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Modification of Human Hemoglobin with Dibromosalicyl Esters of N-Biotinyl-5-Aminoisophthalate, Trifluoroacetyl-Isoleucyl-Glycil-5-Aminoisophthalate and Trifluoroacetyl-Phenylalanyl-5-Aminoisophthalate

by

Sanda Crapatureanu

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 Anca and Andy
Abstract

Modification of Human Hemoglobin with Dibromosalicyl Esters of
N-Biotinyl-5-Aminoisophthalate, Trifluoroacetyl-Isoleucyl-Glycyl-5-
Aminoisophthalate and Trifluoroacetyl-Phenylalanyl-5-Aminoisophthalate

Master of Science, 1998
Sanda Crapatureanu, Department of Chemistry, University of Toronto

Specific anchoring of biologically active molecules to human Hb A can be accomplished by using affinity labeling reagents targeting the cationic sites in Hb. Derivatives of d-biotin, isoleucyl-glycine and phenylalanine have been designed and synthesized for anchoring to hemoglobin. These reagents bind to, and subsequently react at specific sites to give stable, cross-linked hemoglobin conjugates. Major reaction products were isolated and structurally characterized by HPLC-MS, SDS-PAGE and proteolytic digestion. The major modified components consisted of hemoglobin cross-linked between the Lys-82 residues of each β chain and Lys-99 of each α chain.

Applications of this methodology may include introduction of novel biological properties on hemoglobin by means of biotin-avidin-biotin bridges and use of hemoglobin as a mean for transport and delivery of organic substances such as peptides which are difficult to develop as pharmaceuticals due to their short half-lives.
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Bis-Tris  bis(2-hydroxyethyl)aminotris(hydroxymethyl)methane
COHb    carbonmonoxy human hemoglobin A
CH$_2$Cl$_2$ dichloromethane
DBBA    bis(3,5-dibromosalicyl) N-biotinyl-5-aminoisophthalate
DBST-Hb 3,5-dibromosalicyl trimesyl-((Lys-$\beta$-82)-(Lys-$\beta$-82)-hemoglobin
DBTIA   bis(3,5-dibromosalicyl) N-[N-trifluoroacetyl-L-isoleucylglycyl]-5-
aminoisophthalate
DBTPA   bis(3,5-dibromosalicyl) N-[N-trifluoroacetyl-L-phenylalanyl]-5-
aminoisophthalate
DBS     dibromosalicylic acid
DCC     dicyclohexylcarbodiimide
DEC-HCl 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
deoxyHb deoxygenated human hemoglobin
DMAc    N,N-dimethylacetamide
4-DMAP   4-dimethylaminopyridine
DMF      N,N-dimethylformamide
DMSO    dimethylsulfoxide
DPG     2,3-diphosphoglycerate
ESI-MS  electrospray ionization mass spectroscopy
g      grams
Hb A    human hemoglobin A
HPLC    high performance liquid chromatography
IHP     inositol hexophosphate
m      molar
min     minute
mol     moles
MOPS   3-(N-morpholino)propanesulfonic acid
mp      melting point
PBS     phosphate buffer saline
ppm     parts per million
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFA     trifluoroacetic acid
TFAnhydride trifluoroacetic anhydride
THF     tetrahydrofuran
TTDS    trimesoyltris(3,5-dibromosalycilate)
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CHAPTER I

Introduction

The chemical modification of proteins has recently been finding application in the preparation of proteins for both diagnostic and therapeutic use. One of these applications includes the modification of proteins to render them useful for drug binding and transport or to produce derivatives with specific binding properties.

The major advantage of protein-conjugated drugs over free drugs is the significant enhancement of pharmacokinetics, that can be achieved. Targeting of drugs to renal sites by coupling to low-molecular-mass proteins such as lysozymes has been achieved. Specific attention has also been given to protein-glycoconjugates-mediated delivery of antisense oligonucleotides and dipeptides to macrophages in order to stimulate tumoricidal activity. Due to their lack of oral bioavailability and sensitivity to proteases in the digestive tract, peptides and peptidomimetics represent a class of targets for the development of injectable, timed release complexes with carrier proteins.

The chemical reactions to modify proteins aim generally either at modifying one type of residue (residue specificity) or a single amino acid residue in the protein. The second approach takes advantage of affinity reagents, which bind the protein with high affinity in the right juxtaposition to produce specific modifications.

The chemical modification of hemoglobin has been used so far to prepare derivatives of this protein, which could be useful as blood substitutes. A novel approach involves the use of hemoglobin as a model system for delivery of molecules other than oxygen or carbon dioxide. Hemoglobin has unique properties that offer attractive therapeutic possibilities: it is naturally removed from circulation by macrophages, it has a long serum half-life and undesired immune responses to it are not expected since it is an autologous protein. The anticipated advantages of hemoglobin-anchored drugs over free drugs, include extended circulation half-lives, diminished toxicity due to reduced volume
of distribution, timed release of the drug. In addition, hemoglobin delivers O$_2$ to the site of drug release.$^4$

However, the use of free human hemoglobin as a drug carrier presents two main disadvantages, which restricts its usefulness. The first of these is related to its greater oxygen affinity compared to normal human blood and therefore a significantly lower oxygen tension at the level of the tissues, due to removal from an environment high in 2,3-diphosphoglycerate; the second is related to the dissociation of free Hb tetramers into $\alpha\beta$ dimers, which are rapidly eliminated through the renal glomeruli and characterized by the absence of cooperativity and a very high oxygen affinity.$^5$

These two problems could be overcome by covalent cross-linking hemoglobin with anionic cross-linkers, which have high affinity toward cationic binding sites such as the 2,3-DPG binding (between the $\beta$ chains), or the central cationic site between the $\alpha$ chains. The most susceptible to these modifications are the $\beta$-Lys-82, $\beta$-Val-1, $\beta$-Lys-144 and $\alpha$-Lys-99 residues. Intramolecularly cross-linked hemoglobins do not dissociate into $\alpha\beta$ dimers, do not pass across the glomerular filter and have a plasma half time in rats four times longer than native hemoglobin.$^6$ The use of cross-linkers, besides stabilizing the tetramers, also provides an opportunity to conjugate biologically active entities to hemoglobin, transforming it into a potential drug delivery system. In principle there are two approaches for achieving this goal:

1. Chemical modification (cross-linking) of hemoglobin with a trifunctional cross-linker that retains a site for reaction with the drug. Using this approach, Kluger and coworkers have synthesized a trifunctional cross-linking reagent TTDS (Trimesoyltris(3,5-dibromosalicylate))$^7$ that reacts with deoxyhemoglobin and carbonmonoxy-hemoglobin at 35°C in 0.1 M MOPS buffer to produce the $\beta82-82\beta'$ cross-linked hemoglobin as the main product (DBST-Hb). The site selectivity of this reagent is determined by the steering effect of the negative charges directing it to the cationic 2,3-DPG binding site of hemoglobin. The nucleophilic lysine or N-terminal valine amino group react with the ester carbonyl of the leaving group. This hemoglobin conjugate, which retains one electrophilic
site at the unreacted 3,5-dibromosalicyl group, is thus converted into a reactive acylating reagent (DBST-Hb) (Figure 1.1.)

![Diagram of DBST-Hb conjugate](image)

**Figure 1.1.** The structure of the DBST-Hb conjugate formed by reacting Hb A with TTDS.

2. Covalent attachment of the drug to a bifunctional cross-linker followed by reaction with hemoglobin. This type of arrangement has not been widely explored. A reagent, sulfo-SBED, produced by Pierce Chemical, in which there are two reactive groups (N-O-acyl succinimide and azido) connected by a short hydrocarbon chain to biotin, has been reacted with soybean trypsin inhibitor (STI). That reaction does not introduce a cross-link. The biotinylated protein can be further conjugated to a second protein through a photoreaction.

An investigation of hemoglobin as a model system for delivery of peptides was carried out by Anderson and coworkers. They designed and tested bioconjugates formed by attaching angiotensin II analogs to recombinant human hemoglobin through a disulfide bond. This controlled release system is intended to function by reductive cleavage of
disulfide-linked drugs by serum endogenous reducing agents such as reduced glutathione (GSH).

A method of attaching small molecules or peptides to a site-specific cross-linker that forms an intramolecular link between two α or β subunits would produce hemoglobin conjugates with potential characteristics of a timed release drug delivery system. This approach would combine the benefits of stabilizing the tetramer, formation of bioconjugates of known structure with that of a timed release of drugs in circulation. Site selective modification of hemoglobin is required in order to minimize the number of products. The desired site specificity can be obtained by using an amino group specific reagent with an appropriate span between the reactive functionalities. A class of affinity reagents which have been most extensively investigated are the 3,5-dibromosalicylate esters of fumaric, succinic and isophthalic acid. Walder et al.\textsuperscript{10} have shown that bis(3,5-dibromosalicyl)fumarate, when reacted with oxyhemoglobin, specifically cross-linked β-Lys-82 and β'-Lys-82 at the DPG binding site and when reacted with deoxyhemoglobin gave a mixture of inter-β cross-linked products and the ββα99-99α' cross-linked derivative. The functional properties (oxygen affinity, cooperativity and alkaline Bohr effect) of the purified inter-β cross-linked hemoglobin obtained in the reaction with oxyhemoglobin were very similar to those of unmodified hemoglobin.\textsuperscript{11} The modified inter-α cross-linked hemoglobin remained highly cooperative as well but its oxygen affinity decreased by approximately 2-fold.\textsuperscript{10}

The trifunctional reagent TTDS synthesized by Kluger and coworkers\textsuperscript{12} proved to be a more selective modifying agent for the ε-amino groups of the Lys-82 residues of each of the β subunits. The addition of a negatively charged functional group increases the size of the reagent and enhances the electrostatic interactions with the DPG binding domain rich in positively charged groups. Therefore, selectivity increases drastically. Cross-linking Hb A under the same conditions with trimesoyl tris(methyl phosphate) gave a triply cross-linked derivative as the main product.\textsuperscript{13} A more selective and efficient reagent has been developed in the same group by adding the fourth 3,5-dibromosalicylate moiety to the core molecule. The reagent cross-linked deoxyhemoglobin selectively between the β-Lys-82 residues of each β subunit with a 100% yield.\textsuperscript{14}
The purpose of this thesis is to explore the feasibility of conjugating biologically active entities such as biotin or small peptides to cross-linked hemoglobin. This is necessary for a hemoglobin-mediated therapy. The strategy used was first to conjugate the molecule of interest to a central isophthalic core to which the 3,5-dibromosalicylate leaving groups were subsequently attached. As a demonstration of the possibilities of application of this methodology, the following derivatives of d-biotin, N-t-Boc-Ile-Gly and N-t-Boc-Phe were synthesized: bis(3,5-dibromosalicyl) N-biotinyl-5-aminoiso-phthalate (DBBA), bis(3,5-dibromosalicyl) N-[N-trifluoroacetyl-L-isoleucylglycyl]-5-aminoisophthalate (DBTIA) and bis(3,5-dibromosalicyl) N-[N-trifluoroacetyl-L-phenylalanyl]-5-aminoisophthalate (DBTPA) (Figure 1.2.). The terminal amino group in DBTIA and DBTPA was trifluoroacetylated in order to elongate the phenylalanine and isoleucylglycine chain with one amide group in the same step in which deprotection of the t-butyl protected carboxyl group was achieved. Biotin was chosen in order to obtain a hemoglobin-biotin conjugate with known structure and specific binding properties for avidin. The synthesized reagents were conjugated to hemoglobin and the efficiency of the cross-linking reaction and the chemical nature of the cross-linked products have been investigated.

![DBBA](image-url)
Figure 1.2. The structures of DBBA, DBTIA and DBTPA.
CHAPTER II

Experimental

2.1. Materials

d-biotin, N-t-Boc-Ile-Gly, N-t-Boc-L-Phenylalanine and avidin were purchased from the Sigma Chemical Co., 3,5-dibromosalicylic acid from Lancaster Synthesis Inc. and 5-aminoisophthalic acid from the Aldrich Chemical Co. Trypsin (Worthington Biochem Corporation) and endoproteinase Glu-C (Boehringer Mannheim GmbH) solutions were freshly prepared. Human hemoglobin A was obtained in the carbonmonoxy form from Hemosol Inc. All other reagents were of the highest grade commercially available. The purity of newly synthesized materials was assessed by NMR spectroscopy, mass spectrometry, infrared spectroscopy and thin layer chromatography. Proton NMR spectra were recorded on Varian Gemini (200 MHz and 300 MHz), Carbon NMR and Fluorine NMR spectra on Unity (500 MHz and 400 MHz) spectrometers. The chemical shifts are reported in ppm and the coupling constants are given in Hertz. Infrared spectra were recorded on a Nicolet 5DX FTIR spectrometer, UV-VIS spectra on a Perkin Elmer Lambda 19 spectrometer. Melting points were recorded on a Buchi apparatus and are uncorrected.
2.2. Synthesis of Bifunctional Cross-Linking Reagents

2.2.1. Synthesis of Bis(3,5-dibromosalicyl) N-Biotinyl-5-Aminoisophthalate.

**Biotin Acid Chloride.** d-Biotin (1.07 g, 4.38 mmol) was dissolved in 20 mL distilled SOCl₂ at room temperature and stirred until all solid dissolved. After an additional 20 min period, SOCl₂ was removed, the residue dried in vacuo and used without further purification.

**N-Biotinyl-5-Aminoisophthalic Acid.** 5-aminoisophthalic acid (0.993 g, 5.48 mmol) was dissolved in 25 mL anhydrous N,N-dimethylacetamide and stirred for 20 min under nitrogen. 4-dimethylaminopyridine (7.14 x 10⁻³ g, 0.584 mmol) was added to this solution and stirred until dissolved. Solution was cooled to 0°C and then added to the biotin acid chloride solution. Reaction mixture was stirred at room temperature for 14 h. A light brown precipitate formed which was filtered, reprecipitated from dimethylformamide with water, washed with hot methanol and dried under vacuum (0.700 g, 1.72 mmol): mp 220-221°C (decomposed); IR (KBr, cm⁻¹) 3391 (m), 2922 (m), 1696 (s), 1452 (m), 758 (m), 697 (s);¹NMR (DMSO-d₆) δ 10.23 (s, 1H, NH), 8.42 (s, 2H, ArH), 8.12 (s, 1H, ArH), 6.43 (s, 1H, NH), 6.35 (s, 1H, NH), 4.25-4.29 (m, 1H), 4.11-4.15 (m, 1H), 3.10-3.12 (m, 1H), 2.77-2.80 (m, 1H), 2.58 (s, 1H), 2.32 (s, 2H), 1.34-1.61 (m, 6H);¹³C NMR (DMSO-d₆) δ 25.01, 28.11, 28.21, 36.26, 40.10, 55.41, 59.20, 61.05, 123.4, 124.3, 131.6, 139.9, 162.7, 166.5, 171.7; MS (Cl) Calculated: 407.45; Found: 408 (MH⁺).

**Bis((1-tert-butoxycarbonyl)-3,5-dibromosalicyl) N-Biotinyl-5-Aminoisophthalate.** N-biotinyl-5-aminoisophthalic acid (1 equiv., 0.177 g, 0.433 mmol), t-butyl-3,5-dibromosalicylate (2.2 equiv., 0.340 g, 0.966 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (4 equiv., 0.334 g, 1.74 mmol), 4-dimethylaminopyridine (0.4 equiv., 0.0250 g, 0.200 mmol) were dissolved in
15 mL anhydrous dimethylformamide and stirred at 4°C for 20 h. The precipitated urea was filtered and the filtrate was diluted with water and ethyl acetate. The aqueous layer was extracted several times with ethyl acetate, the organic layers were pooled, washed with water, then with saturated sodium bicarbonate and again with water, dried over anhydrous magnesium sulphate and evaporated under vacuum (cream solid). The product was purified by flash chromatography (silica gel 60, eluent: acetone) and gave after isolation 0.186 g, 0.173 mmol: \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 10.6 (s, 1H, NH), 8.83 (d, \(J=1.5\), 2H, ArH), 8.48 (t, \(J=1.5\), 1H, ArH), 8.35 (d, \(J=2.4\), 2H, ArH), 8.03 (d, \(J=2.4\), 2H, ArH), 6.46 (s, 1H, NH), 6.38 (s, 1H, NH), 4.31-4.27 (m, 1H), 4.15-4.12 (m, 1H), 3.13-3.10 (m, 1H), 2.79 (dd, \(J=5\), 1H), 2.58 (d, \(J=12.3\), 1H), 2.38 (t, \(J=7.35\), 1H), 1.66-1.59 (m, 1H), 1.31 (s, 18 H); \(^{13}\)C (DMSO-\(d_6\)) \(\delta\) 172.2, 162.7, 162.1, 161.5, 145.6, 141.1, 138.8, 133.0, 129.4, 128.6, 125.0, 124.9, 119.6, 119.1, 83.1, 61.0, 59.2, 55.4, 40.0, 36.3, 28.2, 28.1, 24.8;

**Bis(3,5-dibromosalicyl) N-Biotinyl-5-Aminoisophthalate.** Bis((1-tert-butoxycarbonyl)-3,5-dibromosalicyl)) N-biotinyl-5-aminoisophthalate (0.186 g, 0.173 mmol) was dissolved in 15 mL anhydrous trifluoroacetic acid (precooled at 0°C), stirred at 0°C for 10 min and then 30 min at room temperature. Diethyl ether was added to induce crystallization and the solution was left for 1 h at 4°C. A cream precipitate formed which was filtered, washed with diethyl ether and dried in vacuo (0.163 g, 0.169 mmol): mp 235-236°C (decomposed); IR (KBr, cm\(^{-1}\)) 3368 (m), 3025 (m), 2921 (s), 1708 (s), 1452 (s), 1198 (s), 697 (s); \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 10.56 (s, 1H, NH), 8.77 (s, 2H, ArH), 8.41 (s, 1H, ArH), 8.34 (d, \(J=2.1\), 2H, ArH), 8.10 (d, \(J=2.1\), 2H, ArH), 6.47 (s, 1H, NH), 6.38 (s, 1H, NH), 4.31-4.27 (m, 1H), 4.15-4.10 (m, 1H), 3.24-3.11 (m, 1H), 2.80 (dd, \(J=5\), 1H), 2.58 (d, \(J=12.6\), 1H), 2.37 (t, \(J=7.2\), 2H), 1.65-1.40 (m, 6H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 24.92, 28.15, 28.25, 36.34, 55.43, 59.26, 61.10, 119.36, 119.54, 124.8, 127.6, 129.5, 133.5, 139.05, 139.70, 140.90, 162.4, 162.8, 163.53. MS(ESI) Calculated: 963.29; Found: 963.4
2.2.2. Synthesis of Bis(3,5-dibromosalicyl) N-[N-Trifluoroacetyl-L-Isoleucylglycyl]-5-Aminoisophthalate

N-[N-tert-Butyloxycarbonyl-L-Isoleucylglycyl]-5-Aminoisophthalic Acid

**Dimethyl Ester.** N-t-Boc-Ile-Gly (2.88 g, 9.97 mmol), dimethyl 5-amino-isophthalate (2.11 g, 10.1 mmol) and 4-dimethylaminopyridine (0.125 g, 1.03 mmol) were dissolved in 60 mL anhydrous dimethylformamide under nitrogen. Dicyclohexylcarbodimide (2.05 g, 9.94 mmol) was dissolved in 30 mL anhydrous dimethylformamide and added dropwise to the first solution. Reaction mixture was stirred for 66 h at room temperature under nitrogen. The precipitated N,N-dicyclohexyl urea was filtered, ethyl acetate was added in the filtrate and then water. The aqueous layer was extracted several times with ethyl acetate, the organic layers pooled, filtered again to remove additional precipitated urea and washed with 2M HCl (1200 mL), brine (500 mL), saturated sodium bicarbonate (700 mL) and again brine (250 mL). The organic phase was dried over anhydrous magnesium sulphate, filtered and concentrated under reduced pressure. The mixture was left at 4°C for 24 h, the yellowish precipitate formed was filtered, washed with hexane and dried in vacuo (1.75 g, 3.65 mmol): mp 124-125°C; IR (KBr) 3307 (m), 2967 (m), 1730 (s), 1555 (s), 1515 (s), 14440 (s), 1343 (s), 1250 (s), 1168 (s), 1009 (m), 758 (s); 

\[ ^1H \text{NMR (DMSO-}d_6) \delta 10.2 (s, 1H, NH), 8.48 (d, J=1.2, 2H, ArH), 8.34 (t, J=5.4, 1H, NH), 8.14 (t, J=1.5, 1H, ArH), 6.94 (d, J=8.1, 1H, NH), 3.91-3.83 (m, 3H, masked), 3.87 (s, 6H), 1.71-1.69 (m, 2H), 1.35 (s, 9H), 1.16-1.11 (m, 1H), 0.86-0.78 (m, 6H); ^{13}C \text{NMR (DMSO-}d_6) \delta 172.00 (s), 168.33 (s), 165.18 (s), 155.70 (s), 139.62 (s), 130.70 (s), 124.1 (s), 123.5 (s), 78.20 (s), 58.99 (s), 52.50 (s), 42.73 (s), 36.24 (s), 28.11 (s), 24.50 (s), 15.34 (s), 11.00 (s); MS (ESI) Calculated: 479.5; Found: 480.4 (MH^+).\]

N-[N-tert-Butyloxycarbonyl-L-Isoleucylglycyl]-5-Aminoisophthalic Acid.

N-[N-tert-butyloxycarbonyl-L-isoleucylglycyl]-5-aminoisophthalic acid dimethyl ester (1.75 g, 3.65 mmol) was dissolved in 100 mL methanol, 20 mL 6N sodium hydroxide were added to this solution and the reaction mixture was stirred under nitrogen for 2 h. After
addition of water and 50 mL 2N HCl to the reaction mixture, a white precipitate formed, which was filtered, washed with water and dried in vacuo (1.58 g, 3.50 mmol): mp 215-216°C; IR (KBr, cm⁻¹) 3329 (m, br), 2970 (m), 1699 (s), 1558 (m), 1252 (m), 667 (w); 
¹H NMR (DMSO-d₆) δ 10.15 (s, NH), 8.43 (d, J=1.5, 2H, ArH), 8.33 (t, J=5.7, 1H), 8.15 (t, J=1.5, 1H, ArH), 6.90 (d, J=8.1,1H, NH), 3.90-3.80 (m, 3H), 1.70-1.68 (m, 2H), 1.35 (s, 9H), 1.14-1.09 (m, 1H), 0.86-0.78 (m, 6H); ¹³C NMR (DMSO-d₆) δ 172.04 (s), 168.24 (s), 155.74 (s), 139.35 (s), 131.87 (s), 124.69 (s), 123.56 (s), 78.24 (s), 59.04 (s), 42.78 (s), 36.31 (s), 28.20 (s), 24.57 (s), 15.40 (s), 11.07 (s); MS(ESI) Calculated: 451.45; Found: 452.4 (MH⁺).

**Bis((1-tert-butoxycarbonyl)-3,5-dibromosalicyl)] N-[N-tert-Butyloxycarbonyl-L-Isoleucylglycyl]-5-Aminoisophthalate.** N-[N-tert-butoxycarbonyl-L-isoleucylglycyl]-5-aminoisophthalic acid (1.58 g, 3.50 mmol), 4-dimethylaminopyridine (8.50 x 10⁻² g, 0.7 mmol) and t-butyl-3,5-dibromosalycilate (2.2 equiv, 2.46 g, 7.00 mmol) were dissolved in 300 mL anhydrous THF and cooled to 0°C in an ice bath. 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.4 equiv, 1.61 g, 8.40 mmol), was dissolved in dry dichloromethane and added via a syringe, under nitrogen, dropwise to the solution. After stirring the mixture at 0°C for 5 min and at room temperature for 48 h, the solvent was evaporated and the residue redissolved in ethyl acetate. The solution was washed successively with saturated sodium bicarbonate and water, evaporated under reduced pressure and the white product used without further purification (1.40 g, 1.25 mmol).

**Bis(3,5-dibromosalicyl) N-[N-Trifluoroacetyl-L-Isoleucylglycyl]-5-Aminoisophthalate.** Bis((1-tert-butoxycarbonyl)-3,5-dibromosalicyl)) N-[N-tert-butyloxycarbonyl-L-isoleucylglycyl]-5-aminoisophthalate (1.40 g, 1.25 mmol) was dissolved in a mixture of trifluoroacetic acid and trifluoroacetic acid anhydride and stirred under nitrogen at room temperature for 2 h. The solvent was evaporated in vacuo, leaving a clear oil as a residue. Addition of ether resulted in the formation of a white precipitate, which was filtered and proved to be an unidentified decomposition product. The desired
compound was precipitated by adding hexane in the filtrate, filtered and dried in vacuo (0.411 g, 0.410 mmol): mp 170-172°C, IR (KBr, cm⁻¹) 3350 (m, br), 2923 (m), 1718 (s), 1558(s), 1452(s), 1199(s), 697 (s); ¹H NMR (DMSO-d₆) δ 10.68 (s, 1H, NH), 9.59 (d, J=8.4, 1H, NH), 8.75 (d, J=1.5, 2H, ArH), 8.68 (t, J=5.7, 1H, NH), 8.44 (t, J=1.5, 1H, ArH), 8.36 (d, J=2.4, 2H, ArH), 8.11 (d, J=2.4, 2H, ArH), 4.08-3.89 (m, 3H), 1.92-1.88 (m, 2H), 1.46-1.40 (m, 1H), 0.83-0.78 (m, 6H). ¹³C NMR (DMSO-d₆) δ 170.69 (s), 168.78 (s), 163.89 (s), 162.79 (s), 156.61 (q, J=145), 146.95 (s), 140.94 (s), 139.42 (s), 133.90 (s), 130.10 (s), 128.04 (s), 125.51(s), 125.27 (s), 119.93 (s), 119.72 (s), 58.13 (s), 43.21 (s), 35.77 (s), 24.98 (s), 15.56 (s), 10.84 (s); ¹⁹F NMR (DMSO-d₆) δ -74.07 (s, CF₃CO); MS (ESI) Calculated: 1003.45; Found: 1004.4 (MH⁺)

2.2.3. Synthesis of Bis(3,5-dibromosalicyl) N-[N-Trifluoroacetyl-L-Phenylalanyl]-5-Aminoisophthalate

N-[N-tert-Butyloxycarbonyl-L-Phenylalanyl]-5-Aminoisophthalic Acid

Dimethyl Ester. N-tert-butoxycarbonyl-L-phenylalanine (1 equiv., 6.21 g, 23.4 mmol), dimethyl 5-aminoisophthalate (1 equiv., 4.89 g, 23.4 mmol) and 4-dimethylaminopyridine (10% mol of acid, 0.29 g, 2.37 mmol) were dissolved under nitrogen in 100 mL anhydrous dimethylformamide. Dicyclohexylcarbodiimide (1 equiv., 4.86 g, 23.6 mmol) was dissolved in 50 mL anhydrous dimethylformamide and added dropwise to the first solution. Reaction mixture was stirred for 3 days at room temperature, producing a white precipitate. The precipitate was filtered and ethyl acetate and water were added to the filtrate. The ethyl acetate layer was washed successively with the following solutions: 500 mL saturated sodium chloride, 500 mL saturated sodium bicarbonate and 500 mL water. During washing and drying the solution over anhydrous magnesium sulphate, a white precipitate formed which was filtered. Evaporation of the solvent under reduced pressure gave a yellow solid. The crude product was purified by flash chromatography over silica gel (10% ethyl acetate in dichloromethane). Analytical TLC of an aliquot showed two spots, the one of lower Rf corresponding to unreacted dimethyl 5-
aminoisophthalate. Crystallization from hot dichloromethane decreased the contamination of product with dimethyl 5-aminoisophthalate and led to a white solid which was dissolved in dichloromethane, washed with 600 mL HCl 2N, water, 250 mL sodium bicarbonate and water, concentrated and reprecipitated with hexane (white solid, 1.58 g, 3.46 mmol):

mp 89-90°C; IR (KBr, cm⁻¹) 3325 (m, br), 2926 (m), 1731 (s), 1677 (s), 1252 (s), 1167 (m), 758 (m), 698 (m); ¹H NMR (DMSO-d₆) δ 10.49 (s, 1H, NH), 8.48 (d, J=1.5, 2H, ArH), 8.16 (t, J=1.5, 1H, ArH), 7.30-7.20 (m, 6H, ArH, NH(masked)), 6.30-6.28 (m, 1H), 3.88 (s, 6H), 3.26-2.84 (m, 2H), 1.31 (s, 9H); ¹³C NMR (DMSO-d₆) δ 171.45 (s), 165.22 (s), 155.40 (s), 139.81 (s), 137.76 (s), 130.64 (s), 129.17 (s), 128.07 (s), 126.34 (s), 124.05 (s), 123.63 (s), 78.20 (s), 56.78 (s), 52.55 (s), 37.13 (s), 28.11 (s); MS (ESI) Calc: 456.5; Found: 456.8

N-[N-tert-Butyloxy carbonyl-L-Phenylalanyl]-5-Aminoisophthalic Acid.

N-[N-tert-butyloxy carbonyl-L-phenylalanyl]-5-aminoisophthalic acid dimethyl ester (1.58 g, 3.46 mmol) was dissolved in 170 mL methanol, 25 mL 6 N NaOH were added and the reaction mixture stirred for 3 h at room temperature. A white precipitate formed upon addition of water and 2N HCl in the solution. The precipitate was filtered, washed with water and dried in vacuo to give 1.11 g, (2.60 mmol) of pure product: mp 280°C (decomposed); IR (KBr, cm⁻¹) 3319 (m, br), 2976 (m), 1708 (s), 1567 (m), 1252 (m), 1166 (m), 761 (m), 699 (m); ¹H NMR (DMSO-d₆) δ 10.42 (s, 1H, NH), 8.43 (s, 2H, ArH), 8.16 (s, 1H, ArH), 7.31-7.18 (m, 6H, ArH, NH (masked)), 4.30-4.28 (m, 1H), 2.99-2.84 (m, 2H), 1.31 (s, 9H); ¹³C NMR (DMSO-d₆) δ 171.15 (s), 166.34 (s), 155.30 (s), 139.40 (s), 137.72 (s), 131.71 (s), 129.11(s), 127.97 (s), 126.24 (s), 124.57 (s), 123.66 (s), 78.12 (s), 56.63 (s), 37.24(s), 28.08 (s); MS (ESI) Calculated: 428.4; Found: 429 (MH⁺).

Bis((1-tert-butoxy carbonyl)-3,5-dibromosalicyl)) N-[N-tert-Butyloxy carbonyl-L-Phenylalanyl]-5-Aminoisophthalate. N-[N-tert-butyloxy carbonyl-L-phenylalanyl ]-5-aminoisophthalic acid (0.147 g, 0.344 mmol), 4-dimethylaminopyridine (20% mol of acid, 8.39 x 10⁻³ g, 6.87 x 10⁻² mmol) and t-butyl-3,5-dibromosalicylate (2.2 equiv., 0.266 g,
0.756 mmol) were dissolved in 20 mL anhydrous THF with stirring and the mixture cooled at 0°C. 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.4 equiv., 0.158 g, 0.824 mmol) was dissolved in 10 mL dry dichloromethane and added dropwise under nitrogen to the above solution at 0°C over a period of 10 min. Reaction mixture was stirred for 5 min at 0°C and then for 63 h at room temperature, under nitrogen. The solvent was evaporated under reduced pressure leaving a slightly yellow residue which was redissolved in ethyl acetate. The ethyl acetate phase was washed with a saturated solution of sodium bicarbonate (400 mL), then with 200 mL saturated sodium chloride and 200 mL water and evaporated under reduced pressure. The white solid obtained was used without further purification (0.182 g, 0.162 mmol).

**Bis(3,5-dibromosalicyl) N-[N-Trifluoroacetyl-L-Phenylalanyl]-5-Aminoisophthalate.** Bis((1-tert-butoxycarbonyl)-3,5-dibromosalicyl) N-p-tert-butyloxy carbonyl-L-phenylalanyl]-5-aminoisophthlate (0.182 g, 0.162 mmol) was dissolved in a mixture of trifluoroacetic acid and trifluoroacetic anhydride under nitrogen and stirred for 2 h. The solvent was evaporated and the residue stirred in ether until a white precipitate was obtained. The precipitate was filtered and proved to be a decomposition product. The filtrate was evaporated in vacuo leaving the product as a cream solid (0.874 g, 0.892 mmol): mp 205-206°C; IR (KBr, cm⁻¹) 3330 (m, br), 3083 (m), 1713 (s), 1557 (m), 1446 (m), 1202 (s), 739 (m), 701 (m); ¹H NMR (DMSO-d₆) δ 10.93 (s, 1H, NH), 9.98 (d, 1H, NH), 8.78 (s, 2H, ArH), 8.47 (s, 1H, ArH), 8.36 (d, J= 2.12, 2H, ArH), 8.11 (s, 2H, ArH), 7.31-7.18 (m, 5H, ArH), 4.72 (m, 1H), 3.21-3.00 (m, 2H); ¹³C NMR (DMSO-d₆) δ 169.36 (s), 163.39 (s), 162.28 (s), 158.47 (s), 156.59 (s), 146.50 (s), 140.21 (s), 138.91 (s), 136.98 (s), 133.43 (s), 131.53 (s), 129.64 (s), 129.09 (s), 128.18 (s), 127.61 (s), 126.64 (s), 125.29 (s), 119.43 (s), 119.24 (s), 55.7 (s), 41.5 (s), 36.4 (s). ¹⁹F NMR (DMSO-d₆) δ -69.72 (COF₃); MS (ES) Calculated: 980; Found: 1003.4 (MNa⁺).
2.3. Cross-Linking Reaction of Human Deoxyhemoglobin A

Deoxyhemoglobin solutions for cross-linking reactions were prepared in the following manner: stock solutions of carbonmonoxyhemoglobin (1.0 mL, 1.23 mM) in 50 mM Bis-Tris HCl buffer (pH=6.9) were passed through a Sephadex G-25 column at 4°C equilibrated with 0.1 M sodium borate buffer (pH=9). Deoxyhemoglobin was converted to oxyhemoglobin by photoirradiation under a stream of humidified oxygen for 3 h at 0°C in a rotating flask. Oxyhemoglobin was brought into the deoxy state by continuous purging of wet nitrogen in the rotating flask for 3 h at 37°C. The cross-linking reagent (molar ratio HbA: reagent: 1:2) was dissolved in 0.2-0.3 mL dioxane (passed previously over a neutral alumina column to remove peroxides) and 0.3 mL of 0.1 M sodium borate buffer (pH=9). The dioxane-buffer solution was deoxygenated by passing a stream of nitrogen for 1 h and then added to the hemoglobin solution via a syringe. The reaction mixture was kept rotating at 37°C for up to 24 h under continuous nitrogen flow to maintain the deoxy state. The reaction was monitored by C-4 HPLC and the reaction considered completed when no more α or β chain modification was observed. At the end of the reaction period the flask was cooled on ice, flushed with carbon monoxide and passed through a Sephadex G-25 column equilibrated with 0.1M borate buffer (pH=9) in order to remove unreacted cross-linking reagent.

2.4. Structural Characterization of Modified Hemoglobins

2.4.1. Chromatography

Modified globin chains were identified by analytical reversed-phase HPLC using a 330 Å pore size C-4 Vydac columns (250 x 4.6 mm) and isolated by preparative reversed-phase HPLC using the same type of columns (250 x 12 mm); developers
contained 0.1% trifluoroacetic acid and various gradients of acetonitrile in water, starting at 20% and ending at 60%. The effluent was monitored for absorbance at 220 nm.

Assessment of heterogeneity of the reaction mixture and isolation of single Hb components was accomplished by analytical and semipreparative anion exchange HPLC using a Synchropak AX-300 column (analytical: 250 x 4.5mm, semipreparative: 250 x 10 mm) and a POROS column. Developers for the AX-300 column contained 15 mM Tris, (pH=8) and various gradients of sodium acetate in water starting at 10 mM and ending at 150 mM. The flow rate was 1 mL/min for the analytical analysis and 4mL/min for the semipreparative separation. For the analysis using the POROS column, a pH gradient starting at pH 8.5 and ending at pH 6.5 was used. The effluent was monitored at 420 nm.

Peptide fragments were separated\(^\text{15}\) by reversed-phase HPLC using a C-18 Vydac column (93 x 4.7 mm), developers containing 0.1% trifluoroacetic acid and gradients of acetonitrile starting at 0% and ending at 100% in water, pumped at 1 mL/min. The effluent was monitored at both 220 nm and 280 nm.

2.4.2. SDS-PAGE

Detection of covalent cross-links between two hemoglobin monomers was accomplished by polyacrylamide gel electrophoresis with SDS. A 12 % polyacrylamide gel was used to distinguish between the 16,000 molecular weight monomers and 32,000 molecular weight cross-linked dimers against a molecular weight standard. Prior to electrophoresis, the protein samples were dissolved in 0.5 M Tris-HCl buffer, pH=6.8 which contained 0.05% bromophenol blue, 4% v/v β-mercapto-ethanol, 2% SDS, 10% v/v glycerol and kept for 5 min in boiling water to denature. Approximately 10 µg of protein was applied to each lane of the gel and subjected to electrophoresis for 35 min. The gel was run using the Bio-Rad Mini-PROTEAN II dual-slab cell apparatus at 200 mV. Unmodified hemoglobin and Bio-Rad SDS-PAGE molecular weight standards were used to estimate the molecular weight of the resulting bands after fixation followed by staining with Coomassie Brilliant Blue R.
2.4.3. Mass Spectroscopy

The molecular masses of cross-linked hemoglobins were determined by electrospray ionization mass spectrometry (ESI-MS) coupled to reversed-phase HPLC. The ESI-MS analysis was performed at the mass spectroscopy laboratory, Medical Sciences Building, University of Toronto.

2.4.4. Peptide Pattern Analysis

Unmodified and cross-linked α and β chains were separated and collected using the reversed-phase HPLC procedure outlined above. Organic developers were evaporated under reduced pressure and water removed by lyophilization. The globin chains were then dissolved in 8 M urea to help denature the protein and obtain a more complete hydrolysis and kept for 2 h at room temperature. Since there was no indication that the globins were resistant to hydrolysis, the cysteinyl residues were not oxidized prior to digestion. This solution was diluted to 2M urea with 0.8 M bicarbonate buffer at pH=8.5. Freshly prepared TPCK-treated trypsin solution (2 mg/mL, 4% of mass of total protein) was added and the digestion continued for 24 h at room temperature. The tryptic hydrolysate was heated in boiling water for 2 min and diluted to 1M urea with 0.8 M bicarbonate buffer (pH=8.5). A solution of Staphylococcus aureus V8 endoproteinase Glu-C (1 mg/mL, 2% of mass of total protein) was then added and the mixture digested for 72 h at room temperature. The sample was clarified by filtration through a 0.45-μm filter before injection onto the C-18 reverse-phase HPLC column. Peptide fragments were separated according to the procedure described above. The modified peaks were identified by peptide pattern analysis. The tryptic-Glu-C peptide maps of modified α and β subunits were analyzed by comparison with the peptide maps of unmodified α and β subunits and by comparison with literature data.15
2.4.5. Quantitative Affinity Chromatography

2.4.5.1. Preparation of Affinity Column

4-Nitrophenyl chloroformate Activation of Sepharose CL-4B

All steps in the activation procedure were performed in an ice bath according to the procedure of Wilchek et al.\textsuperscript{16} Sepharose Cl-4B (50 mL) was washed batchwise through a sintered glass funnel with increasing concentrations of acetone in water, starting at 0% and ending at 100 % absolute acetone. To the washed Sepharose gel, an acetone solution (50 mL) containing 4-nitrophenyl chloroformate (0.3 mmol/mL) was added. To this suspension, 50 mL of an acetone solution containing 4 dimethyl-aminopyridine (0.35 mmol/mL) was added dropwise with stirring, and the resin stirred for 90 min. The activated Sepharose was then washed exhaustively with acetone (500 mL), acetone-2-propanol (1:1) (200 mL), 2-propanol (200 mL), 2-propanol-water (1:1) (200 mL), and distilled water until the solution remained clear in the presence of 0.2 N sodium hydroxide. The activated resin (0.737 mmol 4-nitrophenol/g resin) was dried by lyophilization and stored at 4 °C.

Immobilization of Avidin to the p-Nitrophenyl Activated CL-4B Sepharose Column

Avidin (3.83 mg), was dissolved in 6 mL 0.1 M sodium bicarbonate (pH=8.5). To this solution, 2.73 g of activated Sepharose was added and the reaction mixture stirred for 1 h at room temperature and overnight at 4°C.\textsuperscript{17} The resin was then washed successively with water, 0.2 M acetic acid, water, and 0.01 M sodium hydroxide followed by distilled water, 0.1 M sodium bicarbonate and PBS (phosphate buffer saline). The immobilized avidin was stored at 4°C in PBS.
Determination of Availability of Biotin Moiety for Interaction with Avidin

The percentage of biotinylated Hb molecules that are accessible for binding avidin was assessed according to the procedure described by Wilchek.\textsuperscript{18} Purified biotinylated Hb (0.1 mL, 0.3 mM) was applied to a small avidin-Sepharose affinity column (~ 3 mg avidin/mL resin) prepared as described above. The column was eluted with PBS until the absorbance at 420 nm reached a minimal value. The amount of biotinylated Hb in the effluent fraction was determined spectrophotometrically using ε = 192 and the fraction (%) of biotinylated Hb that is available for interaction with avidin was computed from the following equation:

\[
\frac{Hb_{total} - Hb_{effluent}}{Hb_{total}} = \frac{A_{420}^{applied} - A_{420}^{effluent}}{A_{420}^{applied}} \times 100
\]

2.4.6. Measurement of Functional Properties of Cross-Linked Hemoglobin

Conditions for the oxygen-binding curve determination were 50 mM Bis-Tris, pH 7.4, 0.1M Cl\textsuperscript{-}, 37°C and 55 μM heme. The oxygen equilibrium analyzer was composed of a spectrophotometer GBC 916 (Victoria, Australia), an oxygen monitor YSI 5300 (Yellow Spring Instruments), an oxygen probe YSI 5331, a Cole-Parmer N042-15 flowmeter, a reaction cell and an A/D converter RS232 interface. Data were analyzed according to the Adair stepwise oxygenation scheme. The oxygen pressure at half saturation (P\textsubscript{50}) and the Hill coefficient at half saturation (n\textsubscript{50}) were determined.

2.4.7. CD Spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco J-710 system between 200 and 280 nm (1mm pathlength, hemoglobin concentration 0.3 mM in sodium borate buffer pH 9).
CHAPTER III

Results

3.1. Synthesis of Bis(3,5-dibromosalicyl) N-Biotinyl-5-Aminoisophthalate

Biotin acid chloride (1 in Scheme 1) was synthesized from biotin and thionyl chloride. Addition of 5-aminoisophthalic acid to biotin acid chloride in the presence of a catalytic amount of 4-dimethylaminopyridine, afforded the N-biotinyl-5-aminoisophthalic acid (2 in Scheme 1). Refluxing of the resulting diacid in thionyl chloride gave mainly decomposition products. Therefore, the usual way of preparing the 3,5-dibromosalicylate esters from acid chlorides has been replaced by a coupling reaction using the coupling reagent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride. The reaction was carried out at 4°C in order to minimize formation of by-products. After purification of the crude product by flash chromatography, the desired ester: bis((1-tert-butoxycarbonyl)-3,5-dibromosalicyl)) N-biotinyl-5-aminoisophthalate (3 in Scheme 1) was obtained. Deprotection of the carboxylate functionality with anhydrous trifluoroacetic acid gave bis(3,5-dibromosalicyl) N-biotinyl-5-aminoisophthalate (DBBA) (4 in Scheme 1). Its purity was assessed by NMR, IR and low resolution mass spectrometry.
Scheme 1. The synthesis of DBBA from d-biotin
Scheme 1 (cont.). The synthesis of DBBA from d-biotin
3.2. Synthesis of Bis(3,5-dibromosalicyl) N-[N-Trifluoroacetyl-L-Isoleucylglycyl]-5-Aminoisophthalate

The synthetic route for the preparation of bis(3,5-dibromosalicyl) N-[N-trifluoroacetyl-L-isoleucylglycyl]-5-aminoisophthalate (DBTIA) is outlined in Scheme 2. The synthesis of N-[N-tert-butyloxycarbonyl-L-isoleucylglycyl]-5-aminoisophthalic acid dimethyl ester (1) involved the dicyclohexylcarbodiimide-mediated coupling of N-t-Boc-Ile-Gly with dimethyl 5-aminoisophthalate in the presence of catalytic amounts of 4-dimethylaminopyridine. Deprotection of the methyl ester functionality proceeded smoothly in methanol and NaOH and the resulting diacid (2) was converted to its t-butyl 3,5-dibromosalicylate ester (3) via an 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride-mediated coupling reaction. Removal of the t-butyl group with trifluoroacetic acid and trifluoroacetylation of the terminal amino group with trifluoroacetic anhydride in a single step gave DBTIA (4).
Scheme 2. The synthesis of DBTIA from N-t-Boc-Ile-GLy
Scheme 2 (cont.). The synthesis of DBTIA from N-t-Boc-Ile-Gly
3.3. Synthesis of Bis(3,5-dibromosalicyl) N-[N-Trifluoroacetyl-L-Phenylalanyl]-5-Aminoisophthalate

DBTPA was synthesized using N-t-Boc-L-Phe as the starting material (Scheme 3). N-[N-t-Boc-L-Phe]-5-aminoisophthalic acid dimethyl ester (1) was prepared by coupling N-t-Boc-L-Phe with dimethyl 5-aminoisophthalate in the presence of dicyclohexylcarbodiimide and catalytic amounts of 4-dimethylaminopyridine. Demethylation of the resulting amide with NaOH in methanol gave the diacid (2). The bis(salicyl) diester (3) of the diacid (2) was obtained in an 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride-mediated coupling reaction. A mixture of trifluoroacetic acid and trifluoroacetic anhydride was used to remove the tert-butyl group and to trifluoroacetylate the terminal amino group in the same step, giving DBTPA (4).
Scheme 3. The synthesis of DBTPA from N-t-Boc-Phe
Scheme 3 (cont.). The synthesis of DBTPA from N-t-Boc-Phe
3.4. Cross-Linking Results of Human Hemoglobin A with DBBA

Carbonmonoxhemoglobin reacted very slowly with DBBA at pH 9 (0.1 M sodium borate buffer) and 37°C. The reagent was much more reactive toward deoxyhemoglobin under the same conditions: combining of 1 equivalent of hemoglobin and 2 equivalents of the cross-linker led after 3 h to > 75% conversion of deoxyhemoglobin to products. Analysis of the reaction mixture revealed two main products: the ααββ82-82β' hemoglobin derivative obtained by acylation of the ε-amino groups of Lys-82 of each of the β subunits and the ββαα99-99α' derivative obtained by acylation of the ε-amino groups of Lys-99 of each of the α subunits (Figure 3.1). Increasing the reaction time to 14 h led to > 95% conversion of Hb, with an increase in the extent of inter-α cross-linking and almost no modification of the β subunits.

Figure 3.1. Globin chain separation on C-4 reversed-phase column after reaction of deoxyhemoglobin with DBBA (pH 9, 37°C, 3 h)
3.4.1. Analysis of Reaction Products

Products were analyzed by a combination of analytical and semipreparative C-4 reversed-phase HPLC of separated globin chains, anion exchange AX 300 HPLC of intact tetramers and tryptic/Glu-C digest of globin chains with peptide mapping, as previously described. The C-4 HPLC chromatogram (Figure 3.1.) revealed the presence of two newly cross-linked hemoglobin chains (retention times 43 and 75 min) along with the normal peaks for heme, α and β subunits. The peak integration of the unreacted α and β chains as compared to native hemoglobin indicated that the chemical modification took place predominantly in the β chains.

The approximate weight of the dimer globin species was determined by SDS-PAGE after their separation on a semipreparative C-4 column. Under these denaturing conditions, purely ionic interactions can be overcome, and only the covalent cross-links are detectable. The SDS-PAGE analysis of the globin chains clearly indicated the presence of covalent cross-links due to the presence of bands corresponding to dimers of the monomer units with a molecular weight of ~ 32 kDa. The mass of each globin was determined by HPLC-MS. The first major modified globin (β82-82β' in Figure 3.1.) has an experimental M.W. of 32093 Da, consistent with an inter-β cross-linked dimer bearing the N-biotinyl-5-aminoisophthalate moiety (calculated mass: 32093 Da;). The second major modified globin (α99-99α' in Figure 3.1.) has a mass of 32615 Da, consistent with an inter α-α dimer bearing the N-biotinyl-5-aminoisophthalate moiety (calculated mass: 32619 Da). The two distinct peaks eluting at 43 min and 45 min could not be distinguished by their molecular weight and peptide pattern analysis. Their different chromatographic behavior suggests conformational differences between them, though their exact nature could not be identified. The intermediate zones correspond to cross-linked globins having a second molecule of cross-linker attached to a third site with or without one leaving group hydrolyzed, as suggested by their HPLC-MS analysis. Since they made up only a small percentage of the other two products, they were not further analyzed.
The anion exchange HPLC chromatogram of the reaction products is shown in Figure 3.2. The peaks were identified by comparison with the chromatogram of native Hb run in the same conditions, separation of the main fractions, purification by rechromatography on a POROS column and globin chain separation on a C-4 reversed phase column of the purified hemoglobins. Retention times of globin chains separated on C-4 reversed phase column from the purified hemoglobins were compared with those from the reaction mixture for which HPLC-MS analysis was performed. Peak A (Figure 3.2.) corresponds to unmodified Hb, having the same retention time as unmodified Hb. Peak B was identified as being the inter-α cross-linked Hb having in its C-4 chromatogram peaks where the β monomer and αα' dimer occurred and lacking the peak corresponding to the α monomer. Region C and D correspond to the ααβ82-82β' hemoglobin conjugate. The inter-β cross-linked Hb fraction (peak C in Figure 3.2) that was formed in the highest yield was concentrated and further used for functional studies. Its C-4 chromatogram (Figure 3.3.) indicates the presence of the heme, the α monomer and the β-β' cross-linked dimer. Region E contained a mixture of modified hemoglobins corresponding to the minor globins in the C-4 chromatogram (Figure 3.1.).
**Figure 3.2.** Anion exchange HPLC profile on a POROS column for the reaction of DBBA with deoxyhemoglobin (37°C, pH 9, sodium borate buffer, 3 h)

**Figure 3.3.** Globin chain separation on a C-4 column of the inter-β cross-linked Hb (peak C in Figure 3.2.) purified on an AX-300 column.
3.4.2. The Reaction Site

In order to determine the site of modification of the protein chain, the cross-linked dimers (peaks $\beta 82-82\beta'$ and $\alpha 99-99\alpha'$ in Figure 3.1) were separated on a semipreparative C-4 column and subjected to enzymatic digest with trypsin followed by endoproteinase Glu-C. Trypsin hydrolyzes peptide bonds at the C-terminal side of amino acids with positively charged residues (lysine and arginine). Endoproteinase Glu-C further hydrolyses the resulting peptides at the C-terminal side of glutamate residues. Peptide peaks were identified by mapping with the peptide pattern of the native $\alpha$ and $\beta$ globins digest and by comparison with the literature data. The absorbances of peptide fragments were monitored at both 280 nm and 220 nm. The peptide patterns of peaks eluting at 43 min and 45 min (Figure 3.1) are identical to one another (Figure 3.4 and 3.5, respectively). This peptide maps are characteristic of the $\beta$ chain of human hemoglobin with the exception of the absence of $\beta T-9$ and $\beta T-10a'$ peptide fragments and the presence of new peaks which elute at 93.80 min and 94.30 min respectively. These results can be explained by the acylation of the $\beta$-Lys-82 (on the C-terminus of $\beta T-9$) in each of the $\beta$ chains by DBBA, therefore preventing the cleavage of $\beta T-9$ peptide fragment by trypsin. Since the $\beta T-1$ peak was not altered compared to the unmodified $\beta$ globin chains, cross-linking between the $\beta$-Val-1 residue and $\beta$-Lys-82 residue on the two $\beta$ globin chains did not occur. Thus, in the $\beta$ chains only the $\beta$-Lys-82 residues were biotinylated. Similarly, the peptide pattern for the peak eluting at 75 min in the C-4 chromatogram (Figure 3.1) is missing the $\alpha T-11$ and $\alpha T-12$ peak (Figure 3.6). A large peak elutes at 111.81 min. These results suggest the formation of an amide bond between the $\alpha$-Lys-99 (on the C-terminus of $\alpha T-11$) on each $\alpha$ subunit and the cross-linker. This newly formed amide bond prevents trypsin from hydrolyzing the peptide bond between $\alpha T-11$ and $\alpha T-12$ peptide fragments. These peptide pattern data along with the molecular masses determined experimental by HPLC-MS for the globin dimers, indicate that two $\beta$ and two $\alpha$ chains are cross-linked by a N-biotinyl-5-
aminoisophtalate moiety. The tryptic-Glu-C peptide maps of normal β and α chains are presented in Figure 3.7 and Figure 3.8.

Figure 3.4. C-18 reversed phase HPLC chromatogram of the β82-82β' globin dimer (peak eluting at 43 min in the C-4 chromatogram, Figure 3.1.), after trypsin/Glu-C digestion, monitored at 200 nm.
**Figure 3.5.** C-18 reversed phase HPLC chromatogram of the $\beta_82$-$\beta'_8$ globin dimer (peak eluting at 45 min in the C-4 chromatogram, Figure 3.1.), after trypsin/Glu-C digestion, monitored at 200 nm.
Figure 3.6. C-18 reversed phase HPLC chromatogram of α99-99α' globin dimer (peak eluting at 75 min in the C-4 chromatogram Figure 3.1.), after trypsin/Glu-C digestion, monitored at 200 nm.
Figure 3.7. Tryptic-Glu-C peptide map of unmodified β globin chains of human Hb A 
($\lambda$=200nm)

Figure 3.8. Tryptic-Glu-C peptide map of unmodified α globin chains of human Hb A 
($\lambda$=200 nm.)
3.4.3. Functional Properties of Biotinylated Hemoglobin

The oxygen affinity of the DBBA-Hb conjugate (ααβ82-82β': N-biotinyl-5-aminoisophthalate, zone C in Figure 3.2) was measured after its purification on a semipreparative AX 300 ion exchange HPLC column. The oxygenation curve at 25°C and pH 7.4 is shown in Figure 3.9. The DBBA-Hb conjugate has a $P_50$ comparable to that for native hemoglobin under the same conditions\textsuperscript{13}, indicating a similar oxygen affinity. The Hill coefficient at half-saturation was determined to be 3, indicating that oxygen binding remains highly cooperative in this cross-linked hemoglobin conjugate. Functional properties of the DBBA-Hb conjugate at 25°C and pH 7.4, native Hb and whole blood, are summarized in Table 3.1.

\textbf{Figure 3.9.} Oxygen binding curve of DBBA-Hb (pH 7.4, 50mM Bis-Tris, 0.1M Cl\textsuperscript{-1}, 25°C, 55μM heme)
Table 3.1. Functional properties of the $\alpha\alpha\beta^{82-82}\beta'$: N-biotinyl-5-aminoisophtalate conjugate (pH 7.4, 55 $\mu$M heme, 25°C, 0.1M Cl$^-$, 50 mM Bis-Tris)

<table>
<thead>
<tr>
<th></th>
<th>$P_{50}$ (Torr)</th>
<th>$n_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>whole blood$^a,13$</td>
<td>26</td>
<td>3.1</td>
</tr>
<tr>
<td>Hb-A$^{13}$</td>
<td>5.0</td>
<td>3.0</td>
</tr>
<tr>
<td>$\alpha\alpha\beta^{82-X-82}\beta'$ $^b$</td>
<td>4.9</td>
<td>3.0</td>
</tr>
</tbody>
</table>

$^a$ physiological conditions

$^b$ X = N-biotinyl-5-aminoisophtalate

The CD and UV-VIS spectra of the $\alpha\alpha\beta^{82-X-82}\beta'$ and $\beta\beta^{99-X-99}\alpha'$ hemoglobin conjugates and native hemoglobin, shown in Figure 3.10. and Figure 3.11. are virtually identical, indicating that their tertiary structures are not significantly different.
Figure 3.10. CD spectra of native and modified hemoglobins in sodium borate buffer (pH 9). Concentration $3 \times 10^{-4}$ M, molar ellipticity $[\theta]$ in deg cm$^2$ dmol$^{-1}$
Figure 3.11. UV-VIS spectra of native and modified hemoglobins in CO form
3.4.4. Quantitative Affinity Chromatography of Biotinylated Hemoglobin

Biotin has an extraordinarily high affinity for streptavidin and avidin, with reported dissociation constants of $\sim 10^{-15}$ and $\sim 10^{-14}$ M respectively.\textsuperscript{21} The avidins are stable tetramers with 2-fold symmetry, the binding sites being arranged in two pairs on opposite faces of the molecule. They are capable of binding 4 biotins per subunit. The stability of biotin-avidin complexes is greatly enhanced by binding biotin with a total free energy of binding of about 330 kJ/mol of tetramer.\textsuperscript{22} Chemical modification studies indicate that tryptophan, tyrosine and lysine residues are involved in biotin binding. The initial binding is almost irreversible, provided that the biotinyl residue is accessible, and the 2-fold symmetry of the protein ensures that if the avidin is binding to a surface molecule many outwardly directed biotin sites will remain vacant and can be saturated with a second biotinyl ligand without displacement of the avidin from the labeled site.

The following method was used in order to assess the availability of the biotin moiety in the $\alpha\alpha\beta82-82\beta'$: N-biotinyl-5-aminoisophthaloyl conjugate to interact with avidin. Purified, monobiotinylated cross-linked-hemoglobin (fraction C in Figure 3.2., purified by ion exchange rechromatography on an AX 300 column) was added to an avidin-Sepharose affinity column ($\sim 4$ mg avidin / column) in a molar ratio avidin: biotinylated hemoglobin of 2: 1. The column was eluted with PBS buffer (pH 7.4) until the absorbance of the eluent at 420 nm reached a minimal value. The hemoglobin solution was concentrated and the total amount of hemoglobin applied to the column and the amount eluted from the column were determined spectrophotometrically. The fraction (%) of biotinylated hemoglobin that is available for interaction with avidin, computed from the following equation:

$$\frac{Hb_{\text{total}} - Hb_{\text{effluent}}}{Hb_{\text{total}}} = \frac{A_{420\text{applied}} - A_{420\text{effluent}}}{A_{420\text{applied}}} \times 100$$

was determined to be 17%. We can assume that there is no repulsion between the surfaces of hemoglobin and avidin. Avidin is a basic protein with an isoelectric point near
pH 10.5; at physiological pH, it carries a net positive charge. Human hemoglobin A has an isoelectric point at pH 6.7 and carries a net negative charge at physiological pH which should provide for electrostatic attraction for avidin. The low yield in which the biotinylated hemoglobin bound avidin can be explained by either steric hindrance for the interaction or by the inaccessibility of the ureido moiety of biotin to the binding site in avidin. A spacer arm inserted between biotin and the isophtalic core of the cross-linking reagent will probably allow for better accessibility of the attached biotin to avidin.

3.5. Cross-Linking Results of Human Hemoglobin A with DBTIA

3.5.1. Analysis of Reaction Products

The same materials, method and analytical procedures were used as those for cross-linking hemoglobin with DBBA. Deoxyhemoglobin was cross-linked at 37°C, pH 9, in a 1:2 molar ratio to the cross-linker. DBTIA was dissolved in 0.2 mL dioxane and 0.3 mL 50 mM sodium borate and was added to the hemoglobin solution under a stream of nitrogen. The reaction was stopped after 3 h. The C-4 HPLC analysis of the reaction mixture (Figure 3.12.) indicates a complex reaction mixture in which the β-globin chains have reacted almost completely and the α chains only partially.
The identity of the peaks was confirmed by comparing the chromatogram with that for native Hb run under the same conditions and by HPLC-MS (ESI) analysis. The peaks designated α and β in Figure 3.12. correspond to unreacted globin chains. Peak 1 corresponds to a β-β' dimer cross-linked by the isoleucylglycyl-5-aminoisophtalate moiety (calculated M.W. = 32,133.4; found M.W. = 32,137.8); the molecular weight of the species designated 2 is close to that of an inter-β cross-linked dimer having a second molecule of the cross-linker attached by one amide bond to the protein and the second leaving group still present. This dimer is cross-linked between the same residues as dimer 1 as demonstrated by the peptide pattern analysis. The experimental mass for peak 3 was consistent with the mass of an α monomer to which the reagent is attached by one amide bond and having the second leaving group intact (found: M.W. = 15,839, calculated: M.W. = 15,832). The last peak, 4, corresponds to an inter α-cross-linked dimer (calculated M.W. = 30,661.45, found M.W. = 30,664.85).
The reaction mixture was analyzed on an anion exchange AX-300 column as well, under conditions where intact hemoglobin tetramers and modified tetramers are separated (Figure 3.13.). Region A corresponds to unreacted hemoglobin, as determined by comparison with the chromatogram of native hemoglobin run in the same conditions. Region B corresponds to inter α-cross-linked hemoglobin and region C to inter-β-cross-linked hemoglobin, the major product. The assignment was made after purification of the modified hemoglobins to homogeneity by identical rechromatography and comparison of their C-4 chromatograms to that of unmodified hemoglobin.

Figure 3.13. Anion exchange chromatogram (AX-300) of the reaction mixture formed in the reaction of deoxyhemoglobin with DBTIA (pH 9, 37°C, 3h).
3.5.2. The Reaction Site

In order to determine the site of modification of the protein chain, the cross-linked dimers (peaks 1, 2 and 4 in Figure 3.12.) were separated on a semipreparative C-4 column and subjected to enzymatic digest with trypsin followed by endoproteinase Glu-C. The peptide maps of native α and β chains obtained by C-18 reversed-phase HPLC (Figures 3.7 and Figures 3.8) were compared to those for the modified chains (Figures 3.14, 3.15 and 3.16) in order to identify the site of modification.

![Tryptic-Glu-C peptide map of DBTIA-cross-linked β-chains](image)

**Figure 3.14.** Tryptic-Glu-C peptide map of DBTIA-cross-linked β-chains (peak 1 in Figure 3.12)
Figure 3.15. Tryptic-Glu-C peptide map of DBTIA-cross-linked β chains (peak 2 in Figure 3.12)

Figure 3.16. Tryptic-Glu-C peptide map of DBTIA-cross-linked α chains (peak 4 in Figure 3.12)
In the C-18 reversed-phase HPLC of peaks 1 and 2, the peptide fragments βT-9 and βT-10a' are not present and new peaks elute at approximately 85-100 min (see Figures 3.14 and 3.15). These two peptide fragments result from cleavage adjacent to lysine 82. In both chromatograms the fragment β T-1 appears unmodified. These results suggest that only the ε-amino groups of the β-Lys-82 residues have been blocked, the β-Val-1 residue remaining intact. This is consistent with the formation of a bis-amide cross-link between the ε-amino group of the Lys-82 residue of one β chain and the ε-amino group of the Lys-82 residue of the other β chain. Similarly, the peptide pattern for peak 4 in the C-4 chromatogram (Figure 3.12) is missing the αT-11 and the αT-12 peaks (Figure 3.16). These results, combined with the mass spectral data suggest that a bis amide cross-link between the ε-amino groups of two lysines α-99 (on the C-terminus of αT-11) has formed specifically.

3.6. Cross-linking Results of Human Hemoglobin A with DBTPA

The cross-linking method and all analytical procedures were the same as those for cross-linking deoxyhemoglobin with DBBA and DBTIA. The reagent was dissolved in 0.2 mL peroxide-free dioxane and added to the deoxyhemoglobin solution (borate buffer, pH 9) in a 2:1 ratio. The reaction was carried out for 3 hours at 37°C under a continuous stream of humidified nitrogen. At the end of the reaction period, the hemoglobin solution was purified on a Sephadex G-25 column eluted with sodium borate buffer 0.1 M (pH 9), prior to injection on the analytical C-4 reversed phase column. The peaks in the chromatogram (Figure 3.17) were identified by comparison with the retention times of globin chains of unmodified hemoglobin and by HPLC-MS (ESI) analysis.
Figure 3.17. C-4 reversed phase HPLC chromatogram of separated subunits from the reaction of deoxyhemoglobin with DBTPA (pH 9, 37°C, 3h).

Peaks α and β correspond to unmodified globin chains. The electrospray mass spectrometric analysis indicated that the peak designated α99-99α' in Figure 3.17, corresponds to an inter-α cross-linked dimer (M.W. calculated: 30638; M.W. found: 30640). The mass for the globin designated β82-82β' in Figure 3.17, was found to be 32025. The difference between the expected mass of a β-β' cross-linked dimer (32122) and the observed mass is difficult to interpret. Proteolytic hydrolysis of the cross-linked β chains indicated a β-Lys-82 to β'-Lys-82 cross-link by the total disappearance of the βT-9 and βT-10α' fragments in the tryptic/Glu-C digest of the chains (Figure 3.18). The disappearance of the αT-11 and αT-12 fragments in the tryptic/ Glu-C digest of modified α globins indicate a cross-link between α-Lys-99 and α'-Lys-99 (Figure 3.19).
Figure 3.18. Tryptic-Glu-C peptide pattern of DBTPA-cross-linked β globin chains

Figure 3.19. Tryptic-Glu-C peptide pattern of DBTPA-cross-linked α globin chains
CHAPTER IV

Discussion

4.1. The Design of New Reagents

The reagents reported in this study possess two reactive 3,5-dibromosalicylate functionalities as leaving groups and a derivative of d-biotin, N-fluoroacetyl-phenylalanine or N-fluoroacetyl-isoleucylglycine attached as a pendant ligand. Their design was based on previous studies that have shown that these leaving groups react with the highly conserved β-Lys-82 residues of the β-cleft of hemoglobin. The reagents have an isophthalic arrangement of reactive 3,5 dibromosalicylate ester groups with a span of 7.3 Å between the two electrophilic sites. This span is appropriate for cross-linking two diagonally opposed hemoglobin subunits considering the distance between β-Lys-82 and β'-Lys-82 8.1 Å and a distance of 7.61 Å between α-Lys-99 and α'-Lys-99 in deoxy Hb. Because of their negative charges, the reagents would be attracted in much the same way as an affinity-labeling agent to cationic regions such as the positively charged DPG-binding pocket of hemoglobin.
Figure 4.1. The structure of cross-linking reagents with the isophthalic arrangement of two reactive ester sites and a ligand derived from d-biotin, N-fluoroacetyl-isoleucylglicine and N-fluoroacetyl-phenylalanine.
4.2. Reaction Conditions

Very similar reaction patterns were observed when DBBA, DBTIA and DBTPA were reacted with deoxyhemoglobin at pH 9 and 37°C. All three reagents cross-linked hemoglobin β subunits only between β-Lys-82 and β'-Lys-82 and α subunits only between α-Lys-99 and α'-Lys-99. The yield of cross-linking COHb was considerably lower. This result can be explained if conformational differences between the two liganded states of hemoglobin are considered. Lys-99 is located within a cluster of charged residues in the central cavity of the tetramer near the middle of the hemoglobin molecule. It is more than 25 Å from the outer surface. Based on the observation that IHP does not inhibit the reaction between bis(3,5-dibromosalicyl) fumarate and amino groups in the two α-Lys-99 residues in deoxyhemoglobin, Chatterjee et al. suggested that the major route of entry of the bis(3,5-dibromosalicyl) fumarate molecule into the central cavity is between the α chains. In the liganded conformation, the close approach of α-Thr-134 and α'-Thr-134 and of α-Arg-141 and α'-Arg-141 blocks the entrance to the central cavity between the α chains and renders α-Lys-99 totally inaccessible to the cross-linking reagent. The size of the central cavity between the β chains is also very much decreased when oxygen or carbon monoxide is bound to the hemes, restricting the bulky reagents from reaching the nucleophilic residues in the 2,3-DPG binding site.

Cross-linking experiments performed by Brevitt with a related compound, N-[(3,5-bis(3,5-dibromosalicylate) phenyl]-4-pyridinecarboxamide, have shown that the β82-82β' cross-linked hemoglobin conjugate was obtained in higher yield at pH 9 compared to pH 7.2. At pH 9, the ε amino group of Lys-82 is partially protonated. Electrostatic interactions between the charged lysines and the carboxylate group on the reagent bring the two groups into close proximity and correct orientation. Experimental evidence and molecular dynamics simulations suggest that these electrostatic interactions promote the deprotonation of the ε-amino groups by the carboxylate moiety. In the next
step, the nucleophilic attack of the neutral lysine is assisted by the carboxylate anion on the reagent in a general base catalysis reaction as suggested by Kluger (unpublished results). This mechanism could account for the accelerated reaction rate observed at pH 9.

Monitoring the cross-linking reactions showed that the reaction is 77 % complete within 3 hours. Increasing the reaction time to 14 hours led almost exclusively to an increase in the extent of inter-α chain cross-linking with less modification in the β subunits. Based on these results and observations, the reaction conditions for comparing the effectiveness of the three reagents were chosen as follows: deoxyhemoglobin, pH 9, 37°C, 3 hours reaction time.

4.3. Analysis of Reaction Patterns

All three reagents cross-linked the two opposite β chains preferentially between β-Lys-82 and β'-Lys-82 and the two opposite α chains between α-Lys-99 and α'-Lys-99. This similarity in the reaction pattern and selectivity is not surprising since all three reagents have the same 3,5-dibromosalicylate leaving groups. They differ only in the polarity and bulkiness of the pendant ligand. A schematic representation of the reaction between the two lysine residues and DBBA is shown in Figure 4.2. illustrating the location of nucleophilic attack by the lysines.
Figure 4.2. Representation of the reaction between DBBA and the $\beta$-Lys-82 of Hb
The results of cross-linking deoxyhemoglobin A by DBBA, DBTIA and DBTPA are summarized in Table 4.1.

Table 4.1. Yield of modified hemoglobin α and β chains in the reaction between deoxyhemoglobin and DBBA, DBTIA and DBTPA (based on analytical C-4 reversed-phase HPLC results, integration of peaks and normalization to 100% for the sum of the products). Reaction conditions: pH 9, 3 hours reaction time.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>β82-X-82β'</th>
<th>α99-X-99α'</th>
<th>unreacted β</th>
<th>unreacted α</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBBA</td>
<td>21</td>
<td>10</td>
<td>7.4</td>
<td>23</td>
</tr>
<tr>
<td>DBTPA</td>
<td>15.3</td>
<td>11</td>
<td>15</td>
<td>28</td>
</tr>
<tr>
<td>DBTIA</td>
<td>14.3</td>
<td>4.9</td>
<td>0.7</td>
<td>13</td>
</tr>
</tbody>
</table>

DBBA, DBTPA and DBTIA cross-linked hemoglobin in comparable yield. The degree of specificity of Hb modification was lower with these reagents compared to trifunctional or tetrafunctional reagents\textsuperscript{12, 14} having the same reactive leaving groups. Those reagents were tri- or tetra anions with the charges dispersed on their surface. Strong electrostatic interactions between the positive charges in the 2,3-DPG binding site and the negative charges on those reagents promote the close approach and correct orientation for an $S_{N}2$ attack of the ε amino group of lysine. Since the specificity in the cross-linking reaction depends on the relative positions of the reacting groups, the total charge on the reagent is likely to be an important factor in achieving a high degree of selectivity.

The cross-linking reaction of hemoglobin by bis(salicyl) diesters could occur via either a one- or a two-step process. In a one step mechanism, the β-Lys-82 ε amino groups interact simultaneously with the two ester carbonyl carbons of the reagent. This mechanism requires the correct orientation and proximity of several atoms in order the bonds to be formed simultaneously and is therefore unlikely. More likely, the reaction occurs by a two-step mechanism. In the two step reaction, one lysine reacts with the first
ester carbonyl carbon with subsequent bond formation between the other lysine and the second ester carbonyl carbon. Intermediates of this pathway, the monofunctionally modified ("pseudo cross-linked ") Hb species with one DBS group conserved, have been identified in the C-4 chromatograms of DBBA and DBTIA-reacted hemoglobin. These Hb conjugates may be expected to be stabilized by electrostatic interactions between the carboxyl group of the remaining leaving group of the reagent and one or more of the positive charge(s) in the binding sites.

Minor products corresponding to cross-linked species having a second cross-linker molecule attached to the protein by one amide bond and the second leaving group either hydrolyzed or not, were also detected by HPLC-MS analysis in the reaction between Hb and DBBA and DBTIA. The site of attachment of the second cross-linker could be in the DPG binding site or more probably on the protein surface.

A distinctive feature of the reaction between DBBA, DBTIA, DBTPA and Hb is the occurrence of both inter-α and inter-β chain cross-linking. Inter-α chain cross-linking has either not been observed with other dibromosalicylate diesters14 or it occurred at much lesser extent12 compared to the reagents synthesized in this study. All those reagents possess one or two additional charged, bulky 2,3-dibromo-salicylate substituents. The reaction site between the lysines 99 residues of the two α chains is very narrow26 and the size of the reagents may restrict their ability to reach it. Hb conjugates cross-linked between α-Lys-99 present the advantage of preserving the DPG binding site free.

Why do we not observe cross-links between the β-Val-1 residue of one chain and β-Lys-82 of the other chain? This pattern has been encountered with other reagents having the isophthalic arrangement of the dibromosalicylate leaving groups.12 Inspection of structural models of human deoxyhemoglobin A reveals that the amino groups of β-Lys-82 are much nearer to the surface of the protein than are the N-terminal valine amino groups of the β subunits.27 β1-82β' cross-links have been reported with either less bulkier leaving groups (acyl phosphates)28 or with reagents having three negatively charged 3,5-dibromosalicylate leaving groups.12 The additional negative charge on the reagent may favor its access to the positively charged DPG binding site in order to react with β-Val-1.
Kluger and coworkers, reported the synthesis of hemoglobin adducts with biotin, lysine and the tripeptide gly-gly-gly, by reacting 3,5-dibromosalicyl trimesyl-((Lys-β-82)-(Lys-β-82))-hemoglobin (DBST-Hb) with botin hydrazide, lysine and gly-gly-gly. The reactions were carried out for 10 days at 4°C and the conjugates separated in a 32, 66 and 55% yield respectively. The present approach is more versatile in that different organic substances can be derivatized to incorporate reactive 3,5-dibromosalicylalate leaving groups. The hemoglobin cross-linking reaction goes to completion in several hours with an overall yield of 75-85%. The desired intramolecularly cross-linked derivatives can be isolated by preparative ion-exchange chromatography.

Variation in the structure of the pendant group

Comparison of the extent of Hb modification with DBBA, DBTPA and TIDB reveals some differences. DBBA is more efficient in acylating specific amino groups in the β-cleft region though the highest yield of overall hemoglobin modification was achieved with the tripeptide derivative DBTIA. DBTPA and DBTIA have more rigid structures compared to DBBA due to the presence of additional amide bonds in their pendant side chain. A degree of flexibility should be anticipated for the alkyl chain of DBBA as this can adapt an extended or staggered conformation. The rigidity of the side chains of DBTPA and DBTIA might force the leaving groups into a fixed position, which obviously is not a favorable one for interacting with the lysine groups. Another factor which can be responsible for the observed different reactivities are the ion-dipole interactions within the walls of the cationic binding sites between the polar functionalities of the reagents side chain and the ionic protein groups. Such interactions could produce additional conformational shifts in the reactive lysines microenvironment and hence a less favorable stereochemistry of the amino groups β-Lys-82 or α-Lys-99 of the protein to the remaining carboxyl ester. Since the rates of the aminolysis reaction of the reagents are not expected to differ significantly, the observed variations in reactivity are presumably due to differences in the binding affinity of the reagents to the reaction site. Improved reactivities
may be obtained with reagents having an alkane spacer unit between the 5-amino-isophthaloyl core and the ligand.

4.4. Availability of DBBA-Hb Conjugate for Interaction with Avidin

Functionalization of the carboxyl group of biotin preserves its affinity for avidin as indicated by the fact that the biotinylated Hb conjugate cross-linked between the β chains is able to bind Sepharose-immobilized avidin. Presumably, in the inter-β cross-linked hemoglobin conjugate, the urea moiety is not buried within the β cleft. Introducing a spacer arm between the isophthaloyl moiety and biotin may reduce steric hindrance and increase accessibility to avidin. This opens up new possibilities of conjugating hemoglobin molecules to other biotinylated reagents (proteins, peptides or enzymes) using avidin as a bifunctional, biotin-specific spacer unit (Figure 4.3.). Interesting results have been obtained so far by coupling biotinylated proteins through an avidin bridge both as a method to increase the survival in circulation of enzymes able to degrade high levels of metabolites and as an antigen delivery system.30

4.5. Functional Properties of Biotinylated Hemoglobin

The oxygen affinity of DBBA-Hb and the Hill coefficient are very close to those for native Hb (Table 3.1). Kluger and coworkers have shown that there is a linear correlation between the length of the cross-link span and the oxygen affinity of the hemoglobin conjugate31 for a given type of modification. The oxygen binding properties of the ααβ82-82β': N-biotinyl-isophthalate conjugate fit into this correlation for a span of ~ 7Å between the εN of the lysines 82. Similar results have been obtained with other hemoglobin conjugates which contain an isophthalyl cross-link between β-Lys-82 and β'-Lys-82.11 In this case, the cross-link is located on the molecular 2-fold rotation axis of hemoglobin, so that the cross-linking does not introduce any asymmetric distortion into
the tetramer. X-ray crystallographic studies have shown that this cross-linking causes only minor (symmetric) perturbation in the structure of the molecule.\textsuperscript{11} Thus, it is likely that the $\beta_{82-82\beta'}$ cross-link preserves the quaternary structure of native hemoglobin.

Figure 4.3. Schematic illustrating a hemoglobin-avidin-biotinylated ligand complex
CHAPTER V

Conclusions

Derivatives of d-Biotin, N-t-Boc-Ile-Gly and N-t-Boc-Phe have been synthesized and examined for their reactivity with human hemoglobin A. The reagents possess two anionic carboxylate groups which direct them to cationic pockets in hemoglobin to facilitate specific reactions. Analysis of the reaction products showed the same pattern of cross-linking two β monomers between Lys-82 or two α monomers between Lys-99 amino acid residues. Different reactivities were observed which are presumable dictated by different binding affinities of the reagents within the positively charged reaction microenvironment. The oxygen affinity and the Hill coefficient of the hemoglobin conjugate containing an N-biotinyl-isophthalyl cross-link between β-Lys-82 and β'-Lys-82 were very close to the values observed for native Hb A. Biotin bound to hemoglobin showed reduced availability for interacting with avidin, presumably due to steric hindrance and low accessibility to the binding site in avidin.
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