by formate dehydrogenases and similar proteins[10]. Currently, no proteins that would use other types of molybdenum cofactors have been identified in *E. faecalis*. Therefore, it is not known if the MobA homolog has a role in Moco maturation like in *R. capsulatus* or a minor role in Moco selenation. Further characterization of the pathway is needed in order to determine how analogous the process of selenation is to the process of sulfuration.

The fact that supplementation of selenite and molybdate increases biofilm formation in a EF2570-dependent manner is evidence for a connection between this cluster of genes and the pathogenesis of *E. faecalis*[6]. Indeed, many types of infections, such as urinary tract infections, involve biofilm formation as part of the disease process. Urine contains selenite and molybdate at concentration above those that were shown to increase biofilm formation[39]. In addition, uric acid, another urine metabolite, was shown to be the substrate of EF2570 and cause an increase in biofilm formation and growth at a concentration of 1 mM[6,39]. Altogether, the evidence suggests that urine is a cue to *E. faecalis* that it is in the urinary tract and biofilm formation is favorable.

While uropathogenic *E. coli* is still the most common cause of UTIs, *E. faecalis* is also a culprit, with its prevalence increasing after the first hospital visit for UTI[40]. The ability of *E. faecalis* to penetrate and persist in epithelial cells lining the urinary tract also increases the chances of a recurring infection[41]. Overall, further study is needed to determine if selenium metabolism is involved in *E. faecalis* pathogenesis.

In conclusion, EF2568 is a novel selenocysteine lyase from *E. faecalis* located in a cluster of genes putatively related to selenium metabolism. The enzyme specifically reacts with L-selenocysteine and most likely forms a perselenide on the active site cysteine that then be donated to an acceptor protein. The low concentration of L-selenocysteine in the cell and high concentration of L-cysteine means that the protein would rarely turn over. Still, only one protein has been identified so far that uses selenium: EF2570. The studies completed to date suggest that this cluster of genes is related to biofilm formation and possibly to pathogenesis. In addition, the cluster of genes seem to be the first set of genes identified that could be involved in selenation of the molybdenum cofactor used by the SDMH family of enzymes. Characterization of EF2568 has laid a foundation for further study of this pathway and how it relates to the pathogenesis of *E. faecalis*.

APPENDIX: FIGURES AND TABLES

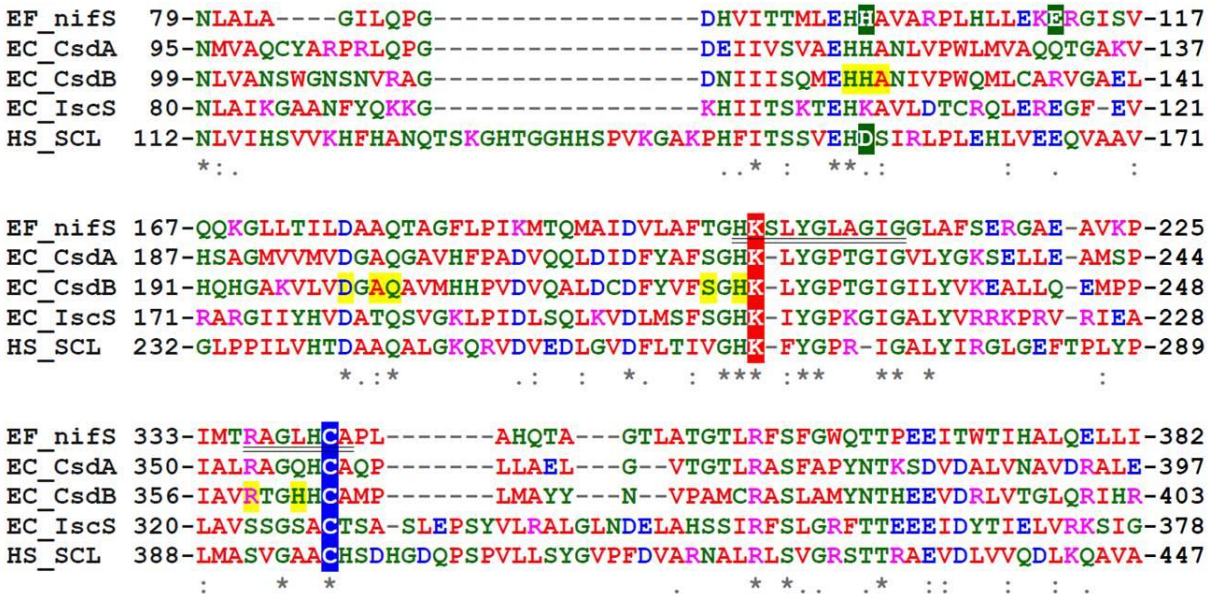

Figure 1: Alignment of EF2568 with NifS-like proteins. The proteins were aligned with Clustal Omega. The lysine highlighted in red is responsible for PLP binding while the cysteine highlighted in blue is a conserved residue shown to have a role in catalysis for the characterized proteins. The aspartic acid residue at position 146 in human selenocysteine lyase is responsible for the substrate specificity (highlighted in green). The putative orthologous residues are highlighted in green for EF2568. The aspartate residue was originally mutated while the histidine will be mutated in further experiments to test its effects on substrate specificity. The yellow highlighted residues are known to be near the active site in CsdB. Finally, the underlined residues are the conserved regions that are used to define NifS-like proteins are group I or group II.

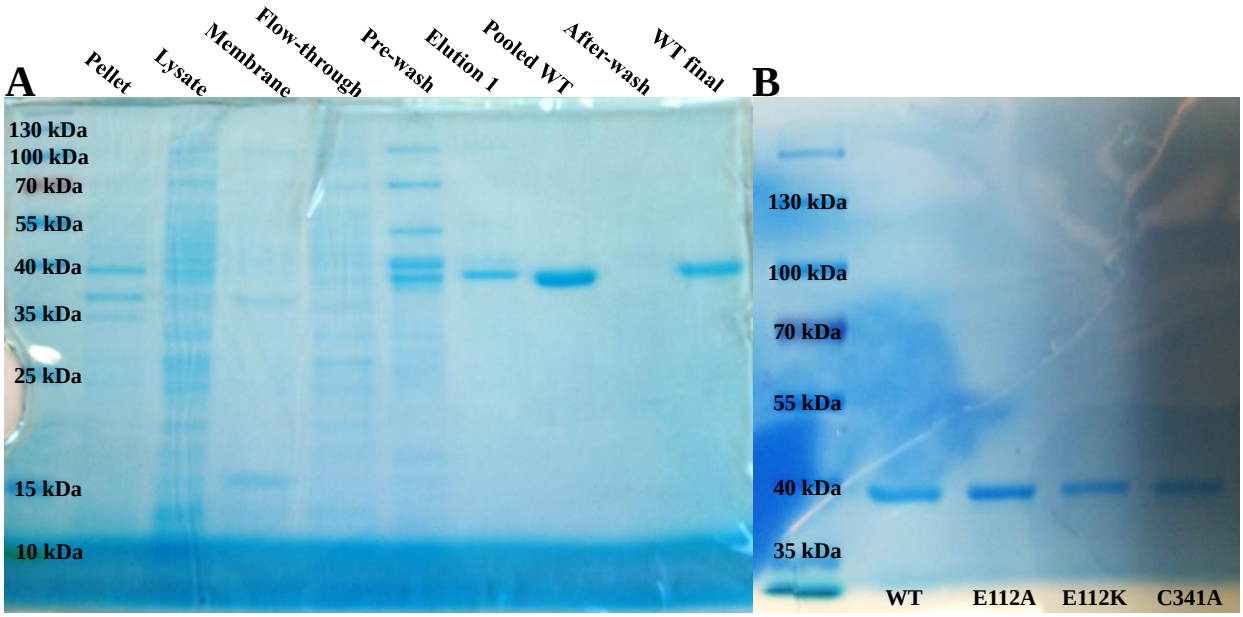

Figure 2: Purification of EF2568 and three mutants. 5 µg of protein from each step of the purification procedure was added to an SDS-PAGE gel. The fractions containing the target WT protein were pooled before addition to the gel filtration column. The final sample shows that the WT was purified to high purity based on the visible inspection of the Coomassie stained gel. The second gel shows that the purification procedure was equally as successful for the three mutants that were also studied.

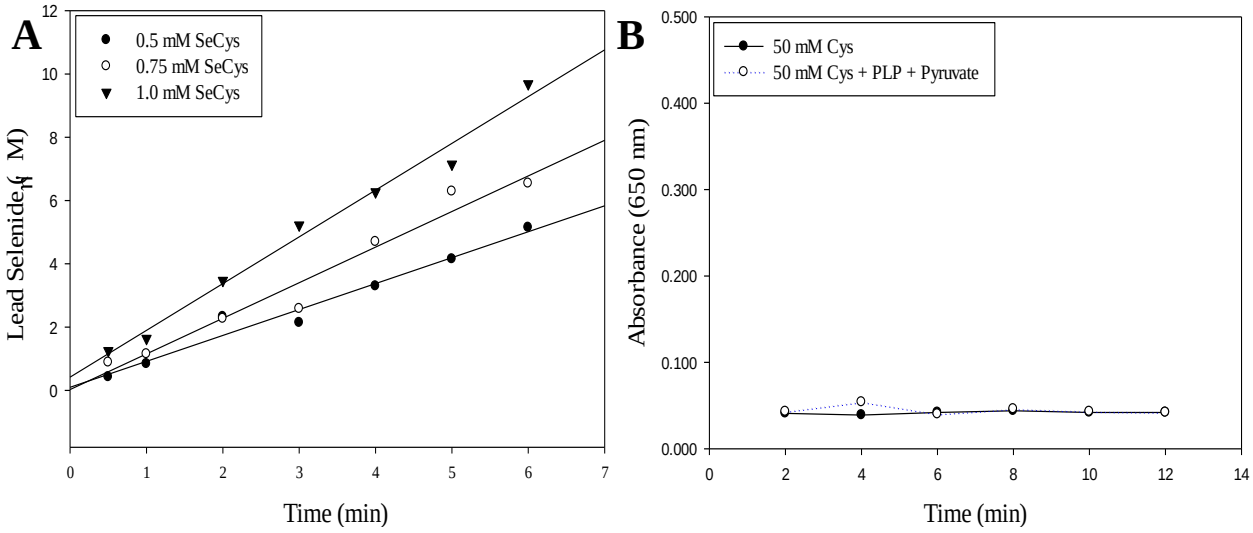

Figure 3: EF2568 reacts specifically with L-selenocysteine. Reaction of EF2568 with L-selenocysteine resulted in the production of selenide that could be detected in complex with lead. The rate and amount of selenide released increased as the amount of L-selenocysteine added to the reaction increased. When the enzyme was reacted with L-cysteine, no sulfide release was detected in the methylene blue assay even with a concentration of 50 mM L-cysteine.

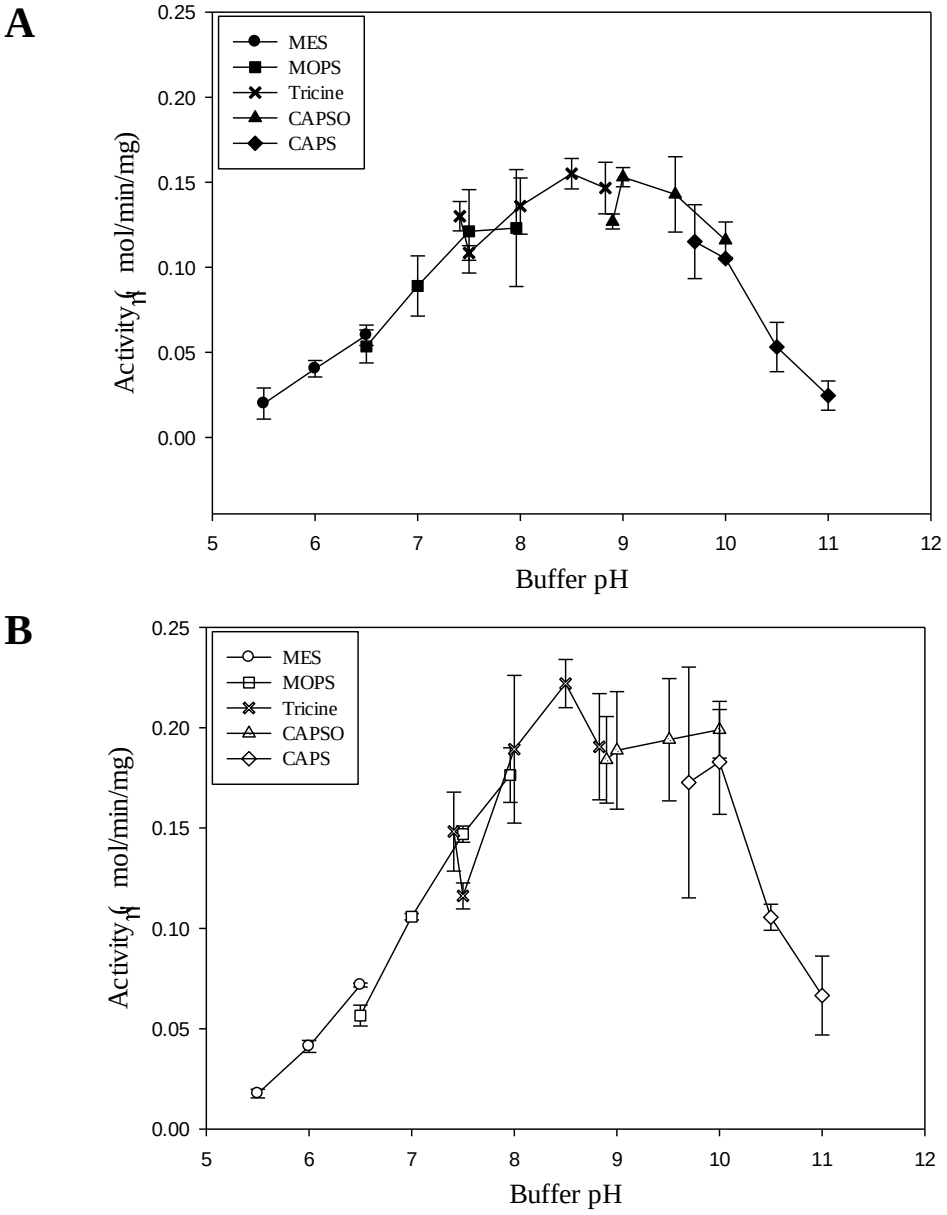

Figure 4: The activity of EF2568 varies with pH of the reaction buffer. The activity of the wild-type of mutant enzymes was tested over a wide pH range in 5 different buffers. The optimal pH for activity was between 8.5 and 9.0 and didn't change for the mutants even though the amount of reactivity did change. The wild-type enzyme data is represented by closed shapes while E112A is signified with open shapes.

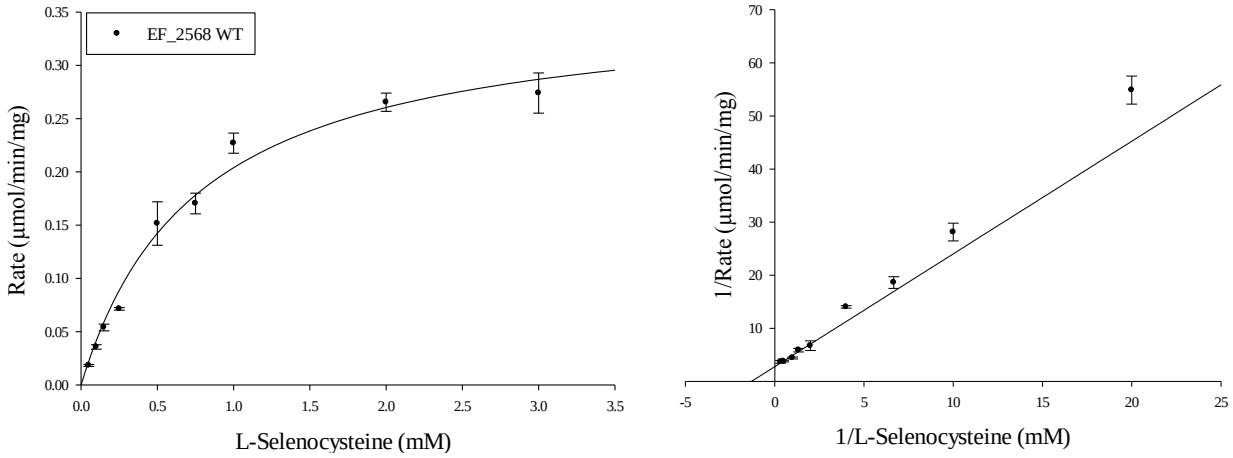

Figure 5: Michaelis-Menten and Lineweaver-Burke Plots. The enzyme activity of EF2568 was determined at concentrations of L-selenocysteine ranging from 0.05 mM to 2.0 mM.

Table 1: Kinetic parameters of EF2568 and its mutants

Enzyme	K_m^{app} (mM)	V_{max}^{app} (μmol/min/mg)	K_{cat} (min^{-1})	K_{cat}/K_m^{app}
Wild-type	1.011	0.376	17.2	17.0
E112A	0.576	0.304	13.9	24.1
E112K	0.610	0.281	12.8	21.0
C341A	1.181	0.216	9.9	8.4

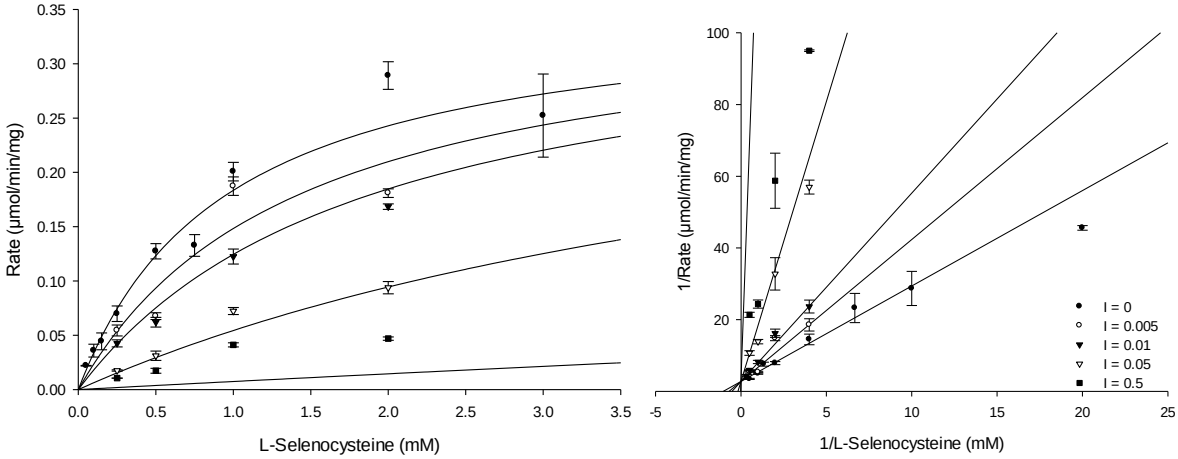

Figure 6: L-Cysteine is a competitive inhibitor of the selenocysteine lyase activity of EF2568. The inhibition by L-cysteine was measured at concentrations from 0.005 to 0.5 mM L-cysteine at four concentrations of L-selenocysteine.

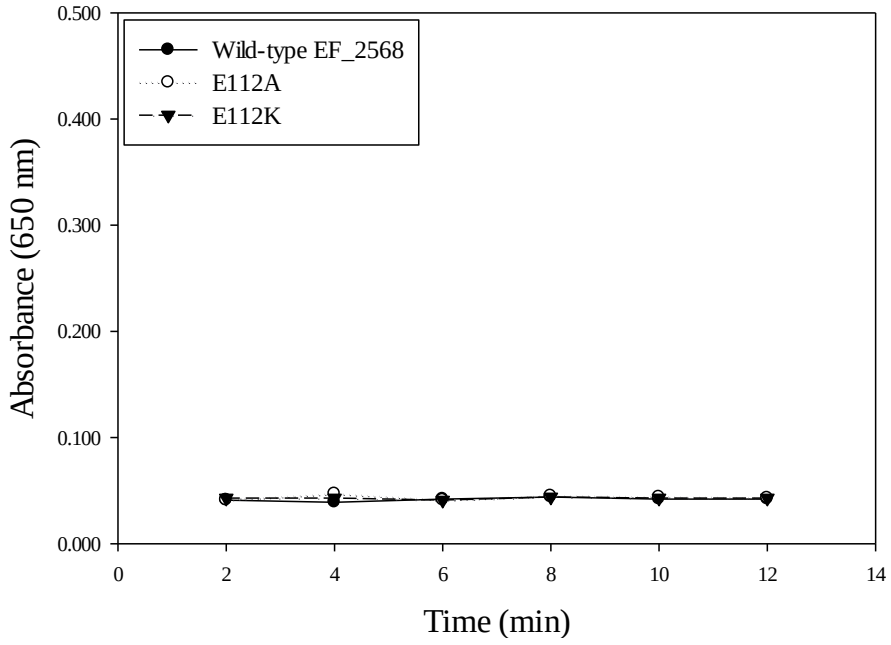

Figure 7: Mutation of position 112 is not sufficient to change substrate specificity of EF2568. The reaction mixtures had PLP and pyruvate added to decrease the possibility of inhibition by abortive transamination. The absorbance for all the samples was similar to the background and did not change for the length of the 12-minute assay.

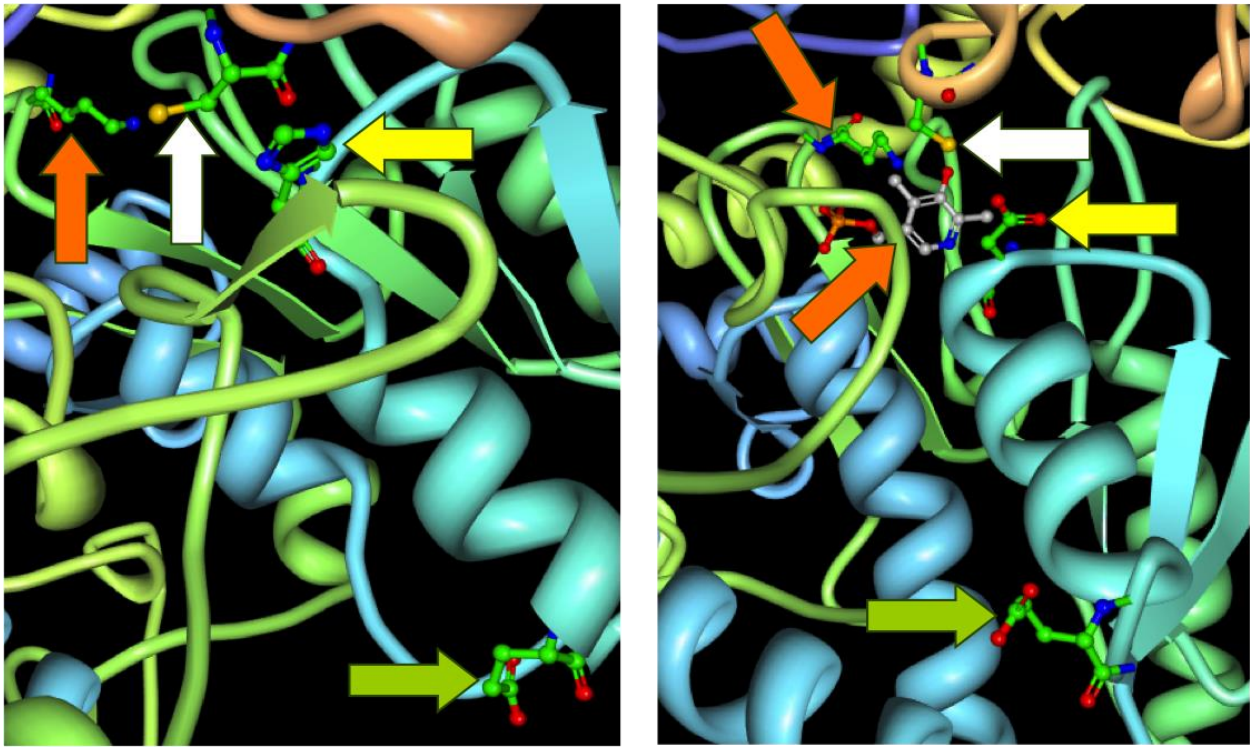

Figure 8: Comparison of the predicted structure of EF2568 with the structure of human SCL. The structure of EF2568 was predicted from the amino acid sequence using ExPASy's SWISS-MODEL homology modeling server (left structure). The structure of the human selenocysteine lyase (right structure) was used as a comparison to determine if homologous residues were located in the same part of the protein. E112 and the aligned homologous residue from hSCL is labeled with the green arrow. The orange arrows point out PLP and the PLP-binding lysine residues. The white arrows label the active site cysteine residue. The yellow arrows point to the D146 residue in the hSCL that has a role in substrate specificity and the aligned homologous residue in EF2568.

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