Controlled Conjugation of [Cu,Zn] Superoxide Dismutase: An Active Tetramer

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

While the catalytically powerful [Cu,Zn] superoxide dismutase (SOD1) possesses great potential as a therapeutic, unfavorable properties in circulation limit its use in clinical medicine. The small, water soluble dimer is rapidly excreted by the kidney. Previous initiatives have been used to increase the mass of the enzyme (PEGylation, liposome encapsulation). This has resulted in highly heterogeneous mixtures of modified SOD1, which are difficult to characterize. Furthermore, these modified proteins have utilized foreign material that has shown to elicit an inflammatory response. We developed an improved strategy that creates a homogenous high molecular weight SOD1 based on combinations of the protein itself. This was accomplished through the addition of a site-specific, azide functionalized cross-linker to unmodified SOD1, followed by the conjugation of SOD dimers using CuAAC and a bis-alkyne linker to form a 64 kDa SOD tetramer. The final product, bis-SOD, presents the fully catalytic activity of the combined proteins.
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Finally and most importantly, I would like to thank my parents, sister and Geoffrey Law. Their unwavering and enthusiastic encouragement is, and will continue to be the motivation behind my academic pursuits.
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List of Abbreviations

Batho: Bathophenanthroline
CD: Circular dichroism
CID: Collision induced dissociation
CuAAC: Cu(I) catalyzed azide-alkyne cycloaddition
Da: Dalton
DBS: 3,5 dibromosalicylate esters
DCM: Dichloromethane
DMAP: 4-(Dimethylamino)pyridine
DMSO: Dimethylsulfoxide
DTPA: Diethylenetriaminepentaacetic acid
DTT: Dithiothreitol
EcSOD: Extracellular superoxide dismutase
EDC: N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide
ESI-MS: Electrospray ionization mass spectrometry
HPLC: High pressure liquid chromatography
LC-ESI MS/MS: Liquid chromatography coupled to tandem mass spectrometry
MWCO: Molecular weight cut-off
PEG: Polyethylene glycol
PDB: Protein data bank
ROS: Reactive oxygen species
SDS- PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC: Size exclusion chromatography
SOD: Superoxide dismutase
SOD1: [Cu,Zn] Superoxide dismutase 1
SOD-N₃: [Cu,Zn] Superoxide dismutase cross-linked with 9
TBTA: Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
THF: Tetrahydrofuran
TFA: Trifluoroacetic acid
THPTA: Tris(3-hydroxypropyltriazolylmethyl)amine
Tris: Tris(hydroxymethyl)aminomethane
TTDS: Trimesoyltris(3,5-dibromosalicylate)
UV-Vis: Ultraviolet-visible
TGase: Transglutaminase
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1 Introduction

1.1 [Cu,Zn] Superoxide Dismutase as a Therapeutic Agent

Molecular oxygen, although essential for life, is reduced in circulation to reactive oxygen species (ROS) that have harmful properties. One type of ROS is the superoxide ion (O$_2^-$), a species that can lead to significant tissue damage$^1$. At physiological concentrations in aerobic organisms, ROS are used in cell signaling pathways and also as defense mechanisms to combat invading pathogens.$^2$ However, at elevated concentrations, ROS can lead to oxidative stress and contribute to the development of various diseases, including cancer, ischemic reperfusion injury and premature aging.$^3$$^4$$^5$ While ROS, such as hydrogen peroxide (H$_2$O$_2$), are relatively long-lived, the superoxide anion is unstable and quickly reacts with surrounding hydrogen donors to form damaging hydroxyl radicals. This property makes superoxide a particularly potent initiator in the cellular oxidative stress cascade.

Superoxide dismutases (SODs) provide an important line of defense against ROS, acting as biological antioxidants. Within the SOD family of enzymes is [Cu,Zn] superoxide dismutase (SOD1). SOD1 is a ~32 kDa homodimer where each monomer contains a Cu$^{2+}$ and Zn$^{2+}$ ion. While the Zn$^{2+}$ ion is essential for structural stability of the protein, the Cu$^{2+}$ ion is responsible for enzymatic function.$^6$ In a two-step process, Cu$^{2+}$ catalyzes the disproportionation of the superoxide anion radical to molecular oxygen and hydrogen peroxide (Figure 1.1).$^7$$^8$

\[
\text{Cu}^{2+} + \text{O}_2^- + \text{H}^+ \rightarrow \text{Cu}^+ + \text{O}_2 + \text{H}^+ \quad (1)
\]

\[
\text{Cu}^+ + \text{H}^+ + \text{O}_2^- \rightarrow \text{Cu}^{2+} + \text{H}_2\text{O}_2 \quad (2)
\]

**Figure 1.1: Disproportionation of superoxide anion by [Cu,Zn] superoxide dismutase**
H$_2$O$_2$ is ultimately transformed into water by the enzymatic action of catalase and peroxidases. With a $V_{\text{max}}$ of $2 \times 10^9$ M$^{-1}$ s$^{-1}$, SOD is among the most catalytically efficient enzyme found in nature, limited only by diffusion of the substrate.$^{9,10}$

This efficient enzyme has promising therapeutic potential as a means to re-establish balance between levels of ROS and antioxidant defense systems in cases of oxidative stress or disease. Cytosolic [Cu,Zn] SOD (SOD1) is particularly favoured over other members of the SOD family of enzymes, as it is localized to the cytosol where oxidants are formed.$^{11}$ As such, cytosolic SOD1 are able to neutralize the superoxide before it leaves the cell, preventing oxidative damage.

However SOD, a small, highly water soluble protein, is rapidly excreted from the kidneys. The circulation half-life of SOD1 is only 5 minutes in mice.$^{12}$ Clinical studies in which supplemental SOD was introduced into physiological systems did not give promising results.$^{13,14,15,16}$ While exogenous SOD possesses excellent catalytic efficiency it probably does not remain in circulation long enough to be an effective therapeutic agent.

### 1.2 State of the Field

Two principal strategies have been used to increase the time prior to clearance of circulating SOD. Both approaches add mass to the dimer, so that the modified protein’s mass is above the molecular weight cut-off of renal filtration (30-50 kDa).$^{17}$ One strategy utilizes non-specific covalent linking of polyethylene glycol (PEG) to the enzyme through lysine acetylation with succinylated PEG (Figure 1.2).$^{18}$
While the attachment of PEG chains significantly increased the mass and half-life of the enzyme (~40 hour circulation time), excessive and non-specific modification yielded a highly heterogeneous product (32-100 kDa) with reduced enzyme activity.\textsuperscript{18} When assessing heterogeneous mixtures, the \textit{in vitro} characterization prior to clinical studies lacks accuracy and purification can become costly for large scale syntheses. In addition, PEG is a non-biodegradable macromolecule that is cleared slowly and its accumulation can be problematic. For example, anaphylactic reactions have been reported after treatment with PEG-modified enzymes.\textsuperscript{19}

Another strategy encapsulates a large amount of native SOD into liposomes. This approach can also delay renal filtration.\textsuperscript{20} A major problem associated with conventional liposomes is that they are recognized by the immune system as foreign substances and can be rapidly removed by phagocytic cells of the reticuloendothelial system.\textsuperscript{21}

In order to increase the viability of [Cu,Zn] SOD as a therapeutic agent, an improved stabilization strategy is needed. Ideally, this strategy must yield a homogeneous product without significantly reducing the activity of the enzyme or introducing potentially toxic materials into circulation.

### 1.3 Bioconjugation and Click Chemistry

Bioconjugation involves the covalent linkage of biomolecules to produce a new complex with improved properties. We proposed that bioconjugation of SOD to another SOD through the
coupling of dimers will form a 64 kDa SOD tetramer with improved properties in circulation. This new mass would place SOD above the filtration threshold of the glomerulus. As well, rather than adding potentially harmful and non-functional mass, size is increased with the protein added to the same protein.

Due to the numerous functional groups and reactivity patterns of amino acids, it is often difficult to site-selectively modify enzymes of interest. Therefore bioconjugation processes must be robust and selective. Our group has previously found success in coupling proteins using Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reactions.\textsuperscript{22,23} In these reactions, azide and alkyne moieties react in a bioorthogonal manner to connect the proteins through formation of a very stable tetrazole ring. The CuAAC reaction requires the Cu(I) catalyst – usually prepared with an appropriate chelating ligand – which maintains the Cu(I) oxidation state and accelerates the reaction. Cu(I) forms a Cu-acetylide intermediate, which in turn reacts with an azide to form a disubstituted tetrazole (Figure 1.3).\textsuperscript{24} This cycloaddition is called a “click” reaction because it meets Sharpless’s definition of being highly selective, high-yielding, and useful for joining two molecules together without the formation of undesired byproducts.\textsuperscript{25}
As a bioconjugation technique, CuAAC has advantages over other coupling techniques such as maleimide/thiol chemistry, which have lengthy reaction times and require use of excess substrate. Furthermore, unlike thiols, azides and alkynes do not occur naturally in biological environments and are therefore orthogonal functionalities. The CuAAC method has been used previously with SOD by Deiters et. al. to conjugate an unnatural amino acid containing an azido side chain. Subsequent assays revealed no significant loss to an enzyme’s activity following the reaction. This indicates a lack of perturbation to the metal ligands of the enzyme despite the presence of excess Cu(I) in solution.
1.4 Chemical Approaches in the Site-Selective Modification of Proteins.

To prevent heterogeneity in the final product, controlling the exact site of modification of the protein is crucial. Site-selective protein modification can be used as a form of control, leading to formation of a highly homogeneous product. This method ensures that the measured biophysical and biochemical properties of the resulting conjugate can be readily correlated to its structure.

Site-directed chemical modification takes advantage of the selectivity of a reagent for a functional group of a side chain of a specific amino acid or group of amino acids in local area. Selectivity is achieved by a combination of the unique chemical properties of amino acid side chains, the microenvironment within the protein, and the reaction conditions. This technique is commonly employed in affinity labeling, where the reagent contains a moiety that mimics an effector ligand of the enzyme, which directs it to the site of interest. This mimicry makes use of all the aspects of selectivity and may yield homogeneous and efficient modifications.

SOD1 has a pI of 4.95 and is therefore negatively charged under physiological conditions. There is, however, a cationic microenvironment that surrounds the Cu active site. Known as a ‘cationic funnel’, this channel serves to maximize the rate of diffusion of the anionic superoxide substrate into the active site. The funnel is lysine- and arginine-rich and includes key charged residues: Lys134, 120 and Arg141 (Figure 1.4). In addition, Glu131 helps focus anionic substrates toward the active site by providing a repulsive negative charge outside the active site channel.
Figure 1.4: X-ray crystal structure of bovine [Cu,Zn] superoxide dismutase (PDB ID: 1CBJ). The light blue and light green ribbons differentiate the identical monomers that form the SOD dimer. Copper and Zinc ions are represented by orange and blue spheres, respectively. Key residues for the cationic funnel surrounding the active site are highlighted in yellow. The image was captured using FirstGlance in Jmol.

Chemical agents that utilize electrostatic effects to impart site-selectivity have been previously used to target the cationic sites of hemoglobin. Those studies employed electron-dense, anionic dibromosalicylate (DBS) leaving groups attached to a cross-linker scaffold (Figure 1.5). The electron-withdrawing bromine substituents on the aromatic ring of the leaving group increase the reactivity toward the ε-amino nucleophiles of lysine residues. Klotz and coworkers noted the substituent effect is much greater with amino groups within the cationic region of hemoglobin. They concluded that the bromine substituents specifically increased
electrostatic interactions between the reagent and protein’s functional groups.\textsuperscript{36} In addition, the anionic carboxylate substituents also enhanced the electrostatic interaction.

![Figure 1.5: Structure of Trimesoyltris(3,5-dibromosalicylate) (TTDS) cross-linker that uses dibromosalicylate leaving groups (highlighted in blue) for site-specific modification of human hemoglobin A.](image)

We proposed that the cationic microenvironment in SOD should interact with DBS groups in a manner similar to hemoglobin. For SOD to participate in CuAAC, site-directing DBS groups could be used to install an azide moiety with control. Meeting this objective would require a bifunctional cross-linker whose purpose is two-fold. First, a cross-link using DBS groups would prevent dissociation of SOD into constituent subunits, extending the circulation half-life. Second, the cross-linking reagent would be able to introduce a new functionality that makes the enzyme available for bioorthogonal conjugation to alkyne-containing species.

As the active site structure is critical to enzyme function, modification of these residues may attenuate catalytic efficiency. Lys120 and Lys134 are residues within the active site that are susceptible to acetylation. A previous study by Argese et. al. demonstrated that modification of these functionally important residues resulted in decreased enzymatic activity by nearly 20-fold.\textsuperscript{37} Further studies by Tainer and co-workers showed that changing the shape of the funnel is
also detrimental to the enzyme. A summary of the distance between these residues, obtained from crystal structures of the enzyme, is outlined in Table 1. The catalytically important lysine residues in the active site of SOD are more than 11 Å apart. In designing the bifunctional cross-linker, a scaffold containing DBS groups at a distance less than 11 Å apart could prevent this undesired modification of the active site.

Table 1: Average distances between catalytically important lysine residues in the active site of bovine [Cu,Zn] superoxide dismutase. Distances were obtained using data from x-ray crystal structures viewed in FirstGlance in Jmol.

<table>
<thead>
<tr>
<th>Residues</th>
<th>Average Distance(Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYS120---LYS134</td>
<td>16</td>
</tr>
<tr>
<td>LYS134---LYS168</td>
<td>11</td>
</tr>
<tr>
<td>LYS134---LYS67</td>
<td>17</td>
</tr>
</tbody>
</table>

1.5 Purpose of This Study

This research was designed to develop a strategy for the preparation of an active, high molecular weight [Cu,Zn] superoxide dismutase through protein-protein bioconjugation. These goals were to be fulfilled in a two-step process. First, a singly cross-linked SOD dimer would be prepared through site-selective acetylation. We proposed that reaction of the enzyme with the bifunctional cross-linker 9 would form an azide-containing SOD (SOD-N₃) (Figure 1.6).
Molecular modeling using Spartan™ software gave a distance of 5 Å between DBS groups. This distance falls well below that of the separation of active site lysine residues, ensuring that chemical modification will not perturb the funnel shape. The rigid structure of the cross-linker is much larger than the superoxide anion. Such a disparity should prevent the reagent from entering the funnel and singularly acetylating lysine residues. Once formed, SOD-N₃ would be suitable for coupling to another SOD dimer using an electron-deficient bis-alkyne linker 10 to form a high MW species through CuAAC coupling (Figure 1.7). The resulting species, referred to as bis-SOD, would then be tested for the retention of enzymatic activity.
Figure 1.7: Strategy for the homogeneous synthesis of high MW SOD. Structure of bis-alkyne linker is inset (10). Reaction of the SOD dimer with azide-activated cross-linker (step 1) yields a SOD-azide (SOD-N$_3$). Coupling of the SOD azide with a bis-alkyne (step 2) produces a tetramer of SOD (bis-SOD).
2 Experimental Procedures

2.1 Materials and Methods

Unless otherwise noted, commercial reagents were used without further purification. Water was doubly distilled and deionized prior to use. THF was distilled and dried over sodium under reflux. Protein concentrations were determined using the Bio-Rad protein assay. Gel electrophoresis was used to analyze the composition of protein mixtures. High pressure liquid chromatography (HPLC) and centrifugal filters fashioned from regenerated cellulose were used to isolate desired components from protein mixtures. UV-Vis spectra were recorded on a UV-Vis spectrophotometer equipped with a thermostatted cell holder. Purified bovine [Cu,-Zn] superoxide dismutase was obtained from MP Biomedicals Canada. High-resolution mass spectrometry was performed at the Advanced Instrumentation for Molecular Structure (AIMS) Laboratory, Department of Chemistry at the University of Toronto. NMR spectra were recorded at 300 MHz ($^1$H), 75 MHz ($^{13}$C).

2.2 Synthesis of Cross-Linking Reagent

Reagent 1 was synthesized according to procedures outlined in a report by Yang.
2.2.1 Synthesis of Cross-Linker 9 2,2′-((5-((4-(azidomethyl)-2-bromophenyl)carbamoyl)isophthaloyl)bis(oxy))bis(3,5-dibromobenzoic acid).

Figure 2.1: Reaction scheme for the synthesis of 9
The synthesis of cross-linker 9 is shown in Figure 2.1. The starting compound, 2-bromo-4-methylaniline (1), was obtained from commercial sources. The amine (3.7 g, 0.02 mol) was dissolved in a 1:1 mixture of THF/H₂O (60 mL) and cooled to 0 °C. Di-tert-butyl dicarbonate was added (5.25 g, 0.024 mol) followed by sodium bicarbonate (6.72 g, 0.080 mol). The mixture was stirred for 10 minutes then left overnight at room temperature. The resulting mixture was refluxed for 5 hours. 200 mL H₂O was added to the mixture, which was then extracted with DCM, dried, and evaporated in vacuo to give a light yellow oil. The desired product was isolated from the starting material via chromatography with 2% ethyl acetate in. Compound 2 was synthesized in 50% yield.

Compound 2 (1.43 g, .005 mol) was dissolved in 10 mL of carbon tetrachloride. N-bromosuccinimide (978 mg, 5 mmol) and benzoyl peroxide (24 mg, 0.10 mmol) were then added. The mixture was heated to reflux and stirred for four hours. The reaction mixture was washed with water, K₂CO₃ (aqueous) and brine and dried over MgSO₄. Filtration and evaporation of solvent in vacuo gave an orange-colored oil (Compound 3).

To install the azide moiety, the product was dissolved in anhydrous DMF (5 mL) to which NaN₃ (650 mg, 10 mmol) was added. The mixture was heated to 65°C and stirred overnight. Water (50 mL) was added to the mixture, which was then extracted with diethyl ether. The ether extracts were further washed with water, dried and then the solvents were evaporated in vacuo to give a light yellow oil. It was separated by chromatography with 2% ethyl acetate in hexanes. Compound 4 was obtained in a 71 % yield.
Deprotection of 4 was carried out by dissolving the material (500 mg, 1.53 mmol) in dry dichloromethane (10 mL) to which TFA (1 mL) was added slowly. The light yellow solution was stirred at room temperature and slowly turned orange. After one hour, saturated sodium bicarbonate in water was added until bubbles ceased to form. The solution was extracted with ethyl acetate and dried over magnesium sulfate. The solvents were removed in vacuo to afford 5 as an orange oil (95% yield).

In the synthesis of compound 6, diethyl 1,3,5-benzene-tricarboxylate (0.5 g, 1.88 mmol) was converted to an acid chloride by refluxing in thionyl chloride (4 mL) for 4 hours. The solution was evaporated, re-dissolved in anhydrous THF (5 mL), and added into a solution of 5 (430 mg, 1.88 mmol) and DMAP (230 mg, 1.88 mmol) in dry THF (10 mL). The mixture was stirred at room temperature for 48 hours, evaporated and re-dissolved in water. The mixture was then extracted with ethyl acetate. The extracts were dried and solvents evaporated in vacuo to give a light oil residue. It was purified by chromatography (silica 20 x 3 mm) with an eluent of ethyl acetate/hexanes (0%-20%) to afford 6 in a 65% yield.

6 (237 mg, 0.50 mmol) was dissolved in methanol/THF (6 mL, 1:1 vol). A solution of KOH in water (0.5 g/mL, 2 mL) was added. The mixture was stirred at room temperature for 1 hour until reaction completion. HCl (2.0 M) was added until the pH of the solution reached 2~3. Water was added and it was extracted with EtOAc. The extracts were dried and solvents were evaporated to give 7 as a white solid (77% yield).

To install the DBS leaving groups onto the linker scaffold 7 (84 mg, 0.20 mmol), tert-butyl 3,5-dibromosalicylate (142 mg, 0.40 mmol) and DMAP (4.9 mg, 40 μmol) were dissolved
in anhydrous THF (20 mL). The solution was cooled in an ice-water bath. EDC (96 mg, 0.50 mmol) in DCM (2 mL) was added through a syringe. The mixture was stirred at 0 °C for 10 minutes and then at room temperature for two days. The solution was evaporated and the residue was dissolved in ethyl acetate. It was washed with NaHCO₃ (sat.) and water, dried, and the solvents were evaporated in vacuo to give a solid residue. Compound 8 was purified by chromatography (silica, 20 × 3 mm) with an eluent of ethyl acetate/hexanes (1:3). The resulting fraction afforded a yield of 70%. ¹H NMR (CDCl₃): δ 9.16 (1H, t, J = 4 Hz), 8.98 (2H, d, J = 4 Hz), 8.53 (1H, s), 8.47 (1H, d, J = 8 Hz), 7.98 (1H, d, J = 4 Hz), 7.88 (1H, d, J = 4 Hz), 7.52 (1H, s), 7.29 (1H, d, J = 8 Hz), 4.27 (2H, s), 1.36 (18H, s). ¹³C NMR: δ 163.1, 162.1, 161.8, 146.7, 139.1, 136.3, 135.5, 133.9, 132.1, 131.0, 128.8, 128.6, 122.3, 120.0, 119.4, 114.5, 15.0, 97.0, 83.5, 53.9, 28.2. ESI-MS: calc. for C₃₈H₃₁Br₅N₄O₉ m/z 1081.8, found 1081.7 [M]+.

To deprotect 8, 100 mg (94 µmol) was dissolved in dichloromethane (4 mL) and TFA (1 mL). The mixture was stirred at room temperature for 1 hour. The solvents were evaporated in vacuo and the residue was left under vacuum for overnight to give 9 as a solid residue. Yield: 81 mg (90%). The structure of the cross-linker was verified through ESI-MS (calc. for C₃₀H₁₅Br₅N₄O₉ m/z 969.7, found 968.7 [M-H]).

### 2.3 Cross-Linking of Superoxide Dismutase

[Cu,Zn] Superoxide dismutase from bovine erythrocytes (9.6 x 10⁻⁹ mol) was dissolved in 0.6 mL of 0.01 M sodium borate buffer (pH 9.0). 4.5 equivalents of cross-linking reagent 9 dissolved in DMSO (0.05 M) were added to the solution. Stocks of the linker were made so that the final volume of DMSO in reaction vessel did not exceed 5%. The cross-linking reaction
proceeded for 12 hours at room temperature in the dark with constant stirring. Excess reagent was removed though selective acetone precipitation of the enzyme. Six volume equivalents of cold acetone (-20 °C) was added to the aqueous solution. The acetone was added in 3 intervals over 30 minutes to prevent damage to the enzyme. All precipitation procedures were performed on ice. The enzyme was collected by centrifugation at 14.8 x 1000 g for 15 min at 4 °C to form a light blue pellet. After air drying for 30 minutes to remove residual acetone, the pellet was re-suspended in deionized water and purified via analytical ion exchange using a SynChropak AX300 column (250 x 4.6 mm). The collected peak was lyophilized immediately and stored at 0 °C.

2.4 Coupling of Cross-Linked Superoxide Dismutase Using CuAAC

To make bis-SOD, SOD-N3 was purified and reacted with a bifunctional alkyne linker 10 (Scheme 1). The coupling reaction was catalysed by Cu(I), which was prepared in situ using an aliquot of CuSO4 under reducing conditions. The reaction was carried out in 0.02 M phosphate buffer (pH 7.4) with SOD-N3 (0.25 mM) in the presence of 2 mM 10 dissolved in DMSO, 3 mM THPTA ligand, and 0.6 mM CuSO4 along with 7 mM ascorbic acid as the reducing agent. The reaction was left in the dark at room temperature for 4 hours, passed through a 0.45 µm filter, and exchanged into 0.1 MOPS buffer (pH 8.0) via three consecutive rounds of centrifugal filtration (15 minutes, 14 x 1000 g) using a 50 kDa MWCO membrane.

Reactions using TBTA followed identical conditions as described above with the exception of the use of a 2:1 Ligand to CuSO4 ratio. TBTA was dissolved in a 1:4 t-butanol:DMSO medium to form a 20 mM stock solution.
For trials employing bathophenanthroline (batho) ligand, a 0.5 mM SOD-N₃ solution (0.02 M phosphate buffer (pH 7.4) was thoroughly purged with a steady stream of N₂ over 1 hour. The reaction was carried out with SOD-N₃ (0.25 mM) in the presence of 2 mM 10 dissolved in DMSO, batho Ligand (1.2 mM), and 0.6 mM CuSO₄ along with 7 mM Ascorbic acid. After the addition of reagent, the vessel was purged, sealed, and left for 4 hours. The mixture was then passed through a 0.45 µm filter and exchanged into 0.1 MOPS buffer (pH 8.0) via three consecutive rounds of centrifugal filtration (15 minutes, 14 x 1000 g) using a 50 kDa MWCO membrane.

2.5 Analysis of Modified Proteins

2.5.1 Chromatography

Anion Exchange Chromatography

Modified proteins for cross-linked superoxide dismutase were analyzed using an analytical anion exchange SynChropak AX-300 (250 X 4.6) column with 1.5 x 10⁻² M Tris, pH 8.0 buffer (A) and 1.5 x 10⁻² M Tris, 1.5 x 10⁻¹ M sodium acetate pH 8.0 buffer (B). The gradient began with 100% buffer A and ended with 100% buffer B over 30 minutes. The effluent was monitored at 254 nm.

Size Exclusion Chromatography

Bis-SOD was analyzed, separated, and collected using semi-preparative size exclusion chromatography (SEC) (Sephadex G-200, 1000 x 35 mm) under slightly dissociating conditions.
The eluent was 37.5 mM Tris, pH 7.4 with 0.5 M magnesium chloride. The effluent was monitored at 254 nm.

2.5.2 Enzymatic Hydrolysis of Native and Cross-linked Superoxide Dismutase

Purified lyophilized protein (3.2 mg) was dissolved in 100 µL 0.1 M sodium bicarbonate (pH 8.5). The sample was placed in a boiling water bath for 5 minutes and then allowed to cool to room temperature. TPCK-treated trypsin in 50 mM acetic acid (4% of protein weight) was then added from a 1 µg/µL stock solution. The mixture was then diluted with 0.1 M ammonium bicarbonate (pH 8.5) to the final protein concentration of 1.0 mg/mL and incubated at room temperature for 24 hours. Upon completion of digestion, the sample was boiled for two minutes to inactivate trypsin. The peptide solution was filtered (0.45 µM) then immediately lyophilized and stored at 0 °C. The digest was run through a LC-ESI MS/MS (positive mode) using a Jupiter 300 C18 column (250 x 2 mm). For the native SOD digest, the solvent for the chromatographic separation of peptides used a gradient over 40 minutes starting with water with 0.1% TFA, and ending with acetonitrile with 0.1% TFA. The modified SOD digest was eluted using a gradient over 60 minutes that started with water with 0.1% acetic acid, and ended with acetonitrile with 0.1% acetic acid. The flow rate for both runs was maintained at 0.2 ml/min.

2.5.3 SDS-PAGE Analysis

The molecular weights of constituent proteins were estimated using polyacrylamide gel (10%, Tris-HCl) electrophoresis under denaturing conditions as described by Laemmli.\textsuperscript{41} Two-dimensional Tris-HCl polyacrylamide gels were comprised of 10% separating gel (pH 8.8) and
5% stacking gel (pH 6.8), both with 10% sodium dodecyl sulfate. Protein samples were treated with 2-mercaptoethanol and sodium dodecyl sulfate. The dimer was further denatured by heating at 95 °C for 5 min and centrifuged for 5 minutes at 2 x 1000 g before loading onto the gel. Gels were run at 140 V for approximately 1 hour. Finished gels were stained with Coomassie Brilliant Blue.

2.5.4 Assay of Superoxide Dismutase Activity.

The method used is as described by Marklund. Air-equilibrated 56 mM Tris-cacodylic buffer with 1.1 mM DTPA (pH 8.2) was prepared by mixing the buffer with air for 24 hours. Stock solutions of lyophilized native and modified SOD were prepared using this buffer. The enzymatic reaction was initiated by the addition of pyrogallol (final concentration 4 mM), which was taken from a 20 mM solution of pyrogallol in 10 mM HCl. The change in absorbance at 420 nm was followed at 25 °C. The optimal enzyme concentration was determined by establishing a value for one unit of the enzyme, which is defined as the amount that inhibits autoxidation of pyrogallol by 50%. Inhibition was determined according to the following equation:

\[ \% \text{ inhibition} = \frac{(A-B)}{A} \times 100 \]

A and B are the initial auto-oxidation rates of pyrogallol in the absence and presence of SOD respectively. The amount of protein required to inhibit pyrogallol autoxidation by 50% was defined as 1 unit of enzyme activity. To obtain the specific activity (units/mg) of SOD, a line was fitted to a set of known concentrations of enzyme against their corresponding % inhibition values. From this graph, the amount of enzyme (mg) needed for 50% inhibition was interpolated. This value was then converted to the overall specific activity of the product (units/mg).
2.5.5 Bio-Rad Protein Assay

The concentration of modified superoxide dismutase was determined using the Bio-Rad® Protein assay. This procedure utilizes a dye that binds to soluble protein containing Tyr, Trp, or Arg residues.\(^{39}\) The absorbance of the dye shifts from 465 to 595 nm upon protein binding. A calibration curve was produced using BSA in the range of 0.1 – 1 mg/mL. The amount of protein in solution was then derived by linear regression analysis with concentrations adjusted by a factor of 4.6. The adjustment factor was required to account for the disparity between the amount of reactive Tyr, Trp and Arg residues in BSA and SOD.

2.5.6 Spectrophotometric Analysis of Modified SOD

Circular dichroism analysis was performed in a Jasco J-710 spectropolarimeter using a quartz cuvette (0.1 or 1.0 cm path length for far and near UV respectively). Spectra reported are an average of 10 runs. A scan speed of 50 nm/min and 1.0 nm bandwidth with a response time of 2 seconds was used. Samples were recorded in 20 mM phosphate buffer pH 7.0 with protein concentrations of 1µM and 50 µM, used for far and near UV respectively.
3 Results

3.1 Cross-linking Reaction of Superoxide Dismutase with 1 and Chromatography Analysis

![Graph showing absorbance over time](image)

**Figure 3.1:** Sephadex G-200 SEC profile of reaction mixture of SOD with 9. Samples were measured under dissociating conditions (0.5 M MgCl₂, 37.5 mM Tris) and monitored at 254 nm.

SOD was reacted with 9 to incorporate an azide moiety through lysine acetylation. The Sephadex G-200 SEC profile of the reaction mixture indicates the formation of cross-linked SOD (Figure 3.1). SOD-N₃, with a molecular weight of ~32 kDa is represented by the first eluting peak, while the second peak is representative of native SOD having dissociated into two 16 kDa subunits. To obtain approximate values for the molecular weights of peaks, the HPLC instrument was calibrated with native SOD and hemoglobin (16 and 32 kDa respectively under dissociating conditions). The best yield of cross-linked SOD was obtained when a 5 mg/mL
solution was reacted with 4.5 equivalents of 9 under basic conditions (pH 9.0, 0.01 M sodium borate buffer). The reaction afforded an 82% yield of SOD-N₃. Similar experiments under more neutral conditions (pH 8.0, 0.01 M sodium borate buffer) failed to form a cross-link. Conversely, experiments performed under concentrated conditions (10 mg/mL) resulted in gross intermolecular cross-links and uncontrolled polymerization of SOD.

SOD-N₃ was separated from unreacted enzyme using AX300 anion exchange chromatography. Modification of the cationic lysine residues caused the enzyme to elute more quickly from the column. As can be seen in Figure 3.2, SOD-N₃ elutes at approximately 3.7 minutes under the modified conditions. This peak was collected, lyophilized, and stored at 0 °C before use as a reagent in click coupling.

![AX300 anion exchange chromatogram (pH 8.0) of SOD before and after modification with 9.](image)

**Figure 3.2:** AX300 anion exchange chromatogram (pH 8.0) of SOD before and after modification with 9.
Both chromatographic profiles of SOD-N₃ indicated that the reaction of SOD with 9 generated a single product. This SOD derivative was modified on at least two lysine subunits to create a protein that does not dissociate under mildly dissociating conditions.

3.2 LC-ESI-MS/MS of SOD-N₃

To identify the site of cross-linking, modified and native hemoglobin species were digested with Trypsin Gold to form a characteristic pattern of peptides. These peptides were separated by C18 reversed phase HPLC and identified through tandem mass spectrometry (ESI-MS/MS). In ESI-MS/MS, the peptide mass of the eluting peaks (MS) and fragmentation spectrum of the peptides (MS/MS) were obtained through collision induced dissociation (CID). In order to identify the HPLC peaks, the data was matched to tryptic fragments of SOD through database searching using Mascot software (Table 2). Using the masses obtained from the MS/MS spectrum, the tryptic fragments were sequenced. The masses found in the ESI-MS spectrum were then assigned a score. The scoring algorithm evaluated how well the sequences of the masses obtained from the MS/MS spectrum matched to the sequence of the actual tryptic fragments. A high score meant that the ESI-MS fragment in question had a mass and amino acid sequence that matched a tryptic fragment found in the software database. The software allowed for post-translational modifications. As noted in the experimental section, different eluting conditions were used for modified and unmodified SOD. The modification of elution conditions provided better separation, revealing new peaks with similar masses to the native digest but different sequence identity.

Comparison of the relative HPLC profiles showed peaks corresponding to trypsin fragment 2 (peak 7) and fragment 3 (peak 6) were reduced upon chemical modification (Figure 3.3). As acetylated lysine residues are unrecognizable by trypsin, the data suggests that the ε-
amino groups of Lys9 from each monomer are chemically modified. Modification at Lys9 was further evidenced by the appearance of a new peak at 21.77 minutes. The peak corresponded to a peptide mass containing the cross-link attached to the two peptides fragments (Figure 3.4). The ESI-MS/MS (Figure 3.5) of the high molecular weight fragment contained tryptic fragments 2 (629.323 Da) and partial fragments of 3(1030.232, 980.196, 173.128 Da). This evidence confirmed that a cross-link was formed, rather than modification of a single Lys9 residue. A separate ESI-MS of the total protein provided a single mass that corresponds to one site of modification (found 31378 [M+2Na]).
Figure 3.3: C18 reverse-phase HPLC chromatogram of the tryptic digests of (a) Native SOD and (b) SOD-N₃.
Table 2: Peptide masses of the native superoxide dismutase digest identified by C18 reversed-phase HPLC whose outflow was analyzed directly by ESI-MS/MS. The PEAKS software used to deconvolute the raw spectrum allowed for post translational modifications.

<table>
<thead>
<tr>
<th>Peak Number</th>
<th>Fragment Number</th>
<th>Tryptic fragment</th>
<th>Mass Found</th>
<th>Mass Calculated</th>
<th>MS/MS Score</th>
<th>Cleaved AA</th>
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<tr>
<td>7</td>
<td>2</td>
<td>4-9</td>
<td>631.3</td>
<td>631.4</td>
<td>39.11</td>
<td>3,9</td>
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<td>6</td>
<td>3</td>
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<td>61.64</td>
<td>23</td>
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<tr>
<td>8</td>
<td>4</td>
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<td>4553.1</td>
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<td>67.86</td>
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</tr>
<tr>
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<td>73</td>
</tr>
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<td>3</td>
<td>7</td>
<td>74-77</td>
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<td>8</td>
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<td>2499.4</td>
<td>97.16</td>
<td>113</td>
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<td>10</td>
<td>114-126</td>
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<td>126</td>
</tr>
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<td>64.39</td>
<td>134</td>
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<td>135-141</td>
<td>661.3</td>
<td>661.3</td>
<td>40.91</td>
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<td>13</td>
<td>141-152</td>
<td>943.6</td>
<td>943.6</td>
<td>46.66</td>
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</tr>
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</table>
Figure 3.4: Structure of cross-linked peptide fragments (MW = 4305 Da).

Figure 3.5: ESI-MS/MS fragmentation spectrum of the cross-linked peptide fragments (Figure 3.4). Peaks corresponding to fragments of tryptic digests 2 and 3 are labeled. All fragments are neutral.
3.2 Click Conjugation of Azide Functionalized SOD to Afford Bis-SOD

![Diagram of SOD-N₃ and Bis-SOD](image)

**Figure 3.6: Conditions for the reaction between azide functionalized SOD and bis-alkyne to form bis-SOD.**

Click reaction mixtures were analyzed by Sephadex G-200 size exclusion analysis. The profile revealed a higher MW SOD species that was consistent with the expected mass of an SOD tetramer (~64 kDa) as seen in Figure 3.6. SEC results showed a yield of ~62%. This value corresponds to a conversion of 76%, as 2 equivalents of SOD-N₃ are required to make one bis-SOD. The chromatogram indicated that only one high MW species was formed under the reaction conditions. This was evidenced by the evolution of a single new peak in the mixture post click coupling (Figure 3.7).
Figure 3.7: Sephadex G-200 SEC profile of reaction mixture of purified SOD-N\textsubscript{3} with 10 to afford bis-SOD. Samples were run under dissociating conditions (0.5 M MgCl\textsubscript{2}, 37.5 mM Tris) and monitored at 254 nm.

Figure 3.8: G-200 SEC profiles of all three forms of SOD under dissociating conditions (0.5 M MgCl\textsubscript{2}, 37.5 mM Tris) and monitored at 254 nm.
To determine the effect of ligand use on conjugation efficiency, Bathophenanthroline (Batho) and TBTA (the water insoluble counterpart to THPTA) were also evaluated (Figure 3.9). Results are summarized in Table 3.

![TBTA](image1.png)  ![THPTA](image2.png)  ![Batho](image3.png)

**Figure 3.9: Structure of ligands utilized in discerning optimal ‘click’ bioconjugation conditions.**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Yield (%)</th>
<th>% Converted</th>
</tr>
</thead>
<tbody>
<tr>
<td>THPTA</td>
<td>62</td>
<td>76</td>
</tr>
<tr>
<td>TBTA</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td>Batho</td>
<td>30</td>
<td>46</td>
</tr>
</tbody>
</table>

**Table 3: click conjugation bis-SOD yields using different ligands as measured by Sephadex G-200 SEC**

3.3 SDS-PAGE Analysis

All gels were obtained under denaturing conditions in the presence of β-mercaptoethanol in developing buffer. SDS-PAGE analysis of the cross-linked species showed bands that migrated at 32 and 16 kDa on the polyacrylamide gel (Figure 3.10). The 16 kDa band corresponded to the native SOD monomer as exemplified by the control lane containing an
unmodified sample of SOD. Using densiometry analysis (ImageJ), it was determined that cross-linked dimers composed approximately 82% of the reaction mixture. This value complemented size exclusion chromatography analysis of samples under dissociating conditions. In the lane containing the click product a band in the 64 kDa region is present, confirming successful formation of bis-SOD. Densiometry analysis of the click reaction mixture was in agreement the yields obtained from Sephadex G-200 SEC.

Figure 3.10: SDS-PAGE analysis of modified superoxide dismutase. From Left: Lane 1, MW standards; Lane 2, SOD1; Lane 3, SOD-N₃ (unpurified); Lane 4, SOD-N₃ (purified); Lane 5, bis-SOD (unpurified).

A faint ~50 kDa band can be seen in Lane 5 of Figure 3.10. The band may indicate the formation of an SOD trimer, a species of modified protein formed when click bioconjugation occurs between a cross-linked and uncross-linked SOD (Figure 3.11)
3.4 Assay of Superoxide Dismutase Activity

Purified bis-SOD was assayed to determine the effects of cross-linking on enzymatic activity. Measurements were carried out using the indirect pyrogallol assay. The activity of the conjugate was evaluated by its ability to inhibit the superoxide dependent auto-oxidation of pyrogallol. Pyrogallol can autoxidize in alkaline solutions to generate a superoxide anion ($\cdot$O$_2^-$). Through a series of intermediates that include semiquinones 11 and 12, the superoxide is consumed to form the UV active purpurgallin (Figure 3.12). By adding SOD superoxide may be prematurely removed, preventing the formation of purpurgallin. The resulting change in the absorbance not only reflects changes in purpurgallin concentration, but also indirectly visualizes the formation of the superoxide anion ($\cdot$O$_2^-$). A lower absorbance indicates a higher inhibition of $\cdot$O$_2^-$ through scavenging by SOD. A unit of enzyme activity is defined as the amount (mg) of enzyme needed to inhibit pyrogallol auto-oxidation by 50%. In order to determine the specific activity (units/mg) of the modified SOD, linear regression analysis was performed which plotted...
the percent inhibition at different protein concentrations (Figure 3.13). The fitted line was used to interpolate the amount of protein that needed for 1 unit of activity. Results from Table 4 confirm that both the cross-linked dimer (SOD-N₃) and bis-SOD retain high catalytic activity despite chemical modification (Figure 3.14).

![Chemical reactions](image)

**Figure 3.12:** Auto-oxidation scheme of pyrogallol.

---

![Graph](image)

**Figure 3.13:** Linear regression analysis of bis-SOD used to determine the enzymatic specific activity. Protein quantities were determined by the Bio-Rad protein assay.
Figure 3.14: Rate profile of autoxidation of 4.0 $10^{-4}$ M pyrogallol in the presence of SOD. All samples contain 5.3 $10^{-7}$ mol/L of the enzyme dimer or 1 unit of SOD.

Table 4: Enzymatic parameters of modified superoxide dismutase

<table>
<thead>
<tr>
<th>SOD species</th>
<th>% Inhibition</th>
<th>% Activity</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>50</td>
<td>100</td>
<td>2078.4</td>
</tr>
<tr>
<td>Bis-SOD</td>
<td>47</td>
<td>94</td>
<td>1919.3</td>
</tr>
<tr>
<td>SOD-N$_3$</td>
<td>41</td>
<td>82</td>
<td>1667.2</td>
</tr>
</tbody>
</table>

3.5 CD Spectral Properties of Modified Superoxide Dismutase

To determine if formation of bis-SOD resulted in any gross alterations to the dimer stability, purified proteins were characterized by circular dichroism (CD) to compare the secondary (far-UV) and tertiary (near-UV) structures. As shown in Figure 3.15, native and modified secondary structures both share a minimum peak at 208nm. This is characteristic of the
β-sheet formations that predominantly comprise the enzyme structure.\textsuperscript{47} The near-UV CD spectrum of the bis-SOD also follows the same shape of native SOD (Figure 3.15).\textsuperscript{48} Both spectra confirm a lack of overall structural change despite chemical perturbation.
Figure 3.15: CD spectra (200-250 nm) of modified SOD compared to native SOD.

Figure 3.16: CD spectra (250-360 nm) of modified SOD compared to native SOD.
4 Discussion

4.1 Site-Specific Cross-Linking

Traditional cross-linking methods can employ the acetylation of the ε-amino groups of lysine residues as a means of site-selective modification. However, with over 20 accessible lysine residues on the surface of the SOD1 dimer, this approach can quickly become problematic.\(^{49}\) Non-specific acetylation may lead to highly heterogeneous products, while acetylation within the active site of the enzyme will diminish biological activity. In the case of the catalytic activity of SOD1, residues Lys134 and Lys120 that reside within the cationic funnel play crucial roles in directing the superoxide anion towards the active site.

To achieve site-selectivity, a cross linker with unique properties was utilized. Electronic steering was used to ensure that acetylation is directed towards the cationic surfaces of the protein, specifically the lysine and arginine-rich cationic funnel adjacent to the protein active site. Electron dense, anionic 1,3-dibromosalicylate leaving groups were used due to their established ability to selectively modify residues near cationic regions.\(^{34}\) This strategy led to the successful cross-linking of SOD1 with azide linker 9, affording SOD-N\(_3\). Subsequent click chemistry with two equivalents of SOD-N3 in the presence of a bis-alkyne linker allowed for the isolation of homogeneous bis-SOD reagents.

Tryptic digests and ESI-MS of the total protein helped determine that the Lys9 residues are the sites of cross-linking in the modified SOD substrates. Lys9 residues lie at the edge of the cationic funnel and have a distance of ~7 Å between the dimer interface. Taking the inherent
flexibility of lysine residues into account, this modification site is consistent with the dimensions of the azide cross-linker 9.

Figure 4.1: X-ray crystal structure of bovine [Cu,Zn] superoxide dismutase (PDB ID: 1CBJ)\textsuperscript{33}. The light blue and light green ribbons differentiate the identical monomers that form the SOD dimer. Copper and Zinc ions are represented by orange and blue spheres respectively. Lys9 is highlighted in yellow with the distance between residues displayed. The image was captured using FirstGlance in Jmol.

As Lys9 was the only basic residue that was successfully modified with 9 over an extended reaction time, it can be said that the anionic character of the linker has a clear directing effect to a specific region of the enzyme surface.

From these experiments it was illustrated that reacting SOD with 9 served as a suitable reagent for controlled cross-linking. Unlike PEG-SOD, the chemical modification was site-
specific and homogenous. In contrast to previous cross-linker designs by Algaic et al., cross-linker 9 does not affect catalytically important residues such as Lys134 (Figure 4.2).

![Structure of maleimide-acyl phosphate bifunctional linker previously used by Algaic et.al. to functionalize and cross-link SOD.](image)

**Figure 4.2: Structure of maleimide-acyl phosphate bifunctional linker previously used by Algaic et.al. to functionalize and cross-link SOD.**

While both acyl phosphates and acyl salicylates are able to site-specifically modify near cationic regions, the disparity in the location of cross-linking originates from the distance between the site-specific leaving groups on the cross-linker scaffold. Structure 13, having a distance of ~15 Å (Spartan), is large enough to perturb the lysine residues within the active site. Additionally the phosphate groups, unlike DBS, are small enough to enter the funnel itself.

With respect to lysine acetylation, it is most advantageous to work in basic conditions. High pH environments increase the concentration of deprotonated amines on the protein surface that can serve as the nucleophile in the acetylation reaction. Although the maleimide/thiol system employed by Algaic et. al. is considered a site-specific conjugation technique, the hydrolysis of
maleimide under basic conditions can lead to decreased product yield. To minimize hydrolysis, reactions by Algaic et. al. were carried out at pH 7.0, affording a 50% yield of cross-linked SOD. In contrast, the azide employed in the present conjugation technique is not susceptible to hydrolysis. As a result, basic conditions were successfully employed to increase product yield by 32%.

4.2 Synthesis and Purification of Bis-SOD

CuAAC ligands have several functions in protein bioconjugation. They accelerate the reaction, maintain the catalytic oxidation state of the metal, and sequester copper ions away from other sites of the protein. However for our purposes, the catalyst must also be water soluble due to the mechanism of conjugation (Figure 4.3). To create the high MW SOD, it is required that both ends of the alkyne react with molecules. However, thermodynamics dictate that it is more likely to observe reactions producing a singly modified linker. In order to promote coupling at both ends of the bis-alkyne linker, phase-transfer catalysis was used. The central bis-alkyne is insoluble in aqueous conditions. Initially, reactions are likely to be limited to the interface between phases, assuring that the first coupling reaction of the bis-alkyne with one SOD-N\textsubscript{3} will occur at a relatively slow rate ($k_1$, Figure 4.3). The second coupling reaction of the resulting soluble SOD–alkyne intermediate with SOD-N\textsubscript{3} takes place in the same phase, necessarily leading to a faster rate ($k_2$). This whole process ensures that both sites of the bis-alkyne linker react to form the bis-tetrazole product (bis-SOD).\textsuperscript{22} Thus, the two steps of the coupling process are cooperative.
Figure 4.3: Model of the acceleration of protein coupling though phase transfer catalysis.

Although Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) is a commonly used ligand for CuAAC, it is not water-soluble and requires an organic co-solvent. The presence of organic solvent likely increased the solubility of the bis-alkyne linker, sequestering it from the first step of the phase transfer catalysis. As a result, low yields were observed for the overall CuAAC reaction.

Batho ligand was also tested for use in the reaction. Unlike TBTA, batho ligand is water soluble. However, a significant drawback was the catalyst’s acute oxygen sensitivity. Cu(I)-batho complexes are strongly electron-rich and highly susceptible to oxidation. The high reactivity of the complex with O₂ can easily consume all of the available reducing agent. In addition, when working with small volumes (~50 µL), creating an oxygen-free environment is difficult. While this result was an improvement over TBTA, excessive purging of the ligand with N₂ did not prove to be ideal for the reaction.

THPTA is a useful ligand with properties in between those of the Batho and TBTA ligands. It is stable under ambient conditions and requires no organic solvent. Thus, THPTA was selected as the ligand most suitable for bis-SOD formation.
As previously stated, SDS-PAGE showed some evidence of trimer formation. This is undoubtedly due to the click conjugation between a cross-linked SOD azide, and one that has been modified at only one lysine site. Since SOD dimers do not readily dissociate under physiological conditions, the trimer should behave as a high molecular weight bis-SOD in circulation.

Many attempts were made to obtain a sample of bis-SOD pure enough for activity studies. The initial approach involved isolation of the desired conjugate from SOD-N$_3$ using Sephadex G-200 size exclusion chromatography. However, the close retention times between the two species caused significant peak overlap. As a result, it was impossible to solely isolate bis-SOD without contamination from the cross-linked dimer. Centrifugal filtration using 50 MWCO filters were used as an alternative purification tool. Chromatography and SDS-PAGE verified that the purification of the SOD using centrifugal filtration was sufficient for activity studies.

4.3 Superoxide Dismutase Activity and Structure

Assays of SOD activity revealed insignificant change in activity with modified SOD. This result indicates that both the reaction conditions and the introduced chemical modifications do not interfere with the catalytic function of SOD. Near-UV CD spectra complemented the activity studies, with no large change in secondary or tertiary structure. More importantly, these results confirmed that the chemical cross-linking has not modified the cationic funnel surrounding the active site. It also appeared that coupling did not block the active site of either dimer.
The decreased activity of SOD-N\textsubscript{3} compared to bis-SOD was an unusual observation. The probable cause is likely related to the competitive inhibitory effect azides have on the active site of SOD when they co-exist in solution.\textsuperscript{53}

4.4 Suggestions for Future Work

To fully evaluate the effectiveness of an active, high molecular weight Cu, Zn superoxide dismutase as a therapeutic agent, further characterization must be carried out. Next steps could include the investigation of the pharmacokinetic properties of bis-SOD. These studies will demonstrate whether the increase in mass correlates with an increased renal retention \textit{in vivo}. Additionally, they will also provide data that can be used to directly compare bis-SOD to other high molecular weight SOD products already used in industry. These studies are traditionally carried out through the intravenous administration of SOD to the tail vein of rats. Samples are then collected at scheduled times and assayed for enzyme activity.\textsuperscript{54}

Additionally, it would be interesting to use this novel strategy to conjugate new antioxidant proteins such as catalase to SOD. A SOD-catalase conjugate could ensure that all ROS (H\textsubscript{2}O\textsubscript{2}) that are over-produced by diseased tissues are neutralized. There is documented success in site-specific modification of catalase using transglutaminase (TGase) (Figure 4.4).\textsuperscript{55} Using TGase, one glutamine residue per subunit of catalase is modified to create a product mixture with high homogeneity. This strategy could be used to attach an azide-functional group to catalase. By coupling catalase-N\textsubscript{3} with SOD-N\textsubscript{3} and a bis-alkyne linker under click chemistry conditions, novel catalase-SOD conjugates could be synthesized.
Figure 4.4: Site-specific modification of catalase using transglutaminase. There is a single accessible glutamine residue located on each catalase monomer.

Finally, other bio-orthogonal strategies may be employed to conjugate this antioxidant enzyme to bis-SOD. Unlike bovine Cu, Zn superoxide dismutase, Human Cu, Zn superoxide dismutase contains a structurally and functionally unimportant free cysteine residue, Cys111.\textsuperscript{56} This residue could be reacted with a maleimide-functionalized catalase to make an overall conjugate of catalase-SOD with dual functionality by means of chemical intervention.
In summary, a novel strategy that allows site-specific, covalent conjugation of two SOD dimers was developed. This technique makes possible the formation of a defined, homogeneous, high MW bis-SOD protein suitable for therapeutic applications. Activity studies showed that chemical intervention did not interfere with enzyme activity. In future work, this strategy may be used to explore the development of other SOD conjugates, such as SOD-catalase. Additionally, it would be beneficial to establish the pharmacokinetic properties of bis-SOD in animal models.
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