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HOW A SILENT MUTATION SUPPRESSES THE ACTIVITY AND IRON INCORPORATION IN SUPEROXIDE DISMUTASE

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HOW A SILENT MUTATION SUPPRESSES THE ACTIVITY AND IRON
INCORPORATION IN SUPEROXIDE DISMUTASE

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Chemistry in the
College of Arts and Sciences
at the University of Kentucky

By
Xiaonan Mei

Lexington, Kentucky

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2012

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HOW A SILENT MUTATION SUPPRESSES THE ACTIVITY AND IRON
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05/14/2012

Date

ABSTRACT

HOW A SILENT MUTATION SUPPRESSES THE ACTIVITY AND IRON INCORPORATION IN SUPEROXIDE DISMUTASE

A mutation (CTG to TTG) of FeSOD gene was found in *Escherichia coli*. Since they both encode leucine, it is a silent mutation. Site-directed mutagenesis was applied to correct the mutation, and the mutant FeSOD (before gene correction) and wild type FeSOD (after gene correction) were purified. The FeSODs from the two genes were characterized using different assays and spectroscopic methods including EPR and CD. The requirement for the rare codon TTG may result in slowed translation and heavy demand on a scarce tRNA. Cultures expressing wild type FeSOD are better able to grow for long times after addition of IPTG and more mature to incorporate Fe atoms to the active sites than are cultures expressing the mutant gene. Moreover, the wild type FeSOD has more activity than the mutant. To our knowledge, this is the first time that a silent mutation has been demonstrated to affect metal incorporation into a metalloenzyme.

KEYWORDS: Iron-containing superoxide dismutase, Metalloenzyme, Metal incorporation, Silent mutation, Site-directed mutagenesis, EPR

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TABLE OF CONTENTS

Chapter one: Introduction of iron-containing superoxide dismutase (FeSOD).....	1
Chapter two: Optimization of FeSOD growth and purification.....	7
2.1 Introduction	7
2.2 Materials and Methods	7
2.2.1 Materials and apparatus	7
2.2.2 Methods.....	8
2.3 Results and discussions.....	10
2.3.1 Final concentration of IPTG.....	10
2.3.2 Different amounts of aeration during the LB growth.....	10
2.3.3 Different heat-cut temperatures in the purification process.....	10
Chapter three: Synonymous codons may not be silent	12
3.1 Introduction	12
3.2 Materials and Methods	14
3.3 Results.....	15
3.3.1 Culture vigor and productivity (Growth curves and protein yield).....	15
3.3.3 Iron content of the active sites of FeSOD produced from the two genes.....	18
3.3.4 Multiple forms of FeSOD depending on the gene from which it was produced (Native gel (NBT assay) ⁶⁸	19
3.3.5 Iron content of the different forms of FeSOD (in-gel stain) ⁶⁹	21
3.3.6 Nature of Fe-binding sites in FeSOD produced from the two FeSOD genes EPR spectra.....	21
3.3.7 Secondary and tertiary structure of FeSODs made from the two genes.....	22
3.4 Discussion	22
3.4.1 Rare codon of silent mutation	22
3.4.2 Activity and Fe incorporation.....	23
3.4.3 Structure change by silent mutation	25
3.4.4 Insight of the project	27
CONCLUSIONS	37
REFERENCES.....	38
Vita.....	44

Chapter one: Introduction of iron-containing superoxide dismutase (FeSOD)

Oxygen is essential for cellular respiration in all kinds of aerobic organisms. Oxygen is a spin triplet in its ground state so reactions that generate only closed-shell products (singlets) are formally forbidden. However, one-electron reduction, of oxygen produces a free radical that can undergo spin recombination to yield singlet product. Thus the superoxide ($O_2^{\bullet-}$) intermediate provides a means of reconciling spin¹. In biological systems, $O_2^{\bullet-}$ is generated by several processes². Autoxidation, for example of myoglobin³, hemoglobin⁴, reduced cytochrome C⁵, reduced ferredoxins⁶ or tetrahydropterins⁷ can produce $O_2^{\bullet-}$ upon reaction with O_2 . Some enzymes, such as xanthine oxidase, may produce $O_2^{\bullet-}$ during their catalytic cycles⁸. Some organelles, such as mitochondria and chloroplasts, are also known to generate $O_2^{\bullet-}$. Actually, $O_2^{\bullet-}$ is not as reactive as people thought. It's only reactive within several amino acids where it is generated. However, since $O_2^{\bullet-}$ can initiate a free radical chain reaction, it can generate other more toxic radicals, which are more harmful to the organisms. There are two main radicals $O_2^{\bullet-}$ can generate, one is perhydroxyl radical (HO_2^{\bullet})⁹ and the other is hydroxyl radical (OH^{\bullet}). Haber and Weiss were the first to propose that $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2) can react to produce hydroxyl radical via the reaction $O_2^{\bullet-} + H_2O_2 \rightarrow OH^- + OH^{\bullet} + O_2$. Because it is so reactive, OH^{\bullet} can amplify the potential dangers of $O_2^{\bullet-}$ ¹⁰.

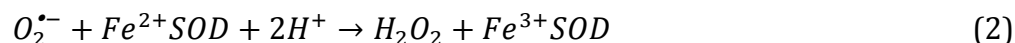
Because of these toxicities, a kind of enzyme called superoxide dismutase (SOD) is used in respiring cells to defend against $O_2^{\bullet-}$ radicals. SOD can enzymatically enhance the rate of $O_2^{\bullet-}$ disproportionation¹¹ ($O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$). There are three types of superoxide dismutase among present organisms and they differ with respect to the metal co-factor they employ as well as the protein fold: copper-zinc SODs (CuZnSODs), manganese or iron SODs (MnSODs or FeSODs), and nickel SOD (NiSODs)¹². Fe- and MnSODs are the most widely distributed SOD of the three enzymes, and they can be found in all five kingdoms of life¹³. MnSODs typically can be found in mitochondria¹⁴ and bacteria where their function is protecting DNA from endogenous oxidative stress. FeSODs are normally

found in bacteria, protists and chloroplasts to defend them from local oxidative stress¹⁵. NiSODs are the most recently identified SODs, being found in *Streptomyces* and related species^{16,17}.

Our group is dedicated to the study of MnSOD and FeSOD. The current project focuses on FeSOD. FeSOD was first discovered by Fridovich's group¹⁸ in 1973 in *Escherichia coli* B, three years after the discovery of MnSOD¹⁹. Early on, FeSODs were found to be dimers with 1-2 atoms of iron per dimer^{18,20}. X-ray crystallography revealed the structure of FeSOD²¹⁻²⁷. The FeSOD monomer is approximately 22 kDa and fully folded into a 3D structure, which is shown in Figure 1.1. Three α -helices a1, a2 and a3 are formed in the N-terminal domain. A short linker with about 10 amino acids connects the N-terminal domain with C-terminal domain. In the C-terminal domain, two α -helices (a4 and a5) are next to the linker, followed by 3 β sheets (b1, b2 and b3). Two more α -helices (a6 and a7) finish the C-terminal domain. Active site iron atom in the monomer is located between N and C-terminal domains. N-terminal provides two ligands to iron. The first α -helix a1 provides His26 as one ligand and α -helix a3 provides His73 as another. The C-terminal domain supplies the other 2 ligands: Asp156 and His60. Besides the four ligands provided by the protein, a coordinated solvent molecule serves as the fifth ligand (Figure 1.2). The five ligands form a trigonal bipyramidal coordination sphere, with His26 and coordinated solvent in the axial positions²⁸. Studies showed that the coordinated solvent molecule is OH⁻ when iron is in the oxidized state (Fe³⁺), and H₂O when iron is in the reduced state (Fe²⁺)^{23,29}. Studies also showed that the 2nd coordination sphere around the active site plays a significant role though hydrogen binding^{25,30-34}(Figure 1.3). Hydrogen bond network is extended from the coordinated solvent in the active site to the 2nd sphere by Gln69, Tyr34, Asn72, Trp122. Among these amino acids, Gln69 plays an important role. By mutating Gln69 to histidine, the former group member Emine Yikilmaz demonstrated that the hydrogen bond between Gln69 and the coordinated solvent molecule could strongly influence FeSOD activity³³.

FeSOD employs a ping-pong mechanism³⁵. The first half reaction involves the oxidation of substrate O₂^{•-} and the reduction of the metal ion co-factor in the active

site. From the substrate's perspective, this reaction only requires electron transfer. Since net charge near the active site of FeSOD is positive, this half reaction is favorable. However the enzyme takes up a proton in conjunction with reduction of the active site Fe^{3+} . On the other hand, simple reduction of $O_2^{\bullet-}$ is not favorable. By providing 2 protons to produce H_2O_2 , FeSOD makes the second half reaction favorable as well.



Although $O_2^{\bullet-}$ is the actual substrate in the reaction, its short lifetime makes it hard to study. Substrate analogues have been studied to understand the binding behavior. One of the substrate analogues is azide (N_3^-). Studies showed that one azide molecule could bind directly to the active site iron and inhibit FeSOD completely^{25,37-40}. Another useful substrate analogue is fluoride (F^-); two F^- ions can bind to the active site iron and one is believed to replace the coordinated solvent^{37,38}.

Electron paramagnetic resonance (EPR) is one of the methods to study the active site electronic structure and coordination geometry of FeSOD, since Fe^{3+} is paramagnetic. EPR spectra have been used to compare active the active sites of FeSODs from different organisms⁴¹. The signal observed near 1500 G when using a microwave frequency near 9 GHz ('X-band') is a signature of the spin transition between $m_s = \pm 3/2$ states of the $S=5/2$ Fe^{3+} .

The reaction of H_2O_2 with $Fe^{3+}SOD$ has also been studied by EPR. Some of the studies indicated that only EPR signal intensity was decreased upon adding H_2O_2 ⁴². On the other hand, some studies showed that H_2O_2 can inhibit FeSOD completely^{43,44}. EPR is also a powerful tool for studying anion binding⁴⁰ and the pH dependence of $Fe^{3+}SOD$ ⁴⁵⁻⁴⁷. It showed that OH^- coordinates to Fe^{3+} when the pH is increased from neutral pH to high pH, and the geometry changed from five-coordinate to six-coordinate.

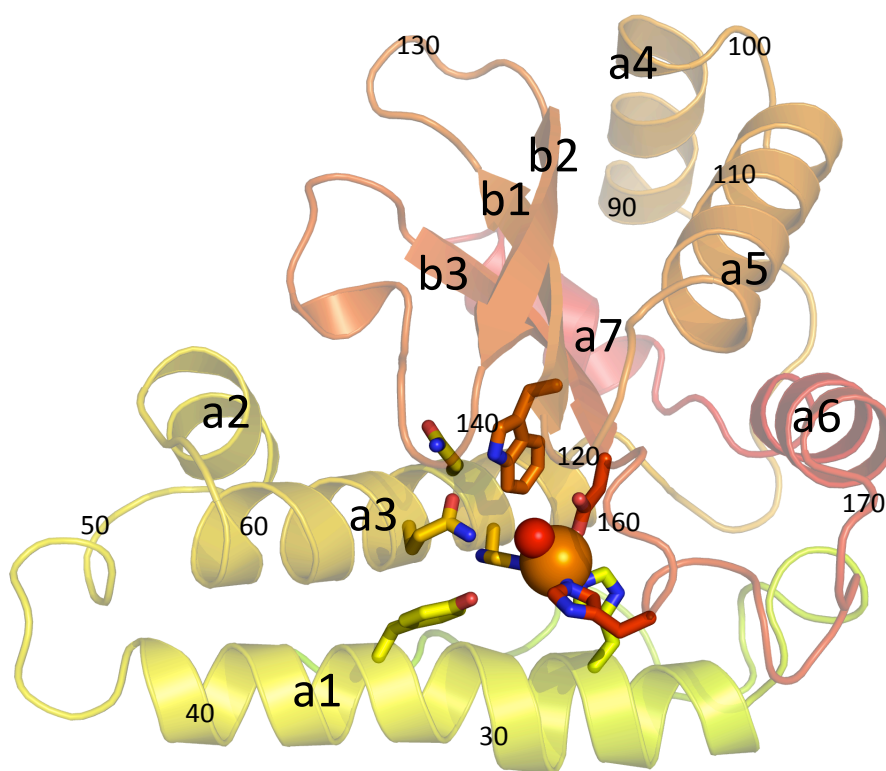


Figure 1.1: Ribbon diagram of a monomer of *E. coli* Fe³⁺SOD showing the Fe³⁺ as a large orange sphere and the heavy atoms of the ligands from C_α out in ball and stick format. Residue numbers are indicated in smaller numbers. Large alphanumeric labels are used to label the different helices and β -strands according to Lah *et al.*²⁵ Based on the coordinates of Fe³⁺SOD published by Lah *et al.*, 1ISB²⁵, and produced by Miller, A. F.²⁸ using MOLSCRIPT.

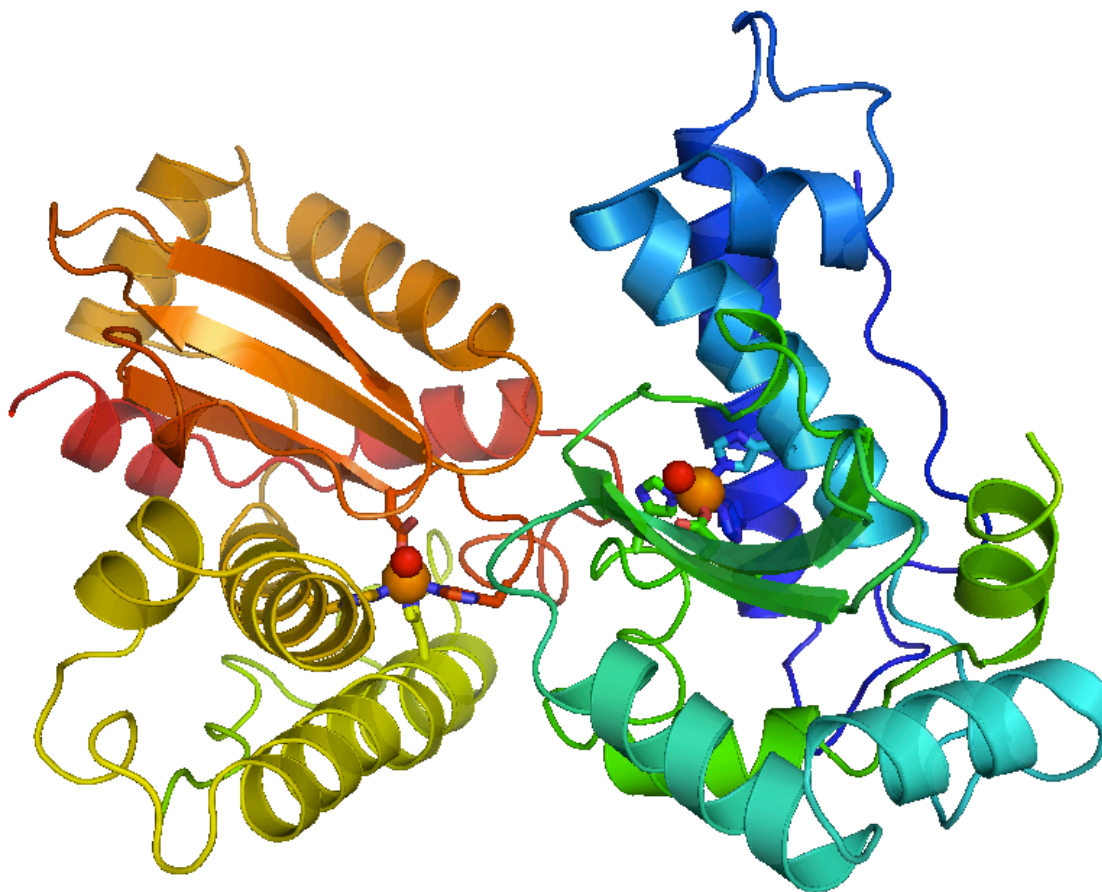


Figure 1.2: Ribbon structure of *E. coli* Fe³⁺SOD dimer showing the Fe³⁺ as a large orange sphere and the heavy atoms of the ligands from C_α out in ball and stick format. Based on the coordinates of Fe³⁺SOD published by Lah *et al.*, 1ISB,²⁵ and produced by Miller, A. F.²⁸ using MOLSCRIPT.

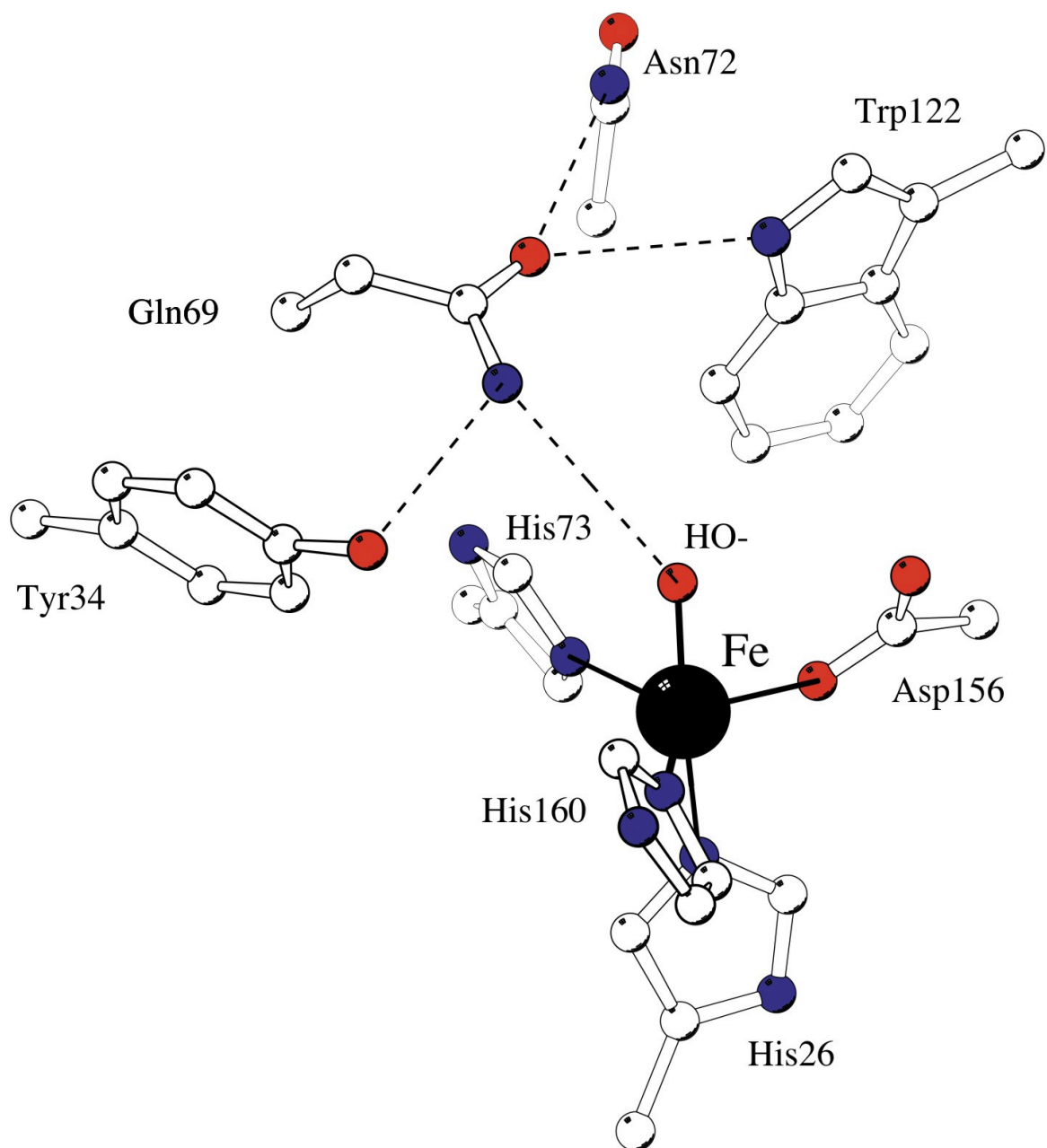


Figure 1.3: The active site including selected second sphere residues in FeSOD with commonly-reported hydrogen bonds. H atoms and backbone atoms are omitted for clarity. Based on the coordinates of Fe^{3+}SOD published by Lah *et al.*, 1ISB,²⁵ and produced by Miller, A. F.²⁸ using MOLSCRIPT.

Chapter two: Optimization of FeSOD growth and purification

2.1 Introduction

The active site Fe of FeSOD is essential for catalytic activity. Thus acquisition of Fe is a key step in maturation for FeSOD. According to the X-ray crystal structure, the mature FeSOD should be a dimer with one iron ion in each of the active sites. Since the first discovery of FeSOD by the Fridovich group in 1973¹⁸, “iron-containing superoxide dismutases have been isolated from a variety of microbial sources, and in each instance 1 to 2 atoms of iron per molecule are found”³⁷. Different growth and purification conditions may affect metal ion uptake, and thereby alter the activity of FeSOD. In the beginning of the project, different culture growth and purification conditions were tested to maximize the metal uptake and enzyme maturation.

2.2 Materials and Methods

2.2.1 Materials and apparatus

The wild type FeSOD gene is encoded on the pRLK1-3 vector. *E. coli* BL21(DE3) harboring plasmid pRLK1-3 was obtained from stock solution prepared by R. L. Koder. The plasmid pRLK1-3 carries kanamycin resistance.

BL21 (DE3) competent cells were purchased from Novagen and stored at -20 °C. Isopropyl β -thiogalactopyranoside (IPTG), kanamycin, and 4-(2- Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) were purchased from Sigma. Bradford reagent and BSA used for protein quantitation were obtained from sigma. All other chemicals were biological grade and purchased from ACROS organics, Research Organics or Sigma-Aldrich.

A Mettler Toledo Model AB54 analytical balance was used for weight measurements. Deionized water used in the experiments was purified using a Millipore Milli-Q Plus System. All bacterial cells were incubated and grown at 37 °C in a National Heinicke Model 3321 incubator. The shaking incubator used is a New

Brunswick Scientific C25 Incubator Shaker. Cells were harvested by centrifugation in a Beckman Coulter J-25I Centrifuge. Bacterial cells were ruptured by passage through a French Press from SLM-AMINCO Spectronic Instruments. An HP8452B diode array spectrophotometer was used to determine the optical density of cells and to measure protein concentrations.

2.2.2 Methods

Protein overexpression was initiated by plating the *E. coli* BL21(DE3)/pRLK1-3 glycerol stock solution on an Luria-Bertani (LB) plate containing 50 µg/mL kanamycin and incubating at 37 °C overnight. A single colony was used to inoculate a tube of 3 mL LB medium (Bactro-tryptone 5g/500mL, Bacto-yeast extract 2.5g/500ml, NaCl 5g/500mL, pH=7.5) containing 50 µg/mL kanamycin. After incubation at 37 °C, shaking at 220 rpm for 12 hours, 100 µL were used to inoculate 100 mL of LB culture containing 50 µg/mL kanamycin. After incubation at 37 °C, shaking at 220 rpm for another 12 hours, 50 mL LB culture was added to 2 L LB medium containing 50 µg/mL kanamycin and then shaken at 37 °C in a 6 L flask. When the optical density at 600 nm (OD₆₀₀) reached approximately 0.6, ferrous-nitrilotriacetate (Fe-NTA) was added to a final concentration of 35 µM and isopropyl β-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce SOD overexpression. The culture was shaken at 37 °C, 220 rpm for another 10-12 hours. The cells were harvested by centrifugation at 4 °C, at 9500 rpm for 15 min. The cells were re-suspended in cell wash buffer (50 mM potassium phosphate buffer, pH=7.4), collected via centrifugation at 9,500 rpm and 4°C for 15 min, and frozen at -20°C until needed. The level of overexpression was tested by running sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of samples collected before and after adding IPTG.

Frozen cells were re-suspended in cell lysis buffer (50 mM potassium phosphate pH=7.4, 0.5% NaCl (w/v), 0.5% KCl (w/v), 1 mM EDTA) with a final concentration 0.2 mg/mL 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF). Approximately 3 ml of cell lysis buffer were used for each 1 g of cell paste. The mixture was transferred to a homogenizer and ground to a smooth paste. Cells

were broken by passage through a French Press 3 times at 10000 to 15000 psi. The crude SOD was separated from cell debris by centrifugation at 4 °C, 18000×g for 30 min.

Potassium chloride (KCl) was gradually added to the supernatant while stirring until the concentration of KCl reached 0.1 M (calculation without accounting for KCl's contribution to the volume). The mixture was transferred to a beaker in 50 °C water bath for 3 min with occasional swirling, followed by an ice bath for 15 min without swirling. Precipitated proteins were separated from crude FeSOD by centrifugation at 4 °C, 18000×g for 30 min.

Ammonium sulfate ((NH₄)₂SO₄) was gradually added to the supernatant while stirring until the solution was 50% saturated by ((NH₄)₂SO₄) to precipitate additional undesired proteins. The crude FeSOD and the cell debris were separated by centrifugation at 4 °C, 18000×g for 30 min.

The supernatant was applied to a 100 mL G-25 desalting column, which had been pre-equilibrated with 3 volumes of 5 mM potassium phosphate buffer (pH=7.4). The flow-through fractions were collected in small tubes (each fraction was about 5 mL), and their A₂₈₀ values were measured using an HP8452B diode array spectrophotometer to identify fractions containing protein ($\epsilon_{280} = 101,000 \text{ M}^{-1}\text{cm}^{-1}$ ³⁷). The eluted SOD protein was identified on the basis of its molecular weight by SDS-PAGE and its yellow color. Fractions containing SOD protein were combined and dialyzed against 12 L dialysis buffer (5mM potassium phosphate, pH=7.4) overnight at 4 °C.

The dialyzed protein was concentrated using an Amicon Ultra-15 centrifugal filter device (10k nominal molecular weight limit) by centrifuging at 4 °C at 4000×g for 20 min, to a final volume of less than 10 mL. The protein concentration was determined based on the A₂₈₀ and the active site Fe³⁺ concentration was determined via the A₃₅₀ ($\epsilon_{350} = 1850 \text{ M}^{-1}\text{cm}^{-1}/\text{dimer}$ ³⁷).

2.3 Results and discussions

2.3.1 Final concentration of IPTG

IPTG was used to induce the production of FeSOD protein. Normally, IPTG is used as an inducer in the concentration range of 0.1 mM to 1.5 mM. Different final concentrations of IPTG may be used to modulate the induction strength and to accommodate the features of the expression system^{48,49}. However, there was no published study to show how the concentration of IPTG affects bacterial overexpression of FeSOD. In the experiment, 0.5 mmol/L and 1.0 mmol/L final concentrations of IPTG were used for two otherwise identical bacterial cultures when the optical density at 600 nm (OD_{600}) reached 0.5, to induce the production of FeSOD. The OD_{600} was monitored and plotted against time. The culture with 0.5 mmol/L IPTG produced about 10% more of bacterial cells than 1.0 mmol/L IPTG.

In conjunction with determinations of the optical density, 1 mL of samples were collected and spun down to harvest the bacterial cells in each sample. All the cell pellets were treated with dithiothreitol and SDS to lyse the cells and then loaded onto a SDS-PAGE gel. The results from SDS-PAGE were consistent with the growth curve in that the 0.5 mmol/L final concentration of IPTG induced slightly more FeSOD protein than 1.0 mmol/L IPTG. Therefore, all subsequent growths employed 0.5 mmol/L IPTG as final concentration.

2.3.2 Different amounts of aeration during the LB growth

Since oxygen is an essential ingredient for the growth of most bacteria, the extent of aeration of the growth system may affect the protein yield. However, iron is less soluble in aerobic than anaerobic medium. In this experiment, 2 L LB in a 6 L flask and 1 L LB in a 6 L flask were compared to test the effect of aeration on bacterial growth. The results in Figure 2.1 showed that less aeration corresponding to 2 L of LB medium in a 6 L flask worked better than 1 L of LB medium in a 6 L flask. All subsequent growths were executed using 2 L of LB medium in a 6 L flask.

2.3.3 Different heat-cut temperatures in the purification process

SOD is a robust enzyme and can tolerate higher temperatures than most proteins. Therefore, a heat-cut step was included in the purification process to

eliminate other undesired proteins by Fee *et al.*³⁷ to improve the purity of FeSOD. To optimize the performance of this step, temperatures 50, 60, 70 and 80 °C were compared. Four identical batches of protein solutions from the French press step were treated with one of the above temperatures, each. No matter what the temperature, the majority of FeSOD stayed in the supernatant. Non-denaturing PAGE was used to assess the results (Figure 2.2). Although there was no significant difference among the four temperatures, a larger fraction of FeSOD stayed in the supernatant when the lower temperature was used, and the degree of contamination of FeSOD in the supernatant was comparable to that obtained at higher temperatures. Therefore, 50 °C was employed for the heat-cut in all subsequent purifications. We note that the FeSOD is close to pure already at this step, only small amounts of contamination proteins are removed, so we deem that the step should be performed with the minimal cost to FeSOD.

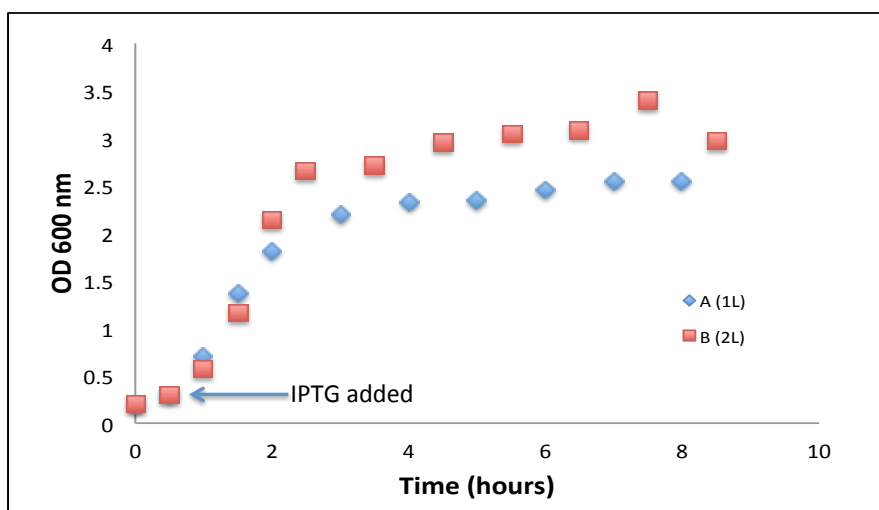


Figure 2.1: Growth curves of pRK3-1/BL21 (DE3) in 2L LB aerobic medium. Red squares indicate 2 L of LB culture in a 6 L flask. Blue diamonds indicate 1 L of LB culture in a 6 L flask.

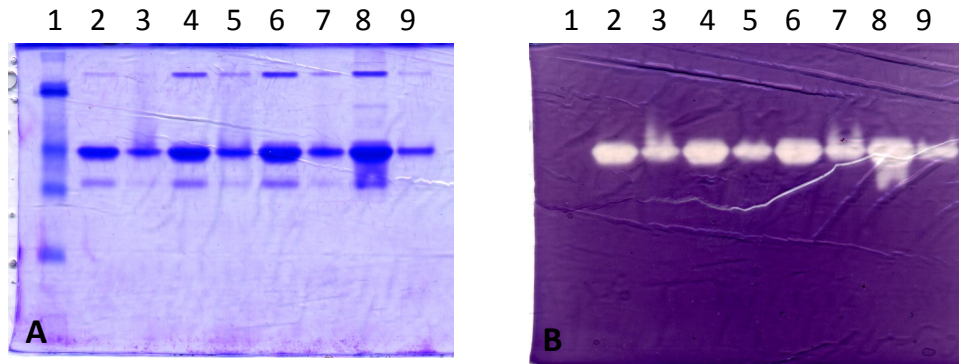


Figure 2.2: Non-denaturing polyacrylamide gel electrophoretograms of wild-type FeSOD. Panel A was stained with Coomassie brilliant blue to visualize all proteins as dark blue bands. Panel B was stained using the NBT assay that produces a violet background everywhere except where there is SOD activity, which is visualized as white regions. In both panels Lane 1 was the prestained SDS-PAGE Standards (Bio-rad, 310006667; Lane 2 was the supernatant at 80°C heat-cut; Lane 3 was the pellet at 80°C heat-cut; Lane 4 was the supernatant at 70°C heat-cut; Lane 5 was the pellet at 70°C heat-cut; Lane 6 was the supernatant at 60°C heat-cut; Lane 7 was the pellet at 60°C heat-cut; Lane 8 was the supernatant at 50°C heat-cut; Lane 9 was the pellet at 50°C heat-cut. Note that in each case there is much more supernatant than pellet so that only a small fraction of FeSOD is lost.

Chapter three: Synonymous codons may not be silent

3.1 Introduction

In 1941, George Beadle and Edward Tatum proposed a hypothesis called “one gene-one enzyme”⁵⁰, which became one of the milestones in the history of molecular biology. In this hypothesis, each gene is responsible for producing a single enzyme. This hypothesis has been modified since it was realized that one gene can code for more than one enzyme. In eukaryotes, a primary transcript can be edited to produce several different polypeptides, so one gene can produce several proteins. Later on, people found out that each amino acid is presented by one or more triplets of nucleotide bases (called “codons”) when translating from mRNAs to proteins. For example, during the translation process, when the mRNA displays the codon CTG,

the corresponding tRNA that has been charged with the amino acid leucine by a tRNA synthase is used by the ribosome to add a leucine to the growing peptide chain. Because there are 64 possible codons possible for encoding 20 amino acids, one same amino acid may have several different codons. In the example above, the amino acid leucine is coded for by 6 different codons.

Early on, it was widely assumed that different codons for one amino acid were used equally, and if a mutation did not change the sequence of amino acids (even with different codons), then it would produce the same result. In fact, so-called synonymous codons (encoding the same amino acid) are utilized to different extents to produce proteins⁵¹⁻⁵³. In another word, there is codon preference⁵⁴ or biased usage⁵⁵ when producing enzymes. Even though it was assumed that so-called "silent" mutations than change which codon is present without changing the amino acid encoded should not cause changes in protein structures and activities since amino acids sequences are not changed, there are many groups dedicated to studying the effects of silent mutations.

As early as the 1980s, studies found that each codon has a cognate tRNA as an adaptor to recognize it, and the populations of the different tRNAs in cells are different⁵⁵⁻⁵⁷. Natural selection tends to use the preferred codons with most abundant tRNA to increase translational efficiency and accuracy⁵⁸⁻⁶⁰. In 1987, Purvis's group proposed that "the way in which some proteins fold is affected by the rates at which regions of their polypeptide chains are translated in vivo"⁶¹. The relationship between codon usage and protein translation rate in *Escherichia coli* was first characterized experimentally in 1989⁶². Not only the traffic of tRNA, but also the altered translation kinetics caused by synonymous codon substitutions may cause the same polypeptide chain to fold into different conformations, and thus alter the activity of the protein⁶³. Recently, more and more studies have shown that silent mutations may cause genetic diseases⁶⁴⁻⁶⁶. Therefore, silent mutations should not be neglected.

In this project, the FeSOD gene was found to contain a spontaneous mutation (C43T). This mutation is silent since CTG and TTG are both codons for leucine.

However, the behavior and activity of the FeSOD from the wild-type and mutant are different, according to my experiments. The same silent codon substitution has recently been studied by another group. The study by Kimchi-Sarfaty *et al.* showed that silent mutations in multidrug resistance-1 (MDR-1) gene may affect the behavior of P-glycoprotein, which is a multiple-transmembrane helix pump that transports different drugs out of cancer cells⁶⁵. They found that silent mutations have a slightly different three-dimensional shape from the wild-type protein. Another recent study showed that a CTG to TTG silent mutation in the immunity-related GTPase family, M (IRGM) gene can alter the binding behavior miR-196 protein and contribute to Crohn's disease⁶⁶.

3.2 Materials and Methods

The correction of the silent mutation in the FeSOD gene was executed by site-directed mutagenesis using the "QuikChange Lightning Kit". Primers for PCR were ordered from Integrated-DNA Technologies (IDT). Pfu Ultra Polymerase, Pfu Ultra HF reaction buffer, XL1-Blue supercompetent cells were purchased from Stratagene. The gel extraction kit, QIAprep Spin miniprep kit and HiSpeed plasmid midi and maxi kits were purchased from QIAGEN. dNTP (deoxy nucleotide triphosphate) mix, Nde I, BamH I enzymes, 100x BSA (bovine serum albumin), NEBuffer 2, T4 ligase buffer and T4 DNA ligase were purchased from Bio-rad. The polymerase chain reaction (PCR) was performed in a GeneAmp PCR System 9700, with settings dependent optimized for the experiment (see below). FeSOD gene sequences were determined by sending plasmid DNA to Retrogen for nucleotide sequence determination based on primers recognizing the T7 promoter and termination sequences. Electron paramagnetic resonance (EPR) was performed using a Bruker 300MX EPR system operating at X-band and an HS resonator cavity.

Site-directed mutagenesis was executed to correct a thymine identified in our overexpression vector back to the cytosine reported for the wild-type (WT) gene.

The forward primer 5'-cac tac cat atg cta aag atg ctc tgg cac cgc aca ttt ctg cgg aaa-3' and reverse primer 5'- ttt ccg cag aaa tgt gcg gtg cca gag cat ctt tag cat atg gta gtg-3' were used for the QuikChange site-directed mutagenesis using the regimen which is described in Figure 3.1. A 51 μ L reaction (total volume) was made in a microamp reaction tube, containing 5 μ L 10X reaction buffer, 2 μ L (76 ng) DNA template, 1.25 μ L(125 ng) forward and reverse primers, 1 μ L dNTP mixture (Stratagene), 1.5 μ L QuikChange® Solution Reagent (Stratagene), 1 μ L QuikChange® Lightning Enzyme (Stratagene), and 38 μ L sterile deionized water. The new 'corrected' FeSOD gene was transformed into BL21 (DE3) competent cells. Cells were spread on an LB plate containing 50 μ g/mL kanamycin to select cells bearing the FeSOD plasmid. After incubation at 37 °C overnight, three single colonies plate were selected and each was used to inoculate a 3 mL LB culture containing 50 μ g/mL kanamycin. After shaking at 37 °C for 12 hours, at 250 rpm, each culture was harvested by centrifugation and plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen). The purified plasmid DNA was double digested with Nde I and BamH I, and analyzed by 1% agarose gel electrophoresis with ethidium bromide (EB) (Figure 3.2). The plasmids with the right size of insert (700 bp) were selected for further characterization.

A strain containing an apparently correct plasmid was grown in a 100 mL LB culture with 50 μ g/mL kanamycin. After incubation at 37 °C for 12 hours, cells were harvested by centrifugation. The plasmid was purified using the QIAprep Spin Miniprep Kit (Qiagen), and sent to Retrogen Inc. for nucleotide sequence determination.

3.3 Results

3.3.1 Culture vigor and productivity (Growth curves and protein yield)

The presence of a mutation in the FeSOD gene was suspected because induction of gene expression caused a sharp loss of culture vigor resulting in lower

cell density after overnight growth. Upon isolation of the plasmid and sequencing of the FeSOD gene a spontaneous cytosine-to-thymine silent mutation was discovered in codon 14. This mutation created a “TTG” codon instead of the WT “CTG” for leucine. Even though this mutation did not alter the identity of the encoded amino acid, which remained leucine, site-directed mutagenesis was performed to convert the TTG codon back to the wild-type CTG because the culture vigor and protein yields were low. Thus the described QuikChange procedure produced a L14L correction. In what follows the product of the spontaneously mutated gene is termed mutant FeSOD and the product of the corrected (WT) gene is termed WT FeSOD. Overexpression of the mutant gene caused the culture density to diminish in prolonged culture (Figure 3.3). In contrast, cultures overexpressing the corrected gene sustained a high density over the same time period indicating that neither the metabolic burden of overexpression nor exhaustion of nutrients was to blame. Since a culture not overexpressing the mutant gene (blue squares in Figure 3.3) did much better than a genetically identical culture overexpressing it (blue diamonds in Figure 3.3), overexpression of the mutant gene causes the culture to lose vigor, not the gene itself. Thus it seemed possible that the protein produced from the mutant gene was detrimental.

Four preparations from before and after mutagenesis were selected to calculate concentrations of iron and protein, and the iron contents (iron per dimer) and the protein yields from 2 L LB growths were determined (summarized in Table 3.1). The FeSOD purified from cultures over-expressing the mutant gene displayed a low iron stoichiometry of only one iron per protein dimer, vs. the expected two. FeSOD produced from the corrected gene contained 50% more Fe and reached the theoretical iron content of 2 per dimer if cultures were allowed to continue growing overnight after induction of FeSOD gene expression, rather than being harvested 4 hours after addition of IPTG. Cultures expressing the mutant gene were not able to be harvested after overnight growth since the cultures had deteriorated so badly that proteins isolated from it could have been subject to degradation and damage. Therefore, it could not be reliably compared with protein isolated from stable cultures. Overnight growth following induction with IPTG almost doubled the yield

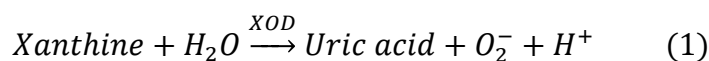
of FeSOD from the corrected gene. Thus the fact that correction of the gene makes overnight induction possible greatly increases the yield attainable from a culture.

Although correction of the gene resulted in greater Fe incorporation, it did not otherwise alter the specific activity of the FeSOD, since the activity on a per-Fe basis was not different for FeSOD from the mutant gene vs. FeSOD from the corrected gene (Table 3.2). Thus the mutant protein that binds Fe does not appear to have any defect (as expected for a silent mutation) although it is less efficient at acquiring Fe. Use of the WT gene however permitted longer growth of the culture and this in turn affects the extent to which harvested protein is mature enzyme. Thus cultures harvested after overnight growth in the presence of IPTG yielded FeSOD of considerably higher quality.

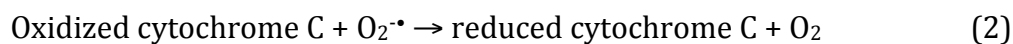
3.3.2 Specific activity of FeSODs produced from the two genes (measured via the cytochrome C-reduction interference assay¹³)

In this assay SOD activity is assessed indirectly via the ability of a sample to interfere with reduction of cytochrome C by $O_2^{\bullet-}$, which is monitored as growth of reduced cytochrome C's optical signal at 550 nm.

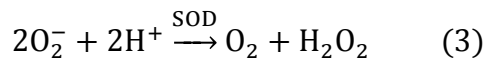
$O_2^{\bullet-}$ was generated by the reaction of xanthine and oxygen catalyzed by xanthine oxidase (XOD).



Oxidized cytochrome C was converted to the reduced form by the $O_2^{\bullet-}$ produced by reaction (2).



When there was superoxide dismutase (SOD) in the system, $O_2^{\bullet-}$ was consumed by SOD according to the reaction (3), which prevented $O_2^{\bullet-}$ from reducing cytochrome C.



Thus the presence of SOD activity resulted in a diminished rate of cytochrome C reduction as monitored at 550 nm. One unit of SOD activity is defined as the amount of SOD that halves a cytochrome c reduction rate equivalent to an absorbance change at 550 nm of 0.125/min.

Figures 3.4 and 3.5 show the decrease in cytochrome C reduction caused by mutant and WT FeSOD. The decreases caused by several different amounts of FeSOD were used to calculate FeSOD activity. The number of activity units per mg protein is used as a measure of the specific activity of FeSOD. Table 3.2 shows the specific activities of different FeSOD preparations. Mutant FeSOD could not defeat superoxide for a long time. The cells from which this FeSOD was isolated were not vigorous and FeSOD activity was around 8000 units/mg. However, correction of the gene and increasing the cultivation time caused FeSOD activity to increase by more than 100%. The resulting specific activity of 19000 is much higher than the published activities, which are in the range of 8000-11000.

The specific activity of FeSOD from overnight inductions is some two-fold higher than that normally reported in the literature³⁷.

3.3.3 Iron content of the active sites of FeSOD produced from the two genes

We used two ways to determine iron concentration in vitro: optical spectroscopy, and the ferrozine assay⁶⁷.

Fe³⁺ in the FeSOD active site has a ligand to metal charge transfer band characterized by an absorption maximum at 350 nm and an extinction coefficient of $\epsilon_{350} = 1850 \text{ M}^{-1}\text{cm}^{-1}$ ³⁷. Based on the absorbance at 280 nm and 350 nm, the concentration of protein and iron can be calculated ($\epsilon_{280} = 1.01 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ ³⁷). Figure 3.6 shows the absorbance spectra of FeSOD produced based on the mutant gene and the WT gene. This method quantifies only Fe³⁺ in SOD.

The other method is the ferrozine assay. Ferrozine, also called 3-(2-pyridyl)-5,6-(4-phenylsulfonic acid)-1,2,4-triazine, is a compound that can form a complex with Fe²⁺. The iron-ferrozine complex displays a stable magenta color and can be quantified by spectrophotometry at 562 nm. This method can detect the total amount of iron (Fe²⁺ and Fe³⁺) in samples after 0.02% ascorbic acid had been used to reduce any Fe³⁺ to Fe²⁺. The protein was precipitated using 11.3% trichloroacetic acid and removed by centrifugation. Mixed ferrozine color reagent (3 mg/mL ferrozine, 3 mg/mL neocuproin) was added to the system to develop a magenta-

colored complex, the concentration of which was determined via the plot of known concentration of standard iron solutions at 562 nm⁶⁷.

Since WT FeSOD is a dimer, theoretically there should be 2 iron ions per dimer. Based on the low Fe³⁺ stoichiometries, the metal uptake was incomplete for mutant FeSOD. There was always approximately one iron per dimer (Table 3.3). After correction of the silent mutation the metal content was improved, and allowing growth to continue overnight after addition of IPTG resulted in a 60% increase in yield and almost stoichiometric iron content. Thus, increasing the cultivation time after addition of IPTG significantly improves both the protein yield and the quality of the FeSOD.

In conclusion, correction of the silent mutation improved the iron content and by permitting a longer cultivation time after addition of IPTG, increased yield of FeSOD we could obtain.

Since the ferrozine assay detects all iron ions regardless of whether they are iron(II) or iron(III), the iron concentrations measured by the ferrozine assay were higher than those obtained from the A₃₅₀, indicating that some of the iron in FeSOD was present as Fe²⁺. The iron content calculated by the ferrozine assay was often more than 2, which means that there was nonspecific binding of iron ions outside of the protein active site. Incubation with 0.5 mM EDTA followed by a desalting using a G25 column was used to remove the extra iron ions. After the treatment, the Fe stoichiometry of mutant FeSOD was 0.63 and that of the WT was 1.40.

3.3.4 Multiple forms of FeSOD depending on the gene from which it was produced (Native gel (NBT assay))⁶⁸

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify the purity of FeSODs produced by mutant gene and wild type gene. Figure 3.7 shows the SDS-PAGE result from both of the FeSODs. According to the SDS-PAGE, both of the purified FeSODs were pure according to the only band in the gels.

Non-denaturing polyacrylamide gel electrophoresis was used to separate SOD from other proteins. FeSOD was localized by soaking the gels in 2.45 mM nitro

blue tetrazolium (NBT) for 20 min, followed by immersion in a solution containing 28 mM tetramethylethylenediamine, 28 μ M riboflavin, and 36 mM potassium phosphate at pH 7.8 for 15 min. The gels were then illuminated using a fluorescent lamp, causing photoexcited riboflavin to reduce O_2 and produce $O_2^{\bullet-}$ (superoxide) that in turn reduced NBT to purple precipitate (blue formazan). However, positions with SOD do not change color, since $O_2^{\bullet-}$ is consumed by SOD. Thus, the positions of active SOD are visible as dye-free zones.

Native gels stained using the NBT assay were used to compare mutant and WT FeSOD. Figure 3.8 shows that mutant FeSOD always migrated as 2 bands despite the fact that mutant protein ran as a single band in SDS PAGE. Although both bands possessed activity, the lower band was always smaller and had less activity than the upper band (Three separate growths and purifications yields mutant FeSOD in which the lower band contained 1/3, 1/3 or 1/10 as much protein as the upper band, whereas the lower bands displayed only 1/10 to 1/30 activity as the upper band). Typical results are shown in Figure 3.8. Even though the lower band concentration was about one half that of the upper band, according to the Coomassie-brilliant-blue-stained non-denaturing gel, the activity of the lower band was much less than one half that of the upper band according to the NBT assay conducted on a duplicate gel. (Dilution series were run for each preparation to permit semi-quantitative assessment of relative amounts of proteins in the different bands).

However, after correction of the gene, there were significant differences with respect to the lower bands. The lower band decreased in significance or even disappeared. And the activity of the lower bands was still much less than that of the upper band, on a per-protein basis. Since our assays of specific activity and Fe content (below) indicated that the WT protein was fully metallated, we could conclude that the upper bands were the native dimers of the wild-type FeSOD.

The silent mutation may change the structure of FeSOD, and affect the Fe content in each FeSOD dimer. Even though these two structures can't be distinguished by SDS-PAGE, they can be separated by the non-denaturing gels. The

structure corresponding to the lower bands in the non-denaturing gels has a lower activity on a per/protein basis than the upper bands and we had found that mutant FeSOD contained less Fe per protein dimer overall. Therefore, we hypothesized that there might be less Fe bound to the lower band protein.

3.3.5 Iron content of the different forms of FeSOD (in-gel stain)⁶⁹

The iron in SOD was localized by immersing the non-denaturing polyacrylamide gels in which FeSOD had already been run in a solution containing 50 mM pH 5.0 sodium acetate, 40 mM hydrogen peroxide (H_2O_2) and 80 mM diaminobenzoic acid dihydrochloride at 35 °C for 30 min. The gels were then rinsed with 7% acetic acid. In this process, any Fe present catalyzes oxidation of diaminobenzoate by H_2O_2 , producing an insoluble pigment in the gels.

Comparison of in-gel iron assay results for mutant and WT FeSODs are shown in Figure 3.9. The gel that was stained with Fe was one of two identical gels, and the other was stained with Coomassie brilliant blue to review the locations of protein bands. Nitroreductase was used as a negative control since there is no iron in nitroreductase. Even though these gels revealed another protein above the band we referred to as the upper FeSOD band, there was no iron in that protein according to the staining applied (Figure 3.9B). Based on the strong staining of the upper bands, there was clearly iron bound to all the dimers of wild-type FeSOD. This was also true of the upper band of mutant FeSOD. The lower band of the mutant FeSOD had a shadowy pigmentation revealing bound iron based on the iron in-gel assay, or at least some metal able to catalyze oxidation of diaminobenzoate. However, comparison of the upper band of lane 8 with the lower band of lane 3 shows that the latter was less Fe on a per-protein basis than does the former. Thus we conclude that the lower band is FeSOD that contains less than the full complement of Fe.

3.3.6 Nature of Fe-binding sites in FeSOD produced from the two FeSOD genes EPR spectra

EPR spectroscopy was used to assess the integrity of those sites that do acquire Fe, for the FeSOD produced based on the mutant gene and the WT gene. Figure 3.10 shows the spectra of two FeSODs at the same protein concentration. The spectra confirm the presence of Fe^{3+} in both active sites. Since the major features in

these two spectra occur at the same field strengths, they indicate that Fe^{3+} adopts the correct coordination number and geometry in the protein from the mutant gene. However, FeSOD produced from the WT gene has more rhombic Fe^{3+} (shoulder peak near 1500 G), which indicates that there is some Fe^{3+} bound outside the active site on FeSOD produced by mutant gene. The active site peak is stronger than the non-active site peak for FeSOD produced by WT gene however the reverse is true in FeSOD produced by the mutant gene. Moreover, the mutant gene FeSOD also had copper signature hyperfine splitting around 3000G than the FeSOD from the WT gene, indicating that the mutant gene FeSOD may have acquired some copper from the medium. Since the protein dimer concentrations for both the SODs were the same, the EPR spectral results were consistent with the optical spectra, which also indicated that the active site Fe^{3+} concentration in FeSOD produced based on the WT gene was twice that in FeSOD produced by the mutant gene (Table 3.1).

3.3.7 Secondary and tertiary structure of FeSODs made from the two genes

The secondary and tertiary structures of FeSODs produced from the mutant gene and the corrected WT gene were assessed by circular dichroism (CD). The far-UV (ultraviolet) CD spectra of these two FeSODs are shown in Figure 3.11. According to the spectra, the FeSODs have similar secondary structures and showed strong features characteristic of α -helical content in the far-UV region. The near-UV CD spectra in Figure 3.12 provide information on the tertiary structure of FeSODs via the intrinsic CD of tryptophans. According to the FeSOD amino acid sequence, there are 11 phenylalanines (F), 9 tyrosines (Y), and 7 tryptophans (W), which make up the absorptions around 280 nm in near-UV CD. However tryptophan has a higher intrinsic optical activity and extinction coefficient so the signal is dominated by tryptophans.

3.4 Discussion

3.4.1 Rare codon of silent mutation

The diminution of culture vitality over longer culture times and the low Fe incorporation into an over-expressed FeSOD was noticed at the beginning of the

project. Upon re-isolation of the overexpression plasmid from these cultures and sequencing of the FeSOD gene, we found out there was a L14L silent mutation.

Overexpression of the wild-type SOD gene had no deleterious effect, so the decline of the culture expressing the mutant gene was not due to the metabolic burden of gene expression *per se*. The only difference between the two genes is that the mutant gene employs a different codon for Leu14, so we conclude that the effects on the culture are due to that codon's identity.

The silent mutation in our FeSOD gene replaces a common codon (CTG accounts for 55% of leucine condons in *E. coli*⁷⁰) with a rare one (TTG accounts for 11% of leucine in *E. coli*). Rare codon use has been shown to cause higher levels of abortive translation resulting in diminished yields of gene product^{56,58-60}. Either the tRNA or the aminoacyl-tRNA synthase needed for translation of that codon may become limiting when a gene employing a rare codon is strongly expressed, as is done when over-producing a protein. Not only will production of the sought-after protein be compromised, but so also will be production of all other proteins dependent on that codon. This can explain why presence of the rare codon does not decrease culture health when the gene is repressed, but causes culture decline when the gene is expressed.

3.4.2 Activity and Fe incorporation

Electrophoresis under non-denaturing conditions revealed that FeSOD preparations produce at least two forms of FeSOD. Of the two dominant forms, the lower band accounts for almost half of FeSOD produced by the mutant gene but only a very minor fraction of the protein for FeSOD produced from the corrected gene. Moreover the lower band contributed a negligible portion of the catalytic activity of FeSOD from the WT gene.

The situation is more complicated for FeSOD produced from the mutant gene. While the lower band does not produce a discrete dye-free zone such as that produced by the upper band, there is extensive streaking between the two positions and the position of the lower band displays a marked increase in the amount of dye. The streak between the positions of the two bands might correspond to protein with

properties intermediate between those in the two bands, but it is interesting to note the streak's virtual absence from FeSOD expressed based on the WT gene. The increase in dye density right at the position of the lower band suggests that this protein actually produces superoxide or otherwise favors some reaction with NBT. Indeed the darkened oval on the bottom edge of the streak can be seen by eye to have a slightly pinker color than the bluer-purple of the dye formed elsewhere in the gel. In any case, the SOD activity of the lower band is lower than expected based on the amount of protein at that position for both FeSODs, and the amount of protein in the lower band is significantly greater for FeSOD based on the mutant gene.

When gels were stained with diaminobenzoic acid to visualize Fe, it was apparent that the lower band possesses much less Fe on a per-protein basis. For example based on the amount of protein in the lower band of FeSOD produced from the mutant gene, one expects similar amounts of Fe in the two bands as well. However the corresponding lane reveals much less Fe in the lower band than in the upper band. The location of the Fe coincides with the apparent pro-oxidant activity of FeSOD produced from the mutant gene.

The large improvement in Fe stoichiometry observed upon extending the post-IPTG growth interval from 4 hours to overnight is consistent with the decreased population of lower band in conjunction with the lower band's lower Fe content. Thus, increased production of a Fe-poor second species (lower band) can account for the lower Fe content of FeSOD from the mutant gene as well as its lower activity.

People may argue that chromosomal SOD may count for some of the activity, since the strain used in the project does express its own chromosomal SOD. However, the SOD from chromosomal gene and plasmid gene can be quantified according to the SDS-PAGE before and after IPTG induction. Before IPTG induction, there was no more than 5% of total SOD producing after IPTG showing in the SDS-PAGE (Figure 3.13), indicating that the chromosomal SOD contribute less than 5% of SOD activity.

Quantitation of Fe showed that FeSOD from the WT gene incorporates iron atoms in every active site when grown overnight following addition of IPTG

whereas the maximum amount of Fe incorporated in FeSOD produced from the mutant gene was only 1 Fe per dimer.

It is also possible that Fe is not bound correctly in FeSOD from the mutant gene. EPR spectra and optical spectra for the FeSODs produced from the mutant gene and the WT gene were compared and consistent with one another. The optical spectrum of FeSOD produced from the mutant gene showed a weaker signal from Fe^{3+} but the absorption maximum was the same as that of FeSOD from the mutant gene, as was the overall shape of the signal. Similarly, the EPR signals of FeSOD from the mutant gene and the WT gene reveal similar coordination environments, but they suggest that a larger fraction of the Fe bound to FeSOD from the mutant gene is not coordinate, and may even be bound outside the active site. Thus the lower amount of Fe present can account for the lower activity of FeSOD produced from the mutant gene, but the presence of a small population of non-native sites may also contribute to the relative lack of activity. Incorrect or incomplete metal ion binding could cause or result from a non-native structure. However the CD spectra do not reveal any difference in the secondary structural content of FeSOD or the environment of the tryptophan side chains.

3.4.3 Structure change by silent mutation

According to the primary sequence of FeSOD, the leucine14 silent mutation is located in the beginning of N-terminus. When ribosome produces the protein, the formation of leucine14 precedes any of the Fe ligand amino acids. From the secondary structure, the side chain of leucine14 is in the middle of a small hydrophobic cluster. This small hydrophobic cluster is located between the two domains, and brings the N-terminal helix and the last helix in C-terminus together. As we know, the Fe active site is located between the two domains, any effect that brings the two domains together will favor the Fe incorporation. We propose that the usage of rare codon TTG may slow down the speed of FeSOD peptide synthesis, since either the tRNA or the aminoacyl-tRNA synthase needed for translation of that codon may become limiting when a gene employing a rare is strongly expressed, as is done when over-producing the protein. At the same time, the N-terminus peptide

before Leu14 may adapt another structure. When synthesis is finished, the hydrophobic cluster formed around the Leu14 may have less ability to bring the two domains together, and then affect the active site Fe incorporation. On the other hand, when the silent mutation has been corrected by site-directed mutagenesis, the speed of peptide synthesis is not hindered, and FeSOD can fold the right second structure, and incorporate the Fe atom well.

Even though is not observed in metalloenzyme like FeSOD, a paper describing another protein in 2007 Science⁶⁵ is in the similar situation as our case. The researchers found out that some cancer patients develop resistance to chemotherapy during cancer treatment, and this led them to discover a silent mutation in P-glycoprotein, called multidrug resistance 1 (MDR1). The rare codon of silent mutation led to a change in the mRNA, and caused the ribosome slow down the speed of protein synthesis⁷¹. The author stated that the N-terminal proregion, which synthesized before the silent mutation may play a role as “intra-molecular chaperone” (IMC), which can change the energy landscape, and further lead the protein to adopt another conformation rather than the native form. Amazingly, the original intention to discover this silent mutation has a surprising resemblance to our case. Just as us, their research “started with an expression problem”⁷². At the beginning of the research, they had a hard time to express large quantities of the protein in bacterial cells. They found out a codon replacement (ATC to ATT) existed, but they were both encode the same amino acid isoleucine. Even though through the past generations, scientists assumed that silent mutations don’t change proteins’ function, more and more clinical data show that these silent mutations can cause some kinds of disorders or diseases. So maybe there is purpose to use frequent codons for highly expressed proteins by nature, which is making sure the protein making and folding process faster and more accurate.

Another convincing example is published in 2011 by Brest *et al.* in Nature. The silent mutation they found is the same with our silent mutation in FeSOD, which is a CTG to TTG mutation for leucine in the coding region of a gene called “immunity related GTPase family, M” (IRGM protein). And they found that this silent mutation is associated with Crohn’s disease. They proposed that the usage of rare codon may

interfere the rhythms of RNA passing through the ribosome, and further interfere the protein folding⁶⁶.

3.4.4 Insight of the project

Silent mutations are assumed to be silent in the past years, since these mutations would not change the protein amino acid sequences. Without knowing the gene's nucleotide sequence, the biochemist Herbert Boyer synthesized the human hormone somatostatin by using *E. coli* favored codons⁷³. And this event became a milestone in the development of biotechnology. Since then, making silent mutations in the gene sequence became a very important and standard practice in pharmaceutical industry. That's why more and more gene therapies, antibodies and vaccines are produced based on adapting synonymous codons. Some of the gene sequences of the constructed proteins may be altered by as much as 80% from their native forms⁷². However, in the past few years, people found out that silent mutations may have effect on human health. Just as the introduction indicated, more and more groups are dedicated to study silent mutations. For example, most recently in the Nature magazine, Brest, P. *et al.* found out the leucine to leucine silent mutation may affect IRGM gene expression and associate with Crohn's disease; and another paper in the Science magazine by Kimchi-Sarfaty *et al.* indicated that silent mutations may cause multidrug resistance protein behave differently in cancer cells. However, neither of the two cases is about metalloenzymes like FeSOD.

Our project mainly focuses on the fact that the Leu14Leu mutation affected the metalloenzyme FeSOD maturation and active site metal Fe incorporation. In order to find out whether there is any research about this field going on in the world, a drastically web-searching was executed through Web of Knowledge. Even though there are 8139 hints about "silent mutation" in the whole timespan, there is no hint related to "silent mutation" and "metal incorporation", "silent mutation" and "metalloenzyme", or "silent mutation" and "maturation". There are only 21 hints related to "silent mutation" and "superoxide dismutase". Among the 21 hints, there are only 7 papers addressed the issue of silent mutations in SOD⁷⁴⁻⁸⁰. All of them are

related to copper/zinc SOD (Cu/ZnSOD). Among these papers, some of them concluded that silent mutation may have effect on the characteristics of Cu/ZnSOD, some of them addressed that the effect remained to be further explained. For example, Campo, S. *et al.* had reported that the silent mutation (GCG to GCT) encoded alanine of Mediterranean population had no significant effect on the protein activity and it may not related to several diseases, such as diabetes and atherosclerosis⁷⁸. On the other hand, another paper⁷⁵ addressed an A140A (GGT to CCA) silent mutation was associated with familial amyotrophic lateral sclerosis disease (FALS). The author proposed that the mutated mRNA might be cytotoxic of the silent mutation. Therefore, whether silent mutations can affect Cu/ZnSOD activity is very controversial.

However, as far as our knowledge, the thesis is the first time to publish the relationship of silent mutation and the metalloenzyme FeSOD maturation/activity. It shows that the silent mutation did affect the FeSOD maturation, Fe incorporation and the protein activity. This new discovery may reveal a new relationship between silent mutations and FeSOD protein. Hopefully, people may not ignore the sound of mutation any more in the near future.

Table 3.1: Metal contents and FeSOD yield from different preparations.

	Gene correction	Duration of growth after addition of IPTG	[Fe]/[dimer]*	Yield from 2L growth/mg
1 st preparation	no	4 hours	1.05	80
3 rd preparation	no	12 hours	0.81	90
5 th preparation	yes	4 hours	1.53	90
6 th preparation	yes	12 hours	1.92	150

*Concentrations were calculated based on A₃₅₀ and A₂₈₀

Table 3.2: Comparison of FeSOD from the mutant and WT gene.

	[Fe]/dimer by A ₃₅₀	Activity (U/mg)*	Activity/Fe (U/mg)*
silent mutant ^a	0.9±0.2	8400	9000
WT (corrected) ^b	1.7±0.2	19000	11000

*SOD activity was measured using the cytochrome C-reduction assay.

a The culture was harvested 4 hours post IPTG.

b The culture was harvested after overnight growth post IPTG.

Table 3.3: Iron content based on the A₃₅₀ and ferrozine assay.^a

	[Fe]/dimer by A ₃₅₀	[Fe]/dimer by ferrozine assay
silent mutant ^b	0.9±0.2	0.6±0.2
WT (Corrected) ^c	1.7±0.2	1.4±0.2

^a Data were collected after EDTA treatment.

^b The culture was harvested 4 hours post IPTG.

^c The culture was harvested after overnight growth post IPTG.

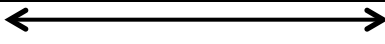
95 °C	95 °C	60 °C	68 °C	68 °C	4 °C
2 min	20 sec	10 sec	210 sec	5 min	∞
	<div style="text-align: center;">  </div>				
	18 cycles				

Figure 3.1: PCR program for site-directed mutagenesis the FeSOD gene

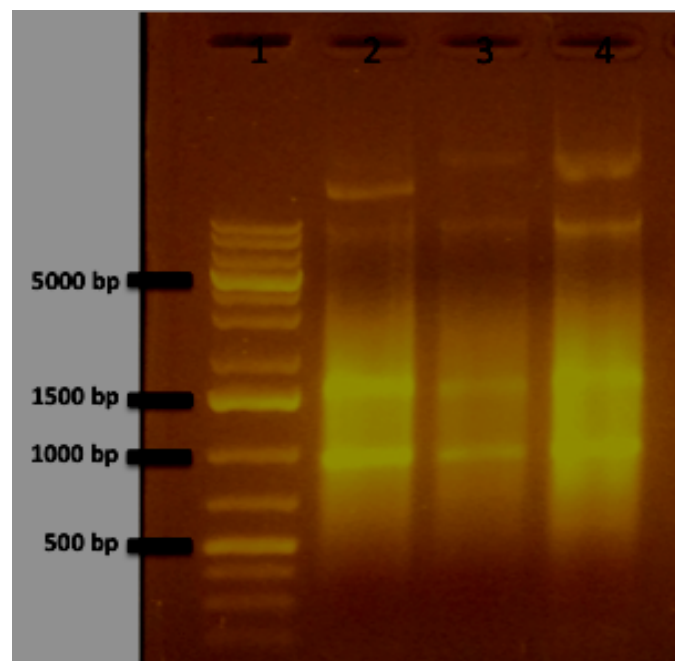


Figure 3.2: 1% agarose gel analysis of FeSOD gene-bearing plasmid after double digestion with Nde I and BamH I. Lane 1, the DNA ladder (GeneRuler™ 1kb DNA ladder from Fermentas). Lanes 2 to 4 were the double digestion of plasmid from 3 cultures inoculated with three colonies, individually.

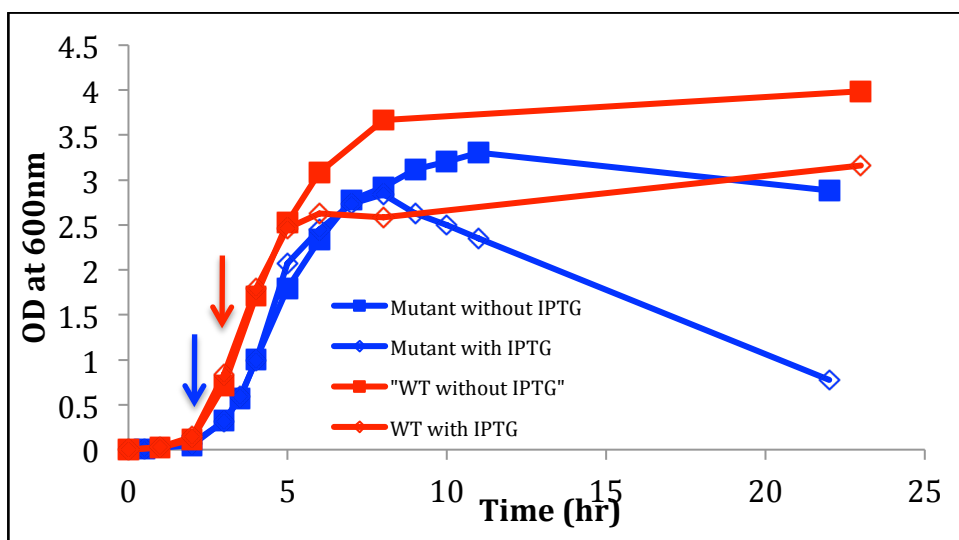


Figure 3.3: Growth curves before and after mutagenesis. The arrows indicate the addition of IPTG.

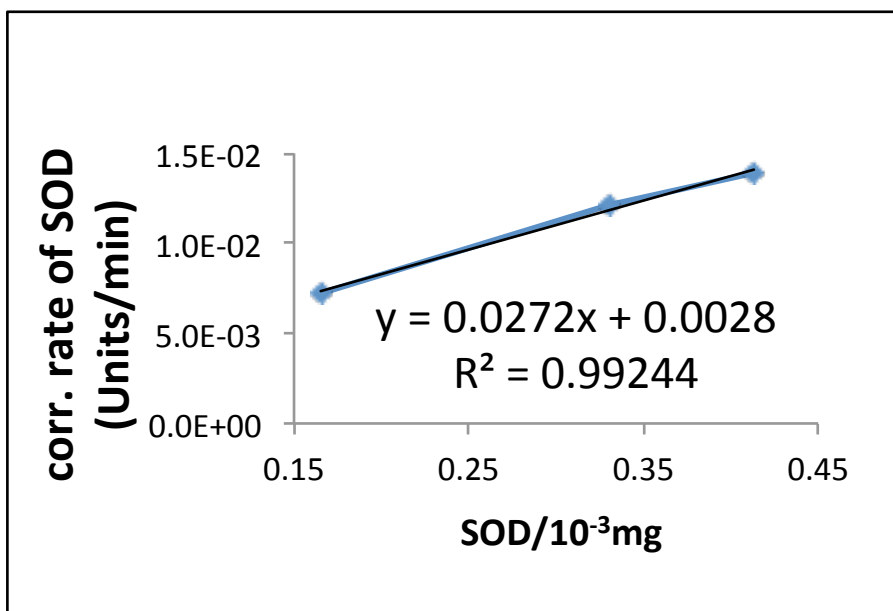


Figure 3.4: Cytochrome C-reduction assay for the mutant gene FeSOD. Decrease in rate of cytochrome C reduction due to superoxide consumption by FeSOD.

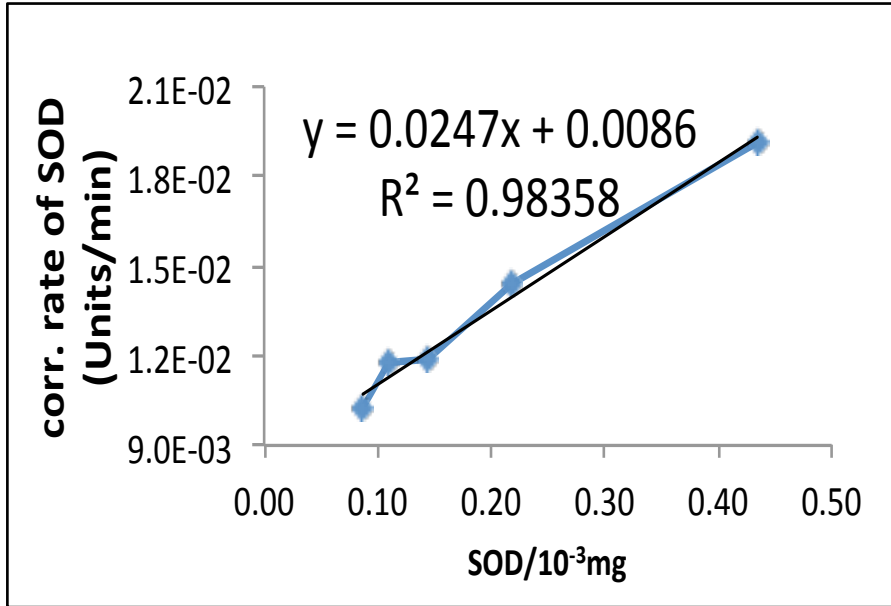


Figure 3.5: Cytochrome C-reduction assay for the WT gene FeSOD. Decrease in rate of cytochrome C reduction due to superoxide consumption by FeSOD.

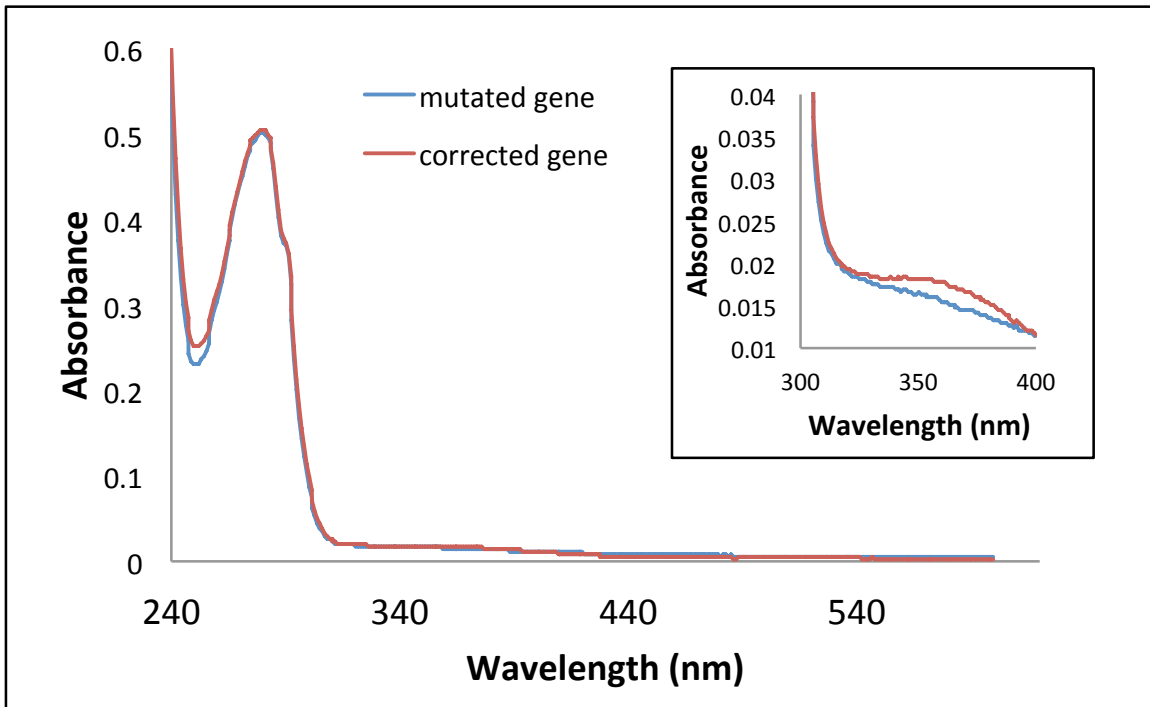


Figure 3.6: Absorbance spectra of FeSOD before and after site-mutagenesis.

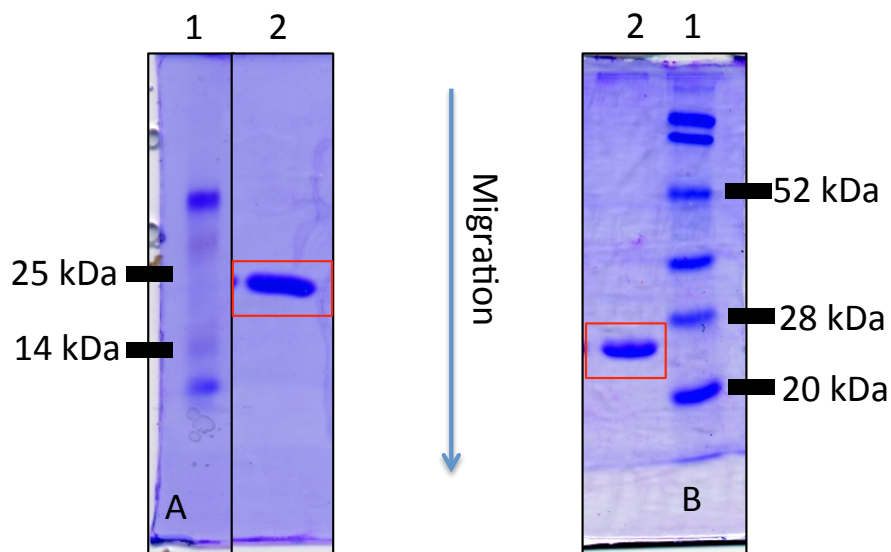


Figure 3.7: SDS-PAGE for FeSODs produced by mutant gene (Panel A) and wild type gene (Panel B). Lane 1 in panel A is Kaleidoscope Prestained Standards (310006790, from BIO-RAD); lane 1 in panel B is Prestained SDS-PAGE Standards (310006667, from BIO-RAD).

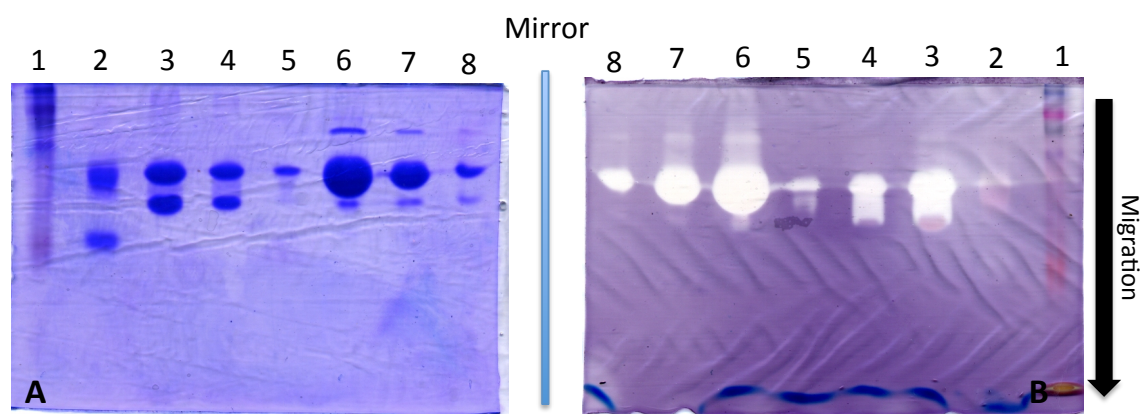


Figure 3.8: Comparison of NBT assay of “before site-directed mutagenesis” preparation and “after site-directed mutagenesis” preparation. Panel A was native gel soaked into Coomassie Blue; Panel B was the gel soaked in NBT buffers. Lane 1 was the molecular weight ladder; Lane 2 was Nitroreductase protein (negative control for iron in-gel assay); Lane 3 was purified FeSOD without dilution from the 1st preparation; Lanes 4 and 5 were 3- and 30- fold dilution of the material loaded in the lane 3; Lane 6 was purified FeSOD without dilution from the 6th preparation; Lanes 7 and 8 were 3- and 30- fold dilution of the material loaded in the lane 6.

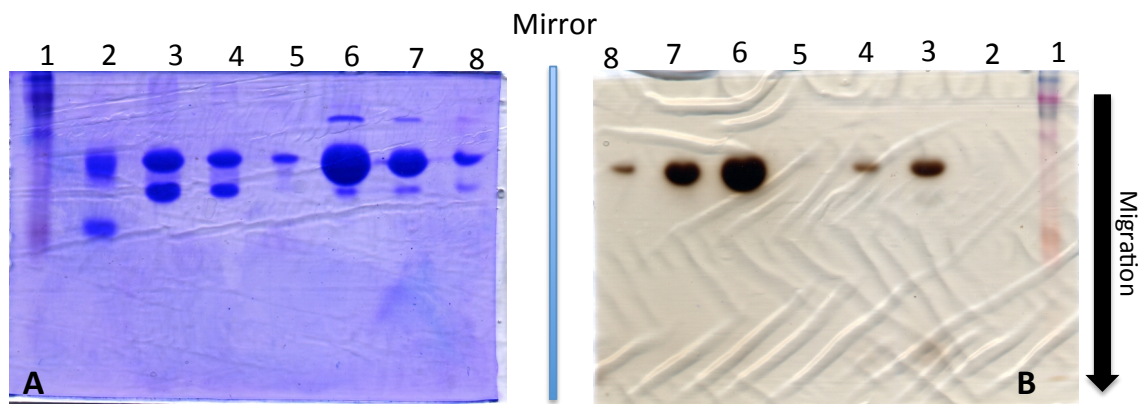


Figure 3.9: Iron in-gel assay for “before site-directed mutagenesis” preparation and “after site-directed mutagenesis” preparation. Panel A was native gel soaked into Coomassie Blue; Panel B was the gel soaked in iron detecting buffers. Lane 1 was the molecular weight ladder; Lane 2 was nitroreductase (negative control); Lane 3 was purified FeSOD without dilution from the 1st preparation; Lanes 4 and 5 were 3- and 30- fold dilution of the material loaded in lane 3; Lane 6 was purified FeSOD without dilution from the 6th preparation; Lanes 7 and 8 were 3- and 30- fold dilution of the material loaded in lane 6.

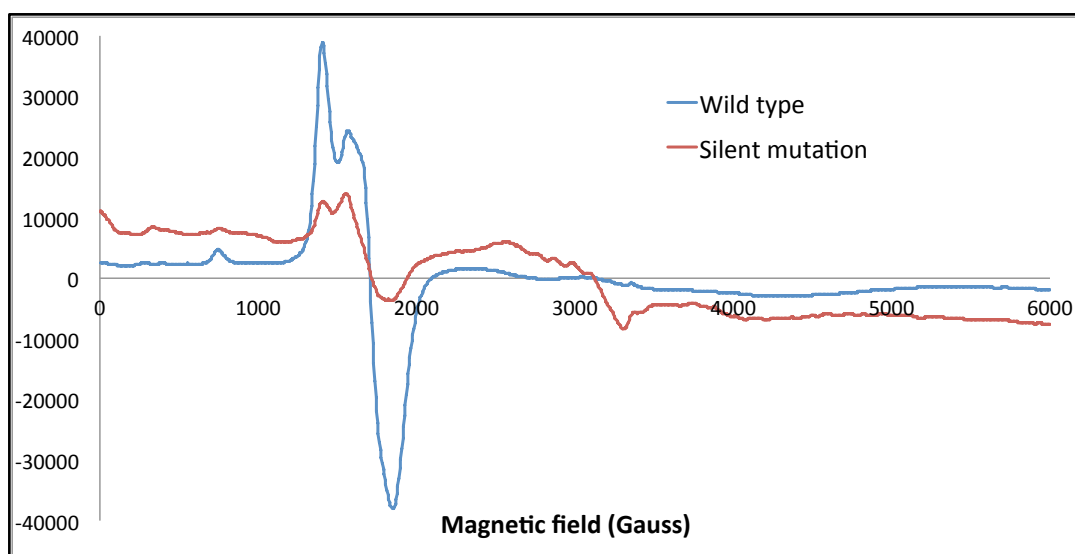


Figure 3.10: EPR spectra for the FeSODs produced based on mutant gene and corrected gene.

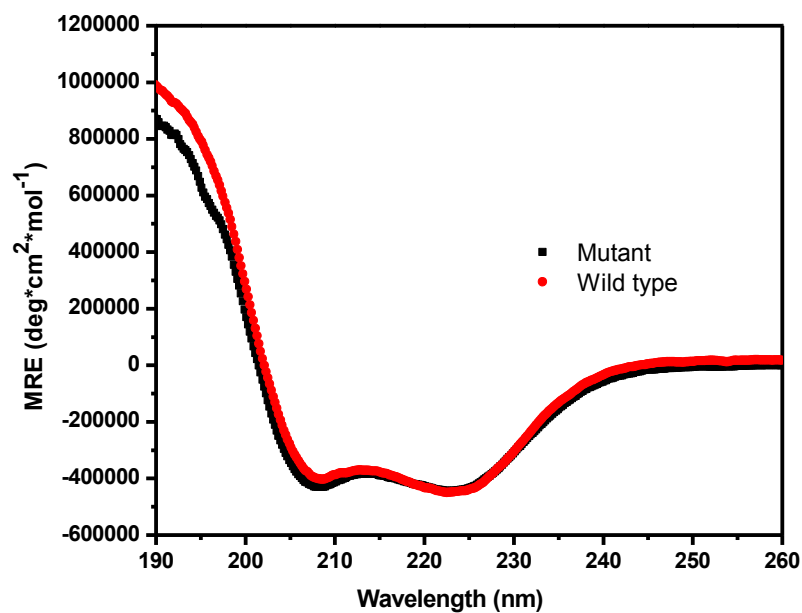


Figure 3.11: Far-UV spectra of FeSODs produced by the mutant and wild-type gene.

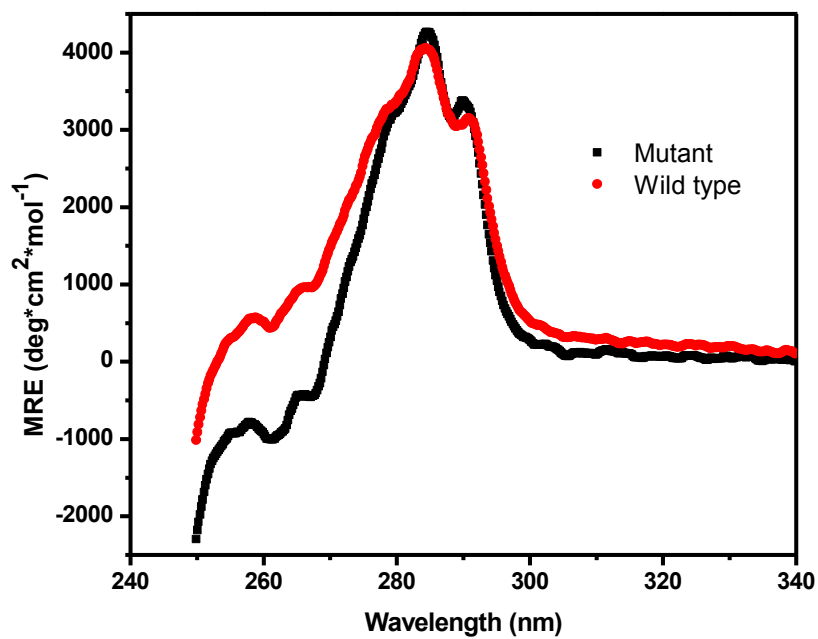


Figure 3.12: Near-UV spectra of FeSODs produced by the mutant and wild-type gene.

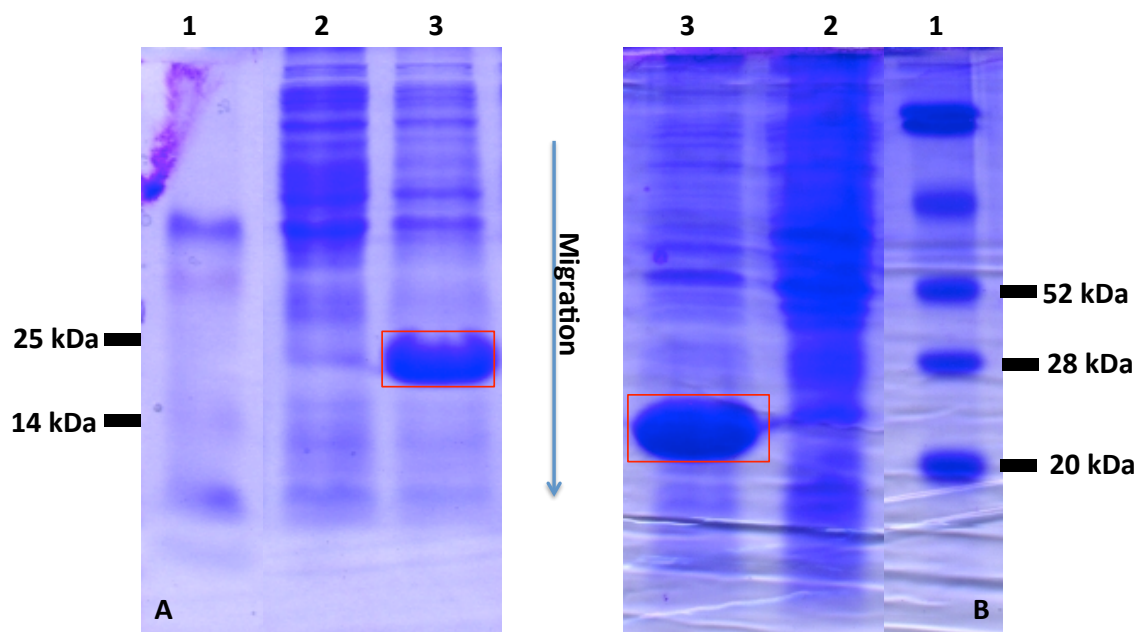


Figure 3.13: SDS-PAGE for FeSODs produced by mutant gene (Panel A) and wild type gene (Panel B) before and after IPTG induction. Lane 1 in panel A is Kaleidoscope Prestained Standards (310006790, from BIO-RAD); Lane 2 in Panel A is FeSOD produced by mutant gene before IPTG induction; Lane 3 in panel A is FeSOD produced by mutant gene after IPTG induction; Lane 1 in panel B is Prestained SDS-PAGE Standards (310006667, from BIO-RAD). Lane 2 in Panel B is FeSOD produced by wild type gene before IPTG induction; Lane 3 in panel B is FeSOD produced by wild type gene after IPTG induction

CONCLUSIONS

In this project, *E. coli* strain pRK3-1 was grown, and FeSOD was purified. In order to obtain good yield and high quality FeSOD, the conditions of growth and purification were optimized. 0.5 mmol/L final concentration of IPTG was used to overexpress the FeSOD gene. Different amounts of aeration also affected the yield of FeSOD. 2 L LB medium in 6 L flask was found to have a good amount of oxygen to improve culture yield.

Even using the optimized condition, the FeSOD activity and Fe content were not satisfied. After sequencing the FeSOD gene, a silent mutation was found, and corrected through the use of site-directed mutagenesis. Upon the gene correction, the iron content was improved from around 1 to close 2, which is the theoretical iron content in FeSOD. The native gels, NBT assay, iron in-gel stain assay, and EPR spectra support this result. CD spectra were applied to prove that the two FeSODs have the similar secondary and tertiary structures.

The purification process eliminates the undesired protein. Different temperature of heat-cut were tested, and 50 °C was used throughout the following preparations. SDS-PAGE gels were used to visualize the purification process and the purity of FeSOD.

After site-directed mutagenesis, overnight growth following addition of IPTG increased both FeSOD yield and its activity. Compared with 5 hours growth post IPTG, overnight growth following the addition of IPTG can provide 60% higher protein yield and 50% higher activity. We speculate that after site-directed mutagenesis and longer time growth, there are more tRNAs to utilize by the cells and able to form correct protein structure and uptake metal iron.

Even there are several papers published recently that silent mutations may affect the structures and activities of proteins, and cause some of diseases^{49,65}, there is no such paper studying metalloenzymes. According to the web search up to date, there is no such study about FSOD, and this thesis may be the first time to publish the relationship between silent mutation and maturation/activity of FeSOD.

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- “Time Matters: Different Growth Time Affects Wild-Type Iron SOD Protein Activity and Yield”, 1st Southeast Enzyme Conference. Atlanta, GA, Apr 10, 2010.
- “Comparisons of Fe-Superoxide Dismutase and Fe-Substituted (Mn) Superoxide Dismutase”, 30th Midwest Enzyme Chemistry Conference. Chicago, IL, Oct 16, 2010.