Hemoglobin Bis-Tetramers: synthesis of cross-linker and reaction with hemoglobin

by

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A thesis submitted in conformity with the requirements for the Degree of Master of Science, Graduate Department of Chemistry University of Toronto.

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to John and my family
I would like to thank Professor Ronald Kluger for his guidance and enthusiasm during my studies.

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Hemoglobin Bis-Tetramers: synthesis of cross-linker and reaction with hemoglobin

Master of Science, 1997

Jodi J.L. Lock-O'Brien, Graduate Department of Chemistry, University of Toronto

Previously, reagents have been developed that react within hemoglobin to interconnect the subunits to prevent dissociation of the αβ dimers. However, there has been no reagent which can react to form these intramolecular cross-links while forming an intermolecular link between hemoglobins. This thesis reports the development of such a reagent and its reaction with hemoglobin to produce dimers of cross-linked hemoglobin, or hemoglobin bis-tetramers.

N,N'-5,5'-bis[bis(3,5-dibromosalicyl)isophthalate]terephthalamide (DBIT) was synthesized. It reacts with hemoglobin to form crosslinked bis-tetramers.
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<tr>
<td>Bis-Tris</td>
<td>bis(2-hydroxyethyl)aminotris(hydroxymethyl)methane</td>
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<td>BTDS</td>
<td>3,5,3',5'-biphenyltetracarbonyl tetrakis(3,5-dibromosalicylate)</td>
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<tr>
<td>Da</td>
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<td>DBIT</td>
<td>N,N'-5,5'-bis[3,5-dibromosalicyloyxycarbonyl]-terephthalamide</td>
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<td>3,5-dibromosalicylic acid</td>
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<tr>
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<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
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<tr>
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<td>DPEE</td>
<td>1,2-Bis(2-[3,5-bis(3,5-dibromosalicyloyxycarbonyl)ethoxy]ethane</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
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<tr>
<td>TFA</td>
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<tr>
<td>TMMP</td>
<td>trimesoyl tris(methyl phosphate)</td>
</tr>
<tr>
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<td>ultraviolet-visible</td>
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Scheme 4. A reaction leading to formation of a triply linked, high molecular weight hemoglobin.
Introduction

Development of a red-cell substitute has become an increasingly important goal in light of heightened concerns about viral and bacterial contaminants in blood products. Beyond these more recent concerns, a red-cell substitute could provide further advantages over red cells in terms of enhanced stability, more flexible storage conditions, universality and the performance of specialized functions, including drug delivery. One approach to developing a red-cell substitute involves the use of hemoglobin as the oxygen delivery machinery. However, the use of red-cell free, unmodified hemoglobin (an ααββ tetramer) has problems associated with its dissociation into αβ dimers and very high oxygen affinity. The αβ dimer has a molecular weight of 32 kDa and is rapidly filtered through the kidneys, potentially causing renal damage.1 Chemically cross-linking tetramers of hemoglobin has proven effective in preventing αβ dimer release2-10 (Figure 1.1).11

The high oxygen affinity of αβ dimers is expected to limit the delivery of oxygen in the body, but the appropriate oxygen affinity for a red-cell substitute has yet to be determined. Applications for substitutes include emergency resuscitation without type matching delays, donor organ perfusion to extend shelf-life, and enhancement of radiotherapy. Each application may require a substitute with a different oxygen affinity.
The optimal therapeutic results in addition to preventing donor release, cross-linking can simultaneously modify the oxygen affinity of hemoglobin. A wide variety of specific, efficient cross-linkers are currently known which can be reacted with hemoglobin to yield products with $P_{50}$'s ranging from 3 to 24 mmHg.\textsuperscript{10}

![Diagram of intramolecular cross-linking between two \( \beta \) globin chains](image)

**Figure 1.1.** Hemoglobin intramolecularly cross-linked between two \( \beta \) globin chains prevents dissociation of tetramer.

Higher molecular weight hemoglobin products are believed to have several potential advantages over intramolecularly cross-linked hemoglobin in a red-cell substitute. These advantages include longer in vivo retention times, increased oxygen delivery at a lower oncotic pressure and, potentially, reduced interference with vascular tension. The most favorable molecular weight for a hemoglobin product has not yet been determined. Current methods for generating higher molecular weight hemoglobin include chemical polymerization using non-specific surface-directed reagents.\textsuperscript{12-18} Studies using mixtures of hemoglobin oligomers have indicated that retention times increase with molecular weight. Dimers and trimers of modified hemoglobin, with nominal molecular weights of 128 and 192 kDa respectively, have been shown to be preferentially retained over cross-linked hemoglobin with a molecular weight of 64 kDa.\textsuperscript{19} However, these non-specific polymerization methods produce heterogeneous
mixtures of hemoglobins having a variety of molecular weights and chemical modifications. This heterogeneity may lead to problems associated with viscosity, oxygen affinity, antigenicity and stability of the tetrameric structure. If molecules within a complex of intermolecularly linked hemoglobins are not also intramolecularly cross-linked, 32 kDa αβ dimers may still be released (Figure 1.2).

![Diagram of hemoglobin complex](image.png)

**Figure 1.2.** Release of an αβ dimer from a high molecular weight hemoglobin due to incomplete intramolecular cross-linking.

Other attempts to increase the molecular weight of hemoglobin include coupling it to larger molecules such as polyethylene glycol and dextran. These complexes have a higher oxygen affinity than unmodified hemoglobin and have a potential complexity due to the attachment of large, nonfunctional moieties.

A cross-linker which reacts specifically to form an intermolecular link between two hemoglobin molecules, and simultaneously forms intramolecular cross-links in each of these hemoglobin molecules, would form a stabilized hemoglobin bis-tetramer. This technique would prevent the release of αβ dimers and generate a high molecular weight hemoglobin product. (Figure 1.3).
Non-specific modification of hemoglobin could be minimized by directing the reagent towards the positively charged DPG binding site. This can be accomplished by incorporating negative charges into the reagent. By selecting the appropriate functional groups, a reagent can react specifically with one site on each β chain, located near the surface of the DPG binding site, to produce a cross-link between the αβ dimers. Based on previous results, the isophthaloyl moiety was chosen as the basic intramolecular cross-linking arrangement. We sought to prepare a reagent which would incorporate this predictable intramolecular cross-linking moiety onto each end of a new reagent core designed to span the distance between two hemoglobin molecules. Such a reagent has 3,5-dibromosalicyl groups esterified to a meta-substituted dicarboxylic acid at each end of a rigid bridging unit of appropriate length. Terephthalamide was chosen to meet these bridging requirements, and N,N'-5,5'-bis[bis(3,5-dibromosalicyl)isophthalate]terephthalamide (DBIT) (Figure 1.4) was synthesized and
hemoglobin to produce hemoglobin bis-tetramers. (Figure 1.5).

**Figure 1.4.** The structure of DBIT.

**Figure 1.5.** A hemoglobin bis-tetramer formed by reaction with DBIT.
Experimental

2.1. Materials

Commercial reagents were utilized without further purification. Solvents were dried prior to use. Buffers were made from water which was doubly distilled and deionized. Other reagents for preparation of buffers and developers for hemoglobin modifications and chromatography were of analytical grade or better. The purity of newly synthesized materials was assessed by a combination of NMR spectroscopy, mass spectrometry, high pressure liquid chromatography and infrared and UV-VIS spectroscopy. Proton and carbon NMR spectra were recorded on 200 MHz and 100 MHz Varian Gemini spectrometers. Infrared spectra were recorded on a Nicolet 5DX FTIR spectrometer. UV-VIS spectra were recorded on a GBC 916 spectrometer. Solutions of human hemoglobin A were obtained from Hemosol, Inc. A purified sample of hemoglobin cross-linked with 5-(isonicotinoylamino)isophthalicyl(3,5-dibromosalicylate) was provided by Dr. Sharon Brevitt.
2.2. Synthesis of cross-linking reagent

2.2.1. Synthesis of N,N'-5,5'-bis[bis-(3,5-dibromosalicyl)isophthalyl]terephthalamide

Bis-(3,5-dicarboxyphenyl)terephthalamide (2.0 g, 4.1 mmol) was stirred and heated to reflux in 10 mL of thionyl chloride for 20 hours under nitrogen. The solvent was evaporated in vacuo to give a white solid. The solid was stirred in 20 mL anhydrous tetrahydrofuran and then added dropwise to a solution of t-butyl 3,5-dibromosalicylate (5.8 g, 16.5 mmol) and potassium t-butoxide (1.9 g, 16.6 mmol) in 60 mL of anhydrous tetrahydrofuran. The resulting mixture was left to stir at room temperature for 20 hours under nitrogen. The solid was then dissolved in 50 mL of diethyl ether, the organic layer washed with distilled water (3x100mL), dried with magnesium sulfate and the solvent removed in vacuo to give a yellow liquid. This product was dissolved in 50 mL of trifluoroacetic acid and stirred at room temperature for 2 hours under nitrogen. Diethyl ether was added to dissolve remaining unreacted 3,5-dibromosalicylic acid and to precipitate additional product. The mixture was left to stir at room temperature for 1 hour under nitrogen. The solution was then filtered to give a white solid (1.7 g, 1.1 mmol). m.p. 228-231°C. $^1$H NMR (ppm, DMSO-d$_6$): δ 11.0 (s, 2H, N-H), 9.07 (d, 4H, J=1.4 Hz, ArH), 8.52 (t, 2H, J=1.4 Hz, ArH), 8.38 (d, 4H, J=2.40 Hz, ArH), 8.21 (s, 4H, ArH), 8.12 (d, 4H, J=2.32 Hz, ArH). $^{13}$C NMR (ppm, DMSO-d$_6$): δ 165.2, 163.3, 162.3, 146.4, 140.6, 138.9, 136.9, 133.4, 129.4,
2.3. Cross-linking reaction of human hemoglobin A

Carbonmonoxy hemoglobin (1.0mL, 1.2μmol) in 50 mM Bis-Tris buffer (pH 6.5) was passed through a Sephadex G-25 column at 4°C equilibrated with 50 mM sodium borate buffer (pH 8.0 or 9.0). For cross-linking carbonmonoxy hemoglobin, the solution was then placed under carbon monoxide flow for 10 minutes, sealed and equilibrated in a 35°C water bath for 15 minutes prior to addition of the cross-linker. To cross-link deoxy hemoglobin, the solution of carbonmonoxy hemoglobin was converted into oxy hemoglobin by irradiation under visible light with flowing oxygen at 0°C for 2 hours. The oxy hemoglobin was deoxygenated under flowing nitrogen at 37°C for 2 hours. For both the deoxy- or carbonmonoxy hemoglobin, the cross-linker was added as a solid (4mg, 2.4μmol) with minimal exposure of the hemoglobin solution to room atmosphere. The reaction mixture was kept rotating at 37°C for 24 hours under continuous water-saturated nitrogen flow with samples being removed at various times for analysis. At the end of the reaction period the flask was flushed with carbon monoxide, then passed through a Sephadex G-25 column equilibrated with 0.1M MOPS buffer (pH 7.2).
2.4. Analysis of modified hemoglobin

2.4.1. Chromatography

The heme and globin chains were separated by reversed phase HPLC using 330-Å pore size C-4 Vydac columns (250 x 4.6 mm for analysis and 250 x 12 mm for preparative) and developers containing 0.1% trifluoroacetic acid and various gradients of acetonitrile, starting at 20% and ending at 60%, in water. The effluent was monitored for absorbance at 220nm.

Peptide fragments were separated by reversed-phase HPLC using a C-18 Vydac column (93 x 4.7 mm). Developers contained 0.1% TFA and various gradients of acetonitrile, starting at 0% and ending at 100%, in water. The effluent was monitored at both 220 nm and 280 nm.

The modified hemoglobin were separated according to molecular weight by semi-preparative gel filtration FPLC using Superdex G75 and G200 HR columns (300 x 10 mm) under both non-dissociating (25mM Tris buffer pH 7.4) and partial-dissociating (25mM Tris buffer pH 7.4, 0.5M MgCl₂) conditions. The effluent was monitored at 220nm.

The hemoglobin solutions were monitored for modification by anion exchange HPLC using both a POROS HQ/H perfusion column (100 x 4.6 mm) and a Synchropak
Tris (25mM) and Tris (25mM). The method used had a flow rate of 2 mL/min, a pH gradient from 6.5 to 8.5 and the effluent was monitored at 410nm.

2.4.2. SDS-PAGE

Polyacrylamide gel electrophoresis, under SDS denaturing conditions was used to separate the individual protein chains according to molecular weight. Prior to electrophoresis, the protein samples (cross-linked hemoglobin, hemoglobin and molecular weight standards) were denatured in boiling water for 5 minutes in 0.5 M Tris-HCl buffer, pH 6.8, which contained 0.05 % bromophenol blue, 4% v/v 2-mercaptoethanol, 2% sodium dodecyl sulfate, 10% v/v glycerol. Approximately 5-10 μg of protein was applied to each lane of the gel. The gel was run using the Bio-Rad Mini-PROTEAN II dual-slab cell apparatus at 200mV. The yield and purity could be estimated by visual comparison of the resolved electrophoretic bands after staining with Coomassie Brilliant Blue. Unmodified hemoglobin and Bio-Rad SDS-PAGE molecular weight standards (broad range) were used to establish the molecular weight of the resulting bands. The gel was then scanned onto a desktop personal computer and analyzed quantitatively using Quantiscan for Windows.
The exact masses of cross-linked hemoglobin were determined by electron spray ionization mass spectroscopy coupled to the reversed-phase HPLC outlined above. The spectroscopy was performed at the mass spectroscopy laboratory, Medical Sciences Building, University of Toronto.

2.4.4. Peptide pattern analysis

Individual \( \beta \) globin chains were separated and collected using the reversed-phase C4 HPLC technique outlined above and the water removed by lyophilization. The globin chains (both unmodified and cross-linked) were then dissolved in 8 M urea and kept at room temperature for 2 hours. Freshly made trypsin solution (2mg/mL, 4% of mass of total globin protein) was then added, the solution diluted with 80 mM ammonium bicarbonate buffer (pH 8.5) to a final urea concentration of 2 M and left to stand at room temperature for 24 hours. The tryptic hydrolysate was then heated in boiling water for 2 minutes and diluted to 1M urea with 80 mM ammonium bicarbonate buffer (pH 8.5). *Staphylococcus aureus* V8 endoproteinase Glu-C solution (2mg/mL, 2% of mass of total globin protein) was then added and the mixture was digested for 72 hours at room temperature. The hydrolysates were filtered through a 0.45-fm filter before injection onto the C18 reverse-phase HPLC column.
were separated by HPLC procedures modified after that of Shelton et. al. using a reversed-phase C18 column as described above. The resulting peptide patterns from modified β globins were analyzed by comparison with the peptide pattern of unmodified β globin. Peaks on the chromatograph for the unmodified β globin digest were identified by comparison with literature.22

2.5. Purification of modified hemoglobin products

2.5.1. Preparative gel filtration FPLC

The modified hemoglobins were separated according to molecular weight by preparative gel filtration FPLC using Superdex G75 and G200 HR columns (300 x 10 mm) under both non-dissociating (25mM Tris buffer pH 7.4) and partial-dissociating (25mM Tris buffer pH 7.4, 0.5 M MgCl₂) conditions. The effluent was monitored at 287 nm. The fastest eluting peak was collected from multiple injections and concentrated by ultrafiltration using an Amicon CF25 25000 MW Centriflow membrane cone. The process was repeated until a single elution peak was obtained and the above analytical techniques were then used for identification.
Both a Sephacel column and an AX-300 HPLC column were used for preparative ion-exchange chromatography. The Sephacel column was run at 4°C using a peristaltic pump. The hemoglobin reaction mixture was first passed through a Sephadex G-25 column equilibrated with distilled water, then loaded onto the Sephacel column equilibrated with glycine (0.2M, pH 7.8) and washed with the same solution. They were then eluted from the column over 48 hours using glycine buffer (0.2M, pH 7.8) and an increasing concentration of KCl (0.03-0.1M). The eluting fractions were combined, concentrated via ultracentrifugation and analyzed using the above methods. Preparative AX300 ion-exchange HPLC was performed in the same manner as the analytical technique outlined above.
Results

3.1. Synthesis of cross-linking reagent

3.1.1. Synthesis of \( \text{N,N'-5,5'-bis[bis-(3,5-dibromosalicyl)isophthalyl]terephthalamide} \)

Bis(3,5-dicarboxyphenyl)terephthalamide was synthesized from terephthaloyl chloride and 5-aminoisophthalic acid in a Schotten-Bauman type reaction described by Aharoni and Edwards\(^\text{24}\) for polyamide synthesis. The tetrakis (carbonyl chloride) was prepared with thionyl chloride and used to prepare the target molecule by an extension of the procedure described by Klotz and co-workers\(^\text{3}\) for the preparation of bifunctional 3,5-dibromosalicylate esters (Scheme 1). The tetrachloride was reacted with a slight excess of the potassium salt of t-butyl 3,5-dibromosalicylate in anhydrous tetrahydrofuran. Deprotection with anhydrous trifluoroacetic acid gave DBIT (Scheme 1). Purity was assessed by NMR, reversed-phase HPLC and low resolution mass spectroscopy.
Scheme 1. The synthesis of DBIT from bis(3,5-dicarboxyphenyl)terephthalamide.
Carbonmonoxy-hemoglobin reacts incompletely with two equivalents of DBIT at both pH 8 and 9, as does deoxy-hemoglobin at pH 9. After 20 hours, between one-half and two-thirds of the β chains remain unmodified. Six equivalents of DBIT drives the reactions to further modify β chains. However, two equivalents of the reagent react with deoxy-hemoglobin at pH 8 to modify 95% of all β chains after 20 hours (Figure 3.1). All subsequent analysis was performed on the cross-linking reaction for deoxy hemoglobin and two equivalents of DBIT at pH 8 and 37 °C.

**Figure 3.1.** C4 reversed-phase HPLC chromatograph separating globin chains following reaction of deoxy Hb with DBIT (pH 8, 37°C).
C4 reversed-phase HPLC separated the heme, unmodified and modified globin chains. The chromatographs indicated that DBIT was specific for modification of the β globin chains. Cross-linking for 20 hours with 2 equivalents of DBIT gave 95% modification of the β globin chains while the α globin chains were not modified. There was no distinct product peak. Rather, several small peaks appeared throughout the product region of the chromatograph.

The Superdex G-75 gel filtration column was calibrated using unmodified hemoglobin and a purified sample of β-β cross-linked hemoglobin (flow rate of 0.6mL/min). It was determined that hemoglobin, a 64 kDa species, eluted at 15.7 minutes under non-dissociating conditions (Figure 3.2). Analysis of the hemoglobin reaction mixture under these conditions indicated the presence of 64 kDa and >64 kDa species in approximately a 2:1 ratio eluting at 15.1 and 13.2 minutes respectively (Figure 3.3). Under partial-dissociating conditions it was determined that the 32 kDa and 64 kDa species eluted at 18.5 and 16.3 minutes respectively (Figure 3.4 and 3.5). Analysis of the hemoglobin reaction mixture indicated the presence of 32 kDa, 64 kDa and >64 kDa species in approximately a 1:3:2 ratio eluting at 18.3, 15.8 and 13.7 minutes respectively (Figure 3.6).
Figure 3.2. G75 size exclusion chromatograph for unmodified Hb under non-dissociating conditions.
Figure 3.3. G75 size exclusion chromatograph for reaction of deoxy Hb with DBIT under non-dissociating conditions.
Figure 3.4. G75 size exclusion chromatograph for unmodified Hb under partial-dissociating conditions.
Figure 3.5. G75 size exclusion chromatograph for purified, cross-linked Hb under partial-dissociating conditions.
Figure 3.6. G75 size exclusion chromatograph for reaction of deoxy Hb with DBIT under partial-dissociating conditions.

The Superdex G200 gel filtration column was as described earlier. Under partial-dissociating conditions with a flow rate of 0.5mL/min, the 32 kDa species eluted at 30.5 minutes (Figure 3.7). Analysis of the hemoglobin reaction mixture indicated the presence of 32 kDa, 64 kDa and >64 kDa species eluting at 30.7, 28.4 and 24.9 minutes respectively (Figure 3.8).
Figure 3.7. G200 size exclusion chromatograph for unmodified Hb under partial dissociating conditions.
Figure 3.8. G200 size exclusion chromatograph for reaction of deoxy Hb with DBIT under partial-dissociating conditions.

SDS-PAGE analysis of the cross-linked hemoglobin reaction mixture indicated the presence of species having a molecular weights of approximately 64, 32, 16 and 15 kDa (Figure 3.9). The well containing unmodified hemoglobin contained only species having molecular weights of 16 kDa and 15 kDa (Figure 3.10). Analysis of purified β-β cross-linked hemoglobin indicated species having molecular weights of 32 kDa and 15 kDa only. The results were calibrated by comparing mobility with the molecular weight standards (Figure 3.11).
Figure 3.9. Density scan of the SDS-PAGE well containing hemoglobin reacted with DBIT.

Figure 3.10. Density scan of the SDS-PAGE well containing unmodified hemoglobin.
Figure 3.11. Density scan of the SDS-PAGE well containing molecular weight standards.

Ion spray mass spectroscopy coupled to C4 reversed-phase HPLC showed products with masses corresponding to the heme, β globin chain and α globin chain. In addition peaks were identified for major product having masses of 16620 Da, 16899 Da and 32468 Da. The mass of 16620 Da corresponds to a product having one β globin chain attached to one molecule of DBIT with one remaining unhydrolyzed DBS ester group (calculated mass of 16620 Da). The mass of 16899 Da corresponds to a product having one β globin chain attached to one molecule of DBIT with two remaining unhydrolyzed DBS ester groups (calculated mass of 16898 Da). The mass of 32468 Da corresponds to a product having two β globin chains attached to one molecule of DBIT with one remaining unhydrolyzed DBS ester group (calculated mass of 32462 Da). Minor products were found having masses of 31718 Da, 17175 Da, 16431 Da, 16153 Da and 15876 Da. These masses correspond to products having combinations of α.
Higher molecular weight species were not identified using this method.

3.4. Purification of hemoglobin bis-tetramers

HPLC analysis of the reaction mixture using POROS columns (seive packing) indicated the presence of several products which were inseparable by ion-exchange chromatography. The higher molecular weight species were separated from the 64 kDa and 32 kDa proteins by collecting the fastest eluting peak from the Superdex G200 gel filtration column, under partial-dissociating conditions. The collected fractions were pooled, concentrated and reinjected. Upon reinjection, the product eluted as one peak. Additional peaks were also collected and concentrated for comparison.

3.5. Analysis of hemoglobin bis-tetramers

SDS-PAGE analysis of this purified higher molecular weight sample indicated that the sample contained primarily 64 kDa and 15 kDa peptides (Figure 3.12). A very small amount of 32 kDa protein was also present.
AX300 anion exchange HPLC indicated that the product was homogeneous, and eluted at 35 min (Figure 3.13). C4 reversed-phase HPLC analysis of the purified sample indicated that unmodified $\beta$ chains were absent and unmodified $\alpha$ chains were present. A product peak eluted at approximately 75 minutes (Figure 3.14).

Figure 3.12. Density scan of the SDS-PAGE well containing purified higher molecular weight hemoglobin.
Figure 3.13. AX300 anion-exchange chromatograph for purified higher molecular weight hemoglobin.
The unmodified β chain peak and the modified β chain product peak were collected from the reversed-phase C4 column and subjected to enzymatic hydrolysis. Individual peptide fragments were separated using a reversed-phase C18 HPLC column. The chromatographic pattern of digested unmodified β chains was compared with digested regions of the modified product peak. Chromatographs for both the 220nm and 280 nm absorbances were used to identify the peptides of the digested unmodified β globin (Figure 3.15, 3.16, 3.17, 3.18). These results indicated that the relative amounts of both the βT-1 and the βT-10a peptides were approximately half of
peaks (Figure 3.17). In addition, several new peaks, with absorbance at 280 nm, appeared in the modified β globin digest chromatograph (Figure 3.18).

**Figure 3.15.** C18 reversed-phase HPLC chromatograph of unmodified β globin chain digest monitored 220 nm.
Figure 3.16. C18 reversed-phase HPLC chromatograph of unmodified β globin chain digest monitored at 280nm.
Figure 3.17. C18 reversed-phase HPLC chromatograph of modified β globin chain digest monitored at 220nm.
Figure 3.18. C18 reversed-phase HPLC chromatograph of modified β globin chain digest monitored at 280nm.
Chapter IV.

Discussion

4.1. Cross-linker design

DBIT is designed to be a site-specific reagent capable of forming bis-tetramers by cross-linking two hemoglobin molecules. Cross-linking can be intramolecular, connecting two sites within the same molecule, or intermolecular, establishing a link between two molecules, or both. Formation of hemoglobin bis-tetramers requires both intermolecular cross-linking, to link two hemoglobin tetramers, and intramolecular cross-linking, to join the dimers of each hemoglobin tetramer and stabilize its structure (Figure 4.1).

Figure 4.1. The structure of a hemoglobin bis-tetramer showing intra- and intermolecular cross-links.
The structure of DBIT is based on previous reagents known to cross-link hemoglobin in a site-specific manner. The isophthalic arrangement of reactive DBS-ester sites has previously been shown to be specific for the amino groups of the Lys-82 and Val-1 residues of the β globin chains of hemoglobin. The amine nitrogen of the protein reacts at the ester carbonyl carbon of the reagent to form a new amide bond between the cross-linker and the protein. The β-Lys-82 and β-Val-1 residues are located in the positively charged DPG binding site. Negatively charged reagents will be attracted to the positively charged DPG binding site. Reagents such as IPMP, TMMP, TTDS and BTDS can have negative charges and all have the isophthalic arrangement of reactive ester sites (Figure 4.3). These reagents react with hemoglobin to establish a cross-link between the two β chains by covalently modifying two of the four available sites. DBIT has two such reactive isophthalic arrangements, thus each end of the reagent should cross-link hemoglobin by reacting within the DPG binding site (Figure 4.2).

**Figure 4.2.** The structure of DBIT.
Figure 4.3. The structure of cross-linking reagents with the isophthalic arrangement of reactive ester sites.

Flexible tetra-functional reagents, even with the two-headed isophthalic structure, have reacted with poor specificity. In particular, DPEE was shown to react with hemoglobin to modify four groups within one DPG binding site, presumably by folding back upon itself (Figure 4.4). This flexibility could also allow the reagent to modify a variety of surface groups on the hemoglobin molecule, thereby jeopardizing its site-specificity. The rigidity of the core of DBIT was intended to prevent the reagent from folding back on itself and reacting with either remaining reactive groups of the DPG binding site or surface groups of the same hemoglobin molecule.
The final consideration in designing a reagent to give bis-tetrameric hemoglobin was to determine the appropriate spacing of the two reactive ends. BTDS, a rigid reagent with the above mentioned two-headed isophthalic structure, did not react with hemoglobin to form bis-tetramers, presumably because the reagent was too short to bridge the distance between two hemoglobin molecules (Figure 4.3). Unfavorable electrostatic repulsion or steric interactions may result from bringing two hemoglobin molecules closely together. Using a divergent dendritic approach to synthesize new reagents facilitates the production of several generations of reagents (Scheme 2). Each generation of cross-linker has progressively longer separations of reactive ends. If necessary, the higher generations of cross-linkers could be reacted with hemoglobin to determine the minimum separation required to form bis-tetramers or to optimize the yield of bis-tetramers. DBIT is the first generation reagent from a dendritic synthetic approach utilizing terephthalic acid as the core and 5-aminoisophthalic acid as the expansion unit (Scheme 2). Since DBIT reacted with hemoglobin to form bis-tetramers, subsequent dendritic generations were not synthesized.
**Scheme 2.** The application of dendritic chemistry to cross-linker synthesis.
4.2. Reaction of DBIT with hemoglobin and analysis of products

Several different techniques are used to analyze the hemoglobin-DBIT reaction products. The conditions used with these techniques can affect the quaternary structure of hemoglobin. The various cross-linked products will be affected differently depending on the type and number of cross-links. Figure 4.5 illustrates the possible reaction products and quaternary structure (with associated molecular weights in kDa) under various conditions. Under non-dissociating conditions, used with some gel filtration and ion-exchange techniques, hemoglobin molecules will remain in tetrameric form. Under partial-dissociating conditions, used with other gel filtration techniques, αβ dimers will dissociate from one another unless covalently attached through a β–β, α–α, or α–β cross-link. Under the dissociating conditions used with C4 reversed-phase HPLC and SDS-PAGE techniques, all globin chains separate from one another unless covalently cross-linked.
Figure 4.5. Possible products resulting from reaction of hemoglobin with DBIT. Shown are the effects of the experimental conditions (non-dissociating, partial-dissociating and dissociating) on the quaternary structure as indicated by the given molecular weight (in kDa) of the various components.

The reaction of DBIT with hemoglobin proceeds more slowly than with previous reagents having similar structures. In particular, BTDS reacts with hemoglobin to modify all β globin chains in 2 hours. The slight differences in structure around the isophthalic ring in DBIT is not expected to cause such a decrease in reactivity. The slower rate of reaction of DBIT may result from steric and electrostatic barriers involved in bringing two hemoglobin molecules together. In addition, access to the
most readily with the deoxy form of hemoglobin and less readily with the carbonmonoxy form. The DPG binding site of hemoglobin is smaller when ligands are bound to heme so the carbonmonoxy hemoglobin site is not as accessible to DBIT as it is with deoxy-hemoglobin.

Reversed-phase HPLC was used to monitor the reaction of DBIT with hemoglobin (Figure 3.1). This is a dissociating technique which separates the heme and various globin chain moieties in the reaction mixture. The cross-linking reaction is stopped when subsequent chromatographs indicate that modification of β globin chains is complete. This can occur because all β chains are modified, as indicated by complete disappearance of the peak at 23 min, or because the size of the peak corresponding to the unmodified β chains is constant over several hours. The size of the peak at 35 minutes, corresponding to unmodified α globin chains, does not change throughout the reaction, indicating that the reagent is selective for β modification only. Product peaks are identified at various retention times indicating several different reaction patterns are accessible to this reagent. HPLC coupled mass spectrometry identified product peaks with a variety of masses. The major product peaks corresponded to products having structures A, B and C in Figure 4.6. Higher molecular weight products having more than two cross-linked globin chains were not identified using this method. The available ESI mass spectrometry techniques were not suitable for ionizing high molecular weight cross-linked globins.
Figure 4.6. Products of DBIT and hemoglobin reaction identified by ESI mass spectrometry (location of remaining DBS groups is subjective).

Size exclusion chromatography is used to separate the reaction products according to molecular weight. The chromatographic medium consists of gel beads having variable sized pores. Smaller proteins can enter these pores and are thereby detained in passing through the column. Larger proteins will not be delayed and will therefore elute faster than smaller proteins. The order of elution is indicative of decreasing molecular weight of proteins in a mixed sample. The effects of the eluent on the quaternary structure of hemoglobin is determined by calibration. The G75 column is calibrated using unmodified hemoglobin and a purified solution of hemoglobin cross-linked between β-subunits. Under non-dissociating conditions both the unmodified hemoglobin and the cross-linked hemoglobin elute with the same retention time,
conditions caused non-cross-linked dimers to dissociate from one another, a different retention pattern would result. Unmodified hemoglobin would elute as a single peak with a retention time corresponding to a molecular weight of 32 kDa (Figure 4.5; partial-dissociating). This retention time would be longer than that of the cross-linked hemoglobin where a covalent bond between β chains would prevent dissociation of dimers. The cross-linked sample would elute with a shorter retention time corresponding to a molecular weight of 64 kDa. Since the two samples elute with the same retention time the column conditions are non-dissociating. The same standards are also used to determine the effects of ‘dissociating’ conditions on the tertiary structure of hemoglobin. Under these conditions the cross-linked hemoglobin elutes as a single peak having a shorter retention time than the single peak of unmodified hemoglobin (Figures 3.4 and 3.5). This is because the cross-linked hemoglobin remains in tetrameric form having a molecular weight of 64 kDa while the unmodified hemoglobin dissociates into αβ dimers having a molecular weight of 32 kDa. If the column conditions are dissociating, the cross-linked hemoglobin would elute as two peaks with retention times corresponding to 32 kDa and 15 kDa, for the separated α chains and the cross-linked β chains respectively (Figure 4.5; dissociating conditions). These results indicated that the ‘dissociating’ column conditions are actually partial-dissociating. The same two conditions are used with the G200 column.

Analysis of the reaction mixture using partial-dissociating, size exclusion chromatography indicates the presence of species with three different molecular
linked hemoglobin with a molecular weight of 64 kDa and hemoglobin αβ dimers with a molecular weight of 32 kDa. The third peak elutes faster that the other two indicating that the third species has a molecular weight higher than 64 kDa. The higher molecular weight species could result from either three or four cross-linked β globin chains with the same number of associated α chains. It can be assumed that the cross-links occur between β chains because the C4 reversed-phase analysis shows very little α chain modification. If three αβ dimers are cross-linked, the molecular weight of the species under partial-dissociating conditions would be 96 kDa (Figure 4.5; partial-dissociating conditions). Similarly, four cross-linked αβ dimers would have a molecular weight of 128 kDa (Figure 4.5; partial-dissociating conditions). The G200 size exclusion column under partial-dissociating conditions would distinguish between species having these molecular weights. Since the chromatograph indicates the presence of only one product with a molecular weight above 64 kDa, only one of the high molecular weight products is possible (Figure 3.8). SDS-PAGE can be used to determine if the 96 or 128 kDa species forms by looking for 45 or 64 kDa peptides, respectively (Figure 4.5; partial-dissociating and dissociating conditions).

SDS-PAGE differentiates between proteins of different molecular weights. The reaction of proteins with β-mercaptoethanol breaks any disulphide bonds and the detergent SDS disrupts all electrostatic interactions resulting in a linear protein. The association of charged SDS molecules along the length of the protein gives each the
mixture is then loaded onto a polyacrylamide gel, which acts as a sieving matrix, and is subjected to an electric field. The various proteins move under the influence of the electric field and the gel slows the progress of the larger proteins. The distance travelled by each protein component is inversely proportional to its molecular weight.

A sample containing known molecular weight markers is run along side the sample in order to prepare a standard curve against which the molecular weight of unknowns can be compared. The conditions used for this technique dissociate α and β globins completely, unless covalently cross-linked, as can be seen from the presence of two bands in close proximity for unmodified hemoglobin (Figure 3.10). These bands correspond to a protein of 16 kDa for β globin chains and 15 kDa for α globin chains (Figure 4.5; dissociating conditions). The absence of high molecular weight species for unmodified hemoglobin indicates that the dissociation of α globins from β globins is complete.

Analysis of the cross-linking reaction mixture indicates the presence of proteins with molecular weights of approximately 64 and 32 kDa in addition to the expected 16 and 15 kDa proteins (Figure 3.9). These results indicate that the reaction does not proceed to completion since some non-cross-linked β globin chains remain in the mixture. The high molecular weight species must result from cross-linked globins because the conditions are dissociating (Figure 4.5). Cross-linked α globin chains were not considered since the C4 reversed-phase analysis shows very little α chain
linked β chains and 64 kDa protein band indicates the presence of four cross-linked β chains. Since each hemoglobin has only two β chains to contribute, the presence of a 64 kDa band indicates intra- and intermolecular bridging, and therefore the formation of hemoglobin bis-tetramers. The molecular weight of the hemoglobin bis-tetramers would be 128 kDa because each of the four β chains would have an associated α chain. Thus the fastest eluting peak from the size exclusion column consists of hemoglobin bis-tetramers with a molecular weight of 128 kDa.

A question remains as to why the triply linked product, having three cross-linked αβ dimers, does not form. There would be two hypothetical routes for formation of the triply linked species. Presumably neither is feasible or else the triply linked product would occur. In the first route, the fourth DBS would be hydrolyzed after the third has reacted, as shown in Scheme 3. This hydrolysis does not happen readily. Possibly the DPG binding site, with DBIT present, is too crowded for a water molecule to approach for the hydrolysis attack, or the reaction of the ester site with a second amine nucleophile on the protein may proceed more quickly than hydrolysis.

The second hypothetical route to formation of the triply linked product is shown in Scheme 4. The DBIT reagent, having one hydrolyzed DBS and one remaining DBS, would react with a hemoglobin tetramer. This reaction does not happen readily because the product is not seen. With one hydrolyzed DBS group, there is only one negatively charged carboxylic acid group at the far end of the reagent. A second negative charge
Two negatively charged groups may be required at the far end for DBIT to be drawn into the positively charged DPG binding site. The requirement for two outlying charges would be most stringent when the reagent has already reacted with a hemoglobin molecule at the other end. The attraction of the negative charges for the positively charged site could then overcome the developing electrostatic interaction between first and the second hemoglobin tetramer.

Scheme 3. A reaction leading to formation of a triply linked, high molecular weight hemoglobin.
Scheme 4. A reaction leading to formation of a triply linked, high molecular weight hemoglobin.

Complete analysis of the reaction products is complicated by the presence of both molecular weight and structural variants. Previous analytical methods exploited the effects of structural variations in cross-linking. For instance, hemoglobin cross-linked between the β-Lys-82 residues of two β globins (ααβ82-82β) could be separated from hemoglobin cross-linked between a β-Lys-82 residue of one β globin and a β-Val-1 residue of another (ααβ82-1β) by ion exchange chromatography. Since it is shown that DBIT can react with hemoglobin to give bis-tetramers, it is possible for the same
(Figure 4.7). For instance, hemoglobin cross-linked between the β-Lys-82 residues of two β chains (ααβ82-82β) could be expected to have similar characteristics to two intermolecular cross-linked hemoglobins with the same intramolecular cross-link between the β-Lys-82 residues of β globin chains (ααβ82-82β – β82-82βαα). These two product may not be easily separated by commonly employed methods. Indeed, both C4 reversed-phase and several ion-exchange chromographic methods failed to resolve the various reaction products. To eliminate complications arising from both molecular weight and structural variants, the reaction mixture is first separated according to molecular weight.

![Diagram of hemoglobin structures](image)

Figure 4.7. Structurally similar hemoglobins with different molecular weights.
The reaction mixture is separated into fractions of hemoglobins of different molecular weights, using the G200 size exclusion column. Each of the three different peaks, attributed to 128, 64 and 32 kDa hemoglobin components, are collected under dissociating conditions (Figure 3.8). Fractions collected from multiple injections are pooled and concentrated. Re-injection of each fraction onto the G200 column indicates that the 128 and 32 kDa fractions are homogeneous while the 64 kDa fraction was heterogeneous, with respect to molecular weight. The 64 kDa fraction has a large peak as well as two smaller peaks attributed to the presence of both 128 and 32 kDa species. Because the three peaks overlap, the contaminating species are most likely collected with the 64 kDa fraction. Since the 128 kDa fraction is of primary interest, further purification attempts are not made on the 64 kDa fraction.

The fractions are then analyzed by SDS-PAGE. The results are consistent with those of the G200 column. The 128 kDa fraction consists of two bands, of approximately equal intensity, having molecular weights of 64 and 15 kDa (Figure 3.12). This is consistent with a sample of pure hemoglobin bis-tetramers. Under SDS-PAGE conditions, the bis-tetramers of hemoglobin would separate into four cross-linked β chains (with a molecular weight of 64 kDa) and unmodified α chains (with a molecular weight of 15 kDa) (Figure 3.12). The 32 kDa fraction consists of two bands, of approximately equal intensity, having molecular weights of 16 and 15kDa. This is consistent with a sample of unmodified hemoglobin. Under SDS-PAGE conditions,
chains (with a molecular weight of 15 kDa). The 64 kDa fraction consisted of several bands having molecular weights of 64, 32, 16 and 15 kDa, indicating that this fraction contains hemoglobin products with several different molecular weights.

4.4. Analysis of hemoglobin bis-tetramers

In order to determine the structural homogeneity of the hemoglobin, the 128 kDa fraction is analyzed by ion-exchange chromatography. If DBIT reacts with hemoglobin to give several bis-tetramers with different cross-linking patterns, they should be separated by ion-exchange. The AX300 ion-exchange column has previously been used to separate modified hemoglobin with different types of cross-links. The AX300 chromatograph for the 128 kDa fraction of hemoglobin modified with DBIT has only one peak (Figure 3.13). The retention time is different than for unmodified hemoglobin. This result indicates that only one cross-linking pattern leads to formation of bis tetramers.

Within the DPG binding site, each β globin chain has two residues most accessible for modification by cross-linkers with the isophthalic arrangement of reactive sites. These are the β-Val-1 and β-Lys-82 residues. Both 1-82 and 82-82 cross-links have been observed with reagents having the isophthalic arrangement. The 1-1 cross-link has not been observed with these reagents so this possibility is unlikely with DBIT. Formation of the bis-tetramers requires at least four covalent modifications, one on
each p-globin chain of two different hemoglobin tetramers. Since DBIT has only four reactive sites, no more than four modifications can occur with one cross-linker. It is possible that a second cross-linker could modify additional residues within the DPG binding site, but the size of the cross-linker makes this unlikely. Mass spectroscopy analysis of the cross-linked globin chains could determine the number of DBIT molecules attached to the cross-linked protein, but attempts to analyze the bis-tetramers were unsuccessful. The available mass spectroscopy techniques were not appropriate for the large 64 kDa protein.

There are many combinations of cross-linking patterns that can give bis-tetramers (Figure 4.8). Since both the reagent and hemoglobin have symmetry properties, some of these possibilities are redundant. There are four possible cross-linking patterns for modification of hemoglobin by one molecule of DBIT resulting in formation of bis-tetramers. A protein digest pattern can distinguish between patterns a and b of Figure 4.8, but patterns c, d and e would give the same digest result.
Figure 4.8. Possible cross-linking patterns for formation of hemoglobin bis-tetramers from reaction with DBIT.

A C4 reversed-phase column is used to separate the modified β chains from the heme and α chains. A broad peak corresponding to modified β is collected from several injections of the 128 kDa hemoglobin fraction. The collected samples are pooled, lyophilized and subjected to hydrolysis by tryptic and gluC enzymes. The resulting
Trypsin hydrolyzes the peptide bonds at the C-terminal side of amino acids with positively charged residues (lysine and arginine). The resulting peptides are further hydrolyzed by endoproteinase Glu-C at the C-terminal side of glutamate residues. In the C18 reversed-phase HPLC peptide pattern chromatograph of the cross-linked β globin, the βT−1 and βT−10a' are reduced by approximately one half compared to the pattern for unmodified β globin. From these results it can be concluded that cross-links occur between the β−Lys-82 residue (found in the βT−10a’ peptide fragment) and the β−Val-1 residue (found in the βT−1 peptide fragment) in both hemoglobin molecules (Figure 4.8; c or d). Alternatively, one hemoglobin molecule could have an intramolecular cross-link between two β−Lys-82 residues and the other molecule an intramolecular cross-link between two β−Val-1 residues (Figure 4.8; e). Either of these cross-linking patterns would result in formation of the bis-tetramer and be consistent with the observed digest results. Previous results have shown that the isophthalic-type cross-linkers have been specific for either 82-82 or 82-1 intramolecular cross-links. Therefore, it is unlikely that one reagent could produce both types of intramolecular cross-links, as seen in structure e of Figure 4.8. More likely, the structure of the hemoglobin bis-tetramer is illustrated by structure c or d of Figure 4.8. A crystal structure of the hemoglobin bis-tetramers would distinguish between these possibilities.
and prevents the tryptic digest from cutting between residues 81 and 82. This should result in a reduction in the βT–9 peptide fragment which corresponds to that of the βT–10a’ peptide fragment. The βT–9 peptide fragment then becomes part of the new peptide peak. This expected reduction of the βT–9 peptide fragment is not obvious due to new product peaks in that area of the chromatograph.

Several new peaks are present in the C18 reversed-phase HPLC peptide pattern chromatograph of the cross-linked β globin (Figure3.17). Many of these new peaks have absorbance at 280 nm, as is expected for peptides attached to DBIT (Figure 3.18). However, only one new product peak is expected if the digested globin chains were structurally pure. The presence of several different product peak indicates that the AX-300 ion exchange column failed to separated the various components present in the bis-tetramer fraction. These components could represent different cross-linking patterns leading to the formation of bis-tetramers or surface modifications of hemoglobin by the excess DBIT present in the reaction. Further purification is necessary before the exact structure or structures of the hemoglobin bis-tetramers can be determined.

4.5. Applications of hemoglobin bis-tetramers

Red-cell Substitute
be promising new candidates for a red-cell substitute. The larger size should prolong retention time relative to that of the unmodified or intramolecularly cross-linked hemoglobin. This method of bis-tetramer preparation is distinct from polymerization methods because the products are more homogeneous and of defined structure. Further studies will indicate if the $P_{50}$ of the bis-tetramers is appropriate for oxygen delivery.

Oxygen Binding

Hemoglobin is a tetramer consisting of four globin chains, two $\alpha$ and two $\beta$, each having an associated heme. Each heme contains a centrally bound Fe(II) ion capable of coordinating one molecule of oxygen. Hemoglobin binds, and releases, four molecules of oxygen in a cooperative manner, as indicated by the sigmoidal shape of its oxygen binding curve. The binding of one oxygen to one heme facilitates the binding of oxygen to the other hemes. Although hemoglobin has been extensively studied, neither the oxygen affinity nor the origin of cooperativity is not completely understood. The study of bis-tetramers of hemoglobin may provide additional information in this area.

Does the hemoglobin display cooperativity within a tetramer? If one molecule of oxygen binds to one heme site, the remaining three sites should have an increased affinity for a second oxygen. But within a bis-tetrameric hemoglobin there would be a competing stoichiometric preference for the second oxygen to bind at one of the four sites of the second molecule. Do the two tetramers communicate with one another and display additional cooperativity among the eight binding sites? Is the three dimensional
A study of the oxygen binding properties of hemoglobin bis-tetramers could provide valuable information about oxygen affinity and the mechanism of cooperativity.
Conclusions

Bis-tetramers of hemoglobin can be formed by intramolecular and intermolecular cross-linking with a reagent have appropriate reactivity, rigidity and length. A novel and effective tetrafunctional protein cross-linker, DBIT, has been designed, synthesized and reacted with human hemoglobin. Analysis of the reaction products showed formation of hemoglobin bis-tetramers. The bis-tetramers have been isolated from the reaction mixture and it has been shown that the bis-tetrameric hemoglobin was modified at the Lys-82 and Val-1 residues of the beta globin chains.

These results extend the concept of protein modification to include controlled and specific intermolecular cross-linking of two protein molecules.
References


