Connecting Proteins by Design. Cross-Linked Bis-Hemoglobin

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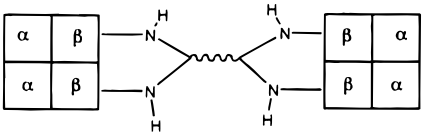
Abstract: A new type of multifunctional reagent creates a specific connection within and between two hemoglobin tetramers, resulting in a cross-linked bis-tetramer ("CLBT"). The tetrafunctional reagent (*N*,*N*′-5,5′-bis[bis(3,5-dibromosalicyl)isophthalyl]terephthalamide**,** DBIT) was prepared by conversion of the corresponding tetraacid to the tetrakis(3,5-dibromosalicylate). Deoxy hemoglobin reacts with DBIT to give a cross-linked bis-tetramer as the major product. Patterns in tryptic digests reveal that there are modifications of amino groups at β-Lys-82 and β-Val-1, indicative of a structure in which each tetramer is cross-linked between these positions. The cross-linked bis-tetramer has a decreased affinity for oxygen and very low cooperativity in oxygen binding. The properties of the material provide insights into the nature of protein−protein interactions.

Chemical modification of proteins presents possibilities for creating new entities that are complementary to those that result from genetic engineering.[1] Site-directed chemical cross-linking produces specifically modified proteins whose functional properties can be related to their altered structures.[2−5] On the basis of the success of these methods, we have sought to extend the possibilities of chemical modification to produce structurally defined connections *between* two proteins. Such bis-proteins would then provide the basis for systematic analysis of protein−protein interactions.

We chose human hemoglobin as the target for this study since its chemical reactivity patterns are well-established and its functional properties are of fundamental interest.[6] The protein is tetrameric ($\alpha\alpha\beta\beta$) and dissociates reversibly into dimers ($\alpha\beta$). Therefore, cross-linking is widely used to maintain the tetrameric structure.[7] Klotz pioneered the use of reagents that introduce specific cross-links within hemoglobin.[8,9] Walder developed a practical procedure for the large-scale production of specifically cross-linked hemoglobin that has desirable oxygen-carrying properties.[10]

An alternative approach to cross-linking hemoglobin uses nonspecific reagents, such as glutaraldehyde, that produce heterogeneous collections of materials. These include variably cross-linked and interconnected proteins.[11−13] Some materials produced in this way are being tested clinically as substitutes in transfusions for red-cells.[14] The oligomeric collections of tetramers that are in these products and others are proposed to have clinical advantages over the cross-linked tetramers.[15] However, since such arrays are neither separated into components nor structurally defined, the specific properties are unknown. Alternatively, hemoglobin could be cross-linked and connected by specific chemical reactions, potentially giving cross-linked bis-hemoglobins with defined structures.[16] The characteristics of a cross-linked bis-tetramer are shown in the generalized structure below. Ideally, such a product should be formed in a single processing step from the reaction of hemoglobin with a reagent.



On the basis of the known reaction patterns of efficient and selective cross-linking reagents,[17,18] we designed doubly connected bis(3,5-dibromosalicyl)isophthalates. These should react preferentially with the ϵ-amino group of each β-Lys-82 and/or the α-amino group of the N-terminal β-Val-1. The tetrafunc-

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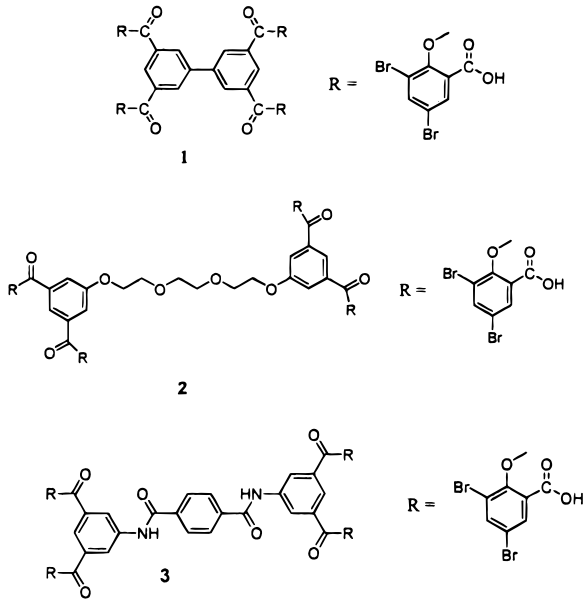
tional reagent derived from biphenyl tetracarboxylic acid (**1**) meets these requirements but it reacts with hemoglobin at only two of its sites,[19] suggesting that a greater separation between reaction sites is needed to connect tetramers. The oligoether reagent (**2**) reacts primarily at all four of its reaction sites but mainly within a single tetramer.[20] This suggests that the flexible hydrophobic reagent reacts in a folded state and a connection reagent should not be able to fold. Molecular modeling indicates that amino groups of the protein that might be cross-linked within a tetramer can be between 5.1 and 7.2 Å apart while the distance between the amino groups in two different tetramers is 10.6−15.9 Å.

Thus, we designed *N*,*N*′-5,5′-bis[bis(3,5-dibromosalicyl)-isophthalyl]terephthalamide **(**DBIT, **3**) a compound that cannot fold onto itself due to its extended sp^2 hydridization. The compound was readily prepared and its reaction with deoxy hemoglobin is rapid and efficient. The resulting products include a cross-linked bis-tetramer of hemoglobin. The specific oxygen-binding properties of the cross-linked bis-tetramer help address the general issue of the effects of protein−protein interactions.



Experimental Section

Materials and Analytical Methods. Commercial reagents were utilized without further purification. Solvents were dried prior to use. Buffers were made from doubly distilled deionized water. Other reagents for preparation of buffers and developers for reactions of hemoglobin and for chromatography were of analytical grade or better. Analysis by combinations of high performance liquid chromatography and spectroscopy (NMR, IR, UV−vis) indicated the purity and identity of newly synthesized materials. Proton and carbon NMR spectra were recorded on spectrometers whose proton resonance is at 200 and 400 MHz. Solutions of human hemoglobin A were obtained from Hemosol, Inc.

Oxygen Binding Analysis. The general method described by Imai[21] as modified by Shih and Jones[22] was adapted to current computational and interfacing procedures.

Synthesis of *N*,*N*′-5,5′-Bis[bis(3,5-dibromosalicyl)isophthalyl]-terephthalamide (3, DBIT). Bis(3,5-dicarboxyphenyl)terephthalamide[23] (2.0 g, 4.1 mmol) was stirred and heated to reflux in 10 mL of thionyl chloride for 20 h under nitrogen. The solvent was removed, leaving a white solid, which was stirred in 20 mL of anhydrous tetrahydrofuran. The resulting solution was added dropwise to a solution of *tert*-butyl 3,5-dibromosalicylate[24] (5.8 g, 16.5 mmol) and potassium *tert*-butoxide (1.9 g, 16.6 mmol) in 60 mL of anhydrous tetrahydrofuran. The mixture was stirred at room temperature for 20 h under nitrogen. The solid was dissolved in 50 mL of diethyl ether and the organic layer washed with distilled water (3 × 100 mL) and dried with magnesium sulfate. The solvent was removed, leaving a yellow liquid. This was dissolved in 50 mL of trifluoroacetic acid and stirred at room temperature for 2 h under nitrogen. Ether was added to dissolve unreacted 3,5-dibromosalicylic acid and to precipitate additional product. The mixture was left to stir at room temperature for 1 h under nitrogen. The solution was then filtered to give a white solid (1.7 g, 1.1 mmol). Mp 228−231 °C. ^{1}H NMR (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, 127.9, 127.5, 126.1, 119.4, 119.2. MS (positive FAB): 1604 (found), 1604 (calcd).

Reaction of DBIT with Hemoglobin. Carbonmonoxy hemoglobin (1.0 mL, 1.2 μmol) in 50 mM bis-Tris (pH 6.5) was passed through a Sephadex G-25 column at 4 °C, equilibrated with 50 mM sodium borate (pH 8.0 or pH 9.0). Carbon monoxide was passed over the solution for 10 min. The sample was sealed and placed in a water bath at 35 °C for 15 min prior to addition of **3**. Deoxy hemoglobin was prepared from a solution of carbonmonoxy hemoglobin, which was converted into oxy hemoglobin by irradiation under visible light in an oxygen flow at 0 °C for 2 h. The oxy hemoglobin was deoxygenated in a flow of nitrogen at 37 °C for 2 h. For reactions with either deoxy hemoglobin or carbonmonoxy hemoglobin, DBIT was added as a solid (0.004 g, 2.4 μmol), avoiding exposure of the hemoglobin solution to the atmosphere. The reaction flask was rotated in a bath at 37 °C for 24 h in a flow of water-saturated nitrogen. Samples were removed periodically for analysis. At the end of the reaction period, the flask was flushed with carbon monoxide, and the contents passed through a Sephadex G-25 column that had been equilibrated with 0.1 M MOPS (pH 7.2) to separate proteins from reagents.

Analysis of Modified Hemoglobin. The procedures are similar to those used previously, following the general methods described by Jones.[25] The heme and globin chains were separated by reversed-phase HPLC by using 330 Å pore C-4 Vydac columns (250 × 4.6 mm (analytical); 250 × 12 mm (preparative)) and developers containing 0.1% trifluoroacetic acid with gradients of acetonitrile, 20% to 60%, in water. The effluent was monitored at 220 nm. Peptide fragments were separated by reversed-phase HPLC with use of a C-18 Vydac column (93 × 4.7 mm). Developers contained 0.1% trifluoroacetic acid and gradients of acetonitrile, 0% to 100%, in water. The effluent was monitored simultaneously at 220 and 280 nm. Modified hemoglobins were separated according to molecular weight by semipreparative gel filtration FPLC with use of Superdex G75 and G200 HR columns (300 × 10 mm) under both nondissociating (25 mM Tris buffer pH 7.4) and partially dissociating conditions[20,26] (25mM Tris, pH 7.4, 0.5 M magnesium chloride). The effluent was monitored at 220 nm. The resulting hemoglobin solutions were monitored for modification by anion exchange HPLC by using both a POROS HQ/H perfusion column (100 × 4.6 mm) and a Synchropak AX300 column (250 × 4.5 mm). The effluent for both columns was a mixture of bis-Tris (25 mM) and Tris (25 mM). The system had a flow rate of 2 mL/min and a pH gradient from 6.5 to 8.5 and was monitored at 410 nm.

SDS-Gel Electrophoretic Analysis. Polyacrylamide gel electrophoresis, with sodium dodecyl sulfate,[27] was used to separate the

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individual protein chains according to molecular weight. Prior to electrophoresis, the protein samples (cross-linked hemoglobin, hemoglobin and molecular weight standards) in 0.5 M Tris, pH 6.8, containing 0.05% bromophenol blue, 4% v/v 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 10% v/v glycerol−water were denatured by being placed in boiling water for 5 min. Approximately 5−10 μg of protein was applied to each lane of the gel. The gel was run with use of a dual-slab cell apparatus at 200 mV. The yield and purity could be estimated by visual comparison of the resolved electrophoretic bands after staining with Coomassie Brilliant Blue. Unmodified hemoglobin and molecular weight standards were used to establish the molecular weight of the resulting bands. The gel was then scanned and analyzed with the program "Quantiscan" (BioSoft, Inc.).

Mass Spectroscopy. The masses of cross-linked hemoglobins were determined by ion-spray mass spectrometry[17,28,29] of the product from the reversed-phase HPLC outlined above.

Peptide Pattern Analysis.[25] Individual β globin chains were separated and collected by using the reversed-phase C4 HPLC technique outlined above and the solvent was removed by lyophilization. The globin chains were then dissolved in 8 M urea and kept at room temperature for 2 h. Freshly made trypsin solution (2 mg/mL, 4% of mass of total globin protein) was then added and 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 h. The tryptic hydrolysate was then heated in boiling water for 2 min and diluted to 1 M urea with 80 mM ammonium bicarbonate buffer (pH 8.5). *Staphylococcus aureus* V8 endoproteinase Glu-C solution (2 mg/mL, 2% of mass of total globin protein) was then added and the mixture was digested for 72 h at room temperature. The hydrolysates were filtered through a 0.45- m filter before injection onto the C18 reverse-phase HPLC column.

The modified peaks were identified by peptide pattern analysis. Peptide fragments were separated by HPLC procedures modified after that of Shelton[30] by 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 chromatogram for the unmodified β globin digest were identified by comparison with literature.

Preparative Gel Filtration Liquid Chromatography. The modified hemoglobins were separated according to molecular weight by preparative gel filtration liquid chromatography (FPLC) by using Superdex G75 and G200 HR columns (300 × 10 mm) under both nondissociating conditions (25 mM Tris, pH 7.4) and partial-dissociating[26] conditions (25 mM Tris, pH 7.4, 0.5 M magnesium chloride). The effluent was monitored at 287 nm. The fastest eluting peak was collected from multiple injections and concentrated by ultrafiltration with use of an Amicon CF25 25000 MW Centriflow membrane cone. The process was repeated until a single elution peak was obtained and the analytical techniques described above were used to identify the product.

Preparative Ion-Exchange Chromatography. 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 with use of a peristaltic pump. The hemoglobin reaction mixture was first passed through a Sephadex G-25 column, which had been equilibrated with distilled water, then loaded onto the Sephacel column equilibrated with glycine (0.2 M, pH 7.8) and washed with the same solution. Products were then eluted from the column over 48 h by using glycine buffer (0.2 M, pH 7.8) and an increasing concentration of potassium chloride (0.03−0.1 M). The eluting fractions were combined, concentrated by using a centrifugal concentrator, and analyzed by using the methods described above. Preparative AX300 ion-exchange HPLC was performed in the same manner as the analytical technique outlined above.

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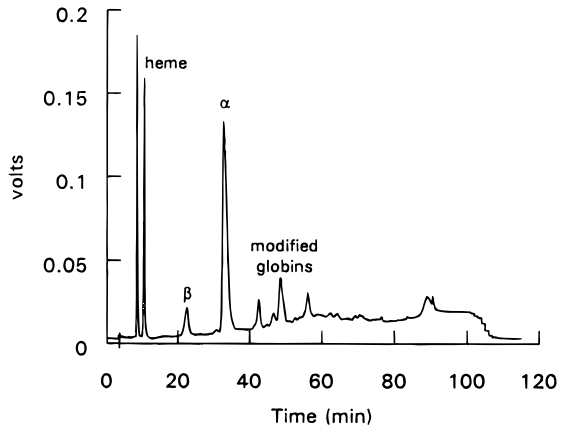
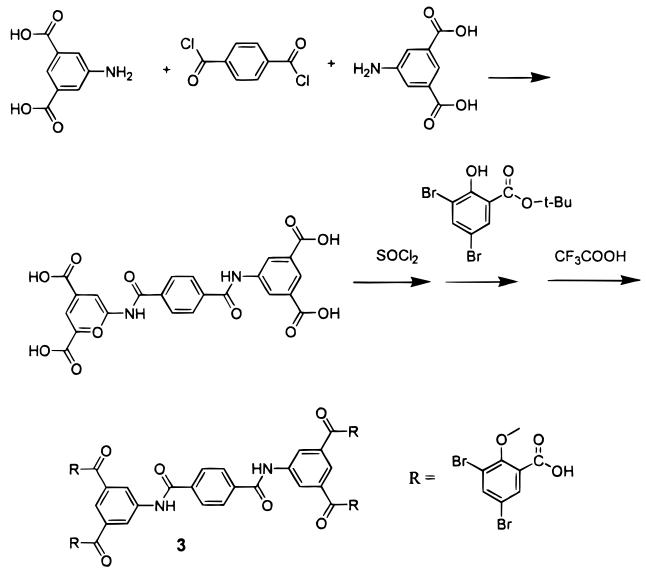


Figure 1. C4 reversed-phase HPLC chromatogram, separating globin chains following reaction of deoxy-Hb with 3 (pH 8, 37 °C).

Scheme 1



Results

The reagent we designed to combine cross-linking and protein connection (DBIT, **3**) was conveniently and efficiently prepared (Scheme 1).

Reactions of DBIT (**3**) with deoxy hemoglobin (deoxy-Hb) and carbonmonoxy hemoglobin (CO-Hb) were conducted in buffered solutions (pH 8 and 9). Combining CO-Hb with up to 20 equiv of **3** gave less than 60% modification of the protein. However, reaction of deoxy-Hb with just 2 equiv of **3** at pH 8, 37 °C for 20 h, modified 95% of the β chains of deoxy-Hb, as shown by HPLC analysis (Figure 1). Reaction at pH 9 was less effective. Therefore, further work was done with deoxy-Hb and **3** at pH 8, 37 °C.

C4 reversed-phase HPLC separates the hemes and unmodified and modified globin chains. The chromatogram in Figure 1 indicates that DBIT selectively modifies the β globin chains, as has been found for other smaller reagents with the same leaving group.[17] There was no distinct product peak from this column. Rather, several small peaks appeared throughout the product region of the chromatogram.

Products were then identified by comparison against known materials: Superdex G-75 gel filtration of unmodified hemoglobin and a purified sample of $\beta-\beta$ cross-linked hemoglobin.[17] The 64 kDa tetrameric hemoglobin eluted at 15.7 min from the column (no magnesium chloride). Analysis of the reaction

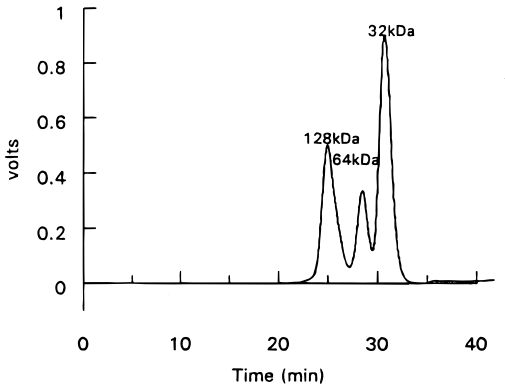


Figure 2. G200 size exclusion chromatogram for reaction of deoxy-Hb with **3** under partial-dissociating conditions.

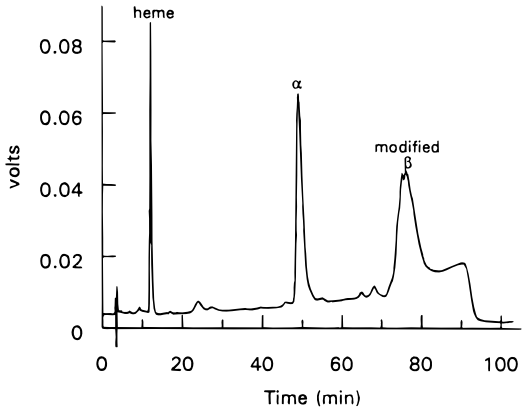


Figure 3. C4 reversed-phase HPLC chromatogram for the purified higher molecular weight hemoglobin oligomers.

mixture under these conditions indicated the presence of 64 and >64 kDa species in approximately a 2:1 ratio eluting at 15.1 and 13.2 min, respectively. Under partial-dissociating conditions (magnesium chloride) the 32 kDa hemoglobin dimer subunits and 64 kDa cross-linked tetramers eluted at 18.5 and 16.3 min, respectively. Analysis of the hemoglobin reaction mixture indicated the presence of 32, 64, and >64 kDa species in ratios of 1:3:2, eluting at 18.3, 15.8, and 13.7 min, respectively. Under partial-dissociating conditions (magnesium chloride) the 32 kDa dimers eluted from the Superdex G200 gel filtration column after the higher molecular weight species. Analysis of the reaction mixture indicated the presence of materials at times expected for 32 and 64 kDa species, along with a peak that we assign to the 128 kDa species (Figure 2).

SDS-PAGE analysis of the cross-linked hemoglobin reaction mixture indicated the presence of species having molecular weights of approximately 64, 32, 16, and 15 kDa. The lane containing unmodified hemoglobin gave peaks with molecular weights only of 16 and 15 kDa, while analysis of a genuine $\beta-\beta$ cross-linked hemoglobin tetramer indicated species having molecular weights of 32 and 15 kDa only.

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 products having masses of 16620, 16899, and 32468 Da. The mass of 16620 Da corresponds to a product having one β globin chain attached to one molecule of **3** with one remaining unhydrolyzed ester group (calculated mass 16620 Da[29]). The peak with mass 16899 Da corresponds to a product having one β globin chain attached to one molecule of **3** with two remaining unhydrolyzed dibromosalicyl ester groups (calculated mass: 16898 Da). The peak with mass 32468 Da corresponds to a product having two β globin chains reacting with **3**, with one remaining unhydrolyzed ester group (calculated mass: 32462 Da). Minor products also appear with masses of 31718, 17175, 16431, 16153, and 15876 Da. These masses correspond to products with combinations of α globin chains and β globin chains having reacted with **3** and leaving up to three residual ester groups.

Purification of Cross-Linked Hemoglobin Bis-Tetramers. HPLC analysis of the reaction mixture with POROS columns (sieve packing) indicated the presence of several products that did not separate by preparative ion-exchange chromatography. Thus, the higher molecular weight species were separated from the 64 and 32 kDa proteins by collecting the first eluting peak from the Superdex G200 gel filtration column, under partial-dissociating conditions. The collected fractions were pooled, concentrated, and injected. The product eluted as a single peak. Additional peaks were also collected and concentrated for comparison.

Analysis of Cross-Linked Hemoglobin Bis-Tetramers. SDS-PAGE analysis of the purified higher molecular weight sample indicated that it contained primarily 64 and 15 kDa peptide chains. Only a trace of 32 kDa protein was present. If the β subunits are cross-linked within a tetramer by a linkage and that connects to a similarly cross-linked tetramer, then the peaks that should be present will be 64 (four connected β subunits) and 15 kDa (unmodified α subunits). AX300 anion exchange HPLC indicated that the product was homogeneous, eluting at 35 min. C4 reversed-phase HPLC analysis of the purified sample indicated that there were no unmodified β chains and there was a sufficiently large proportion of unmodified α chains, consistent with specific β modification (Figure 3).

An unmodified β chain peak and the modified β chain product peak were collected from the reversed-phase C4 column and subjected to tryptic digestion analysis.[25] Individual peptide fragments were separated by 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. Comparison of absorption of each peak at 220 and 280 nm was used to identify the peptides of the digested unmodified β globin. These results indicate that the relative amounts of both the βT-1 and the βT-10a′ peptides were approximately half of that in the unmodified digest. The reduced amount of the peptides indicates that the α-amino group of the β-chain N-terminal valine (in βT-1) is cross-linked to the ϵ-amino group of β-Lys-82.[31] Product peaks obscure the peak of the βT-9 peptide, which also arises from β-Lys-82 (Figure 4; for the native protein digest see ref 2). In addition, several new peaks, with absorbance at 280 nm, appeared in the modified β globin digest chromatogram, consistent with formation of aromatic derivatives (Figure 5).

Thus, we draw a schematic structure of the cross-linked, interconnected hemoglobins (Figure 6). We shall refer to the structure as CLBT, for "cross-linked bis-tetramer". The material corresponding to the 128 kDa peak was collected and its oxygen-binding properties were analyzed (Figure 7).

The oxygen-binding curve shows that the average oxygen affinity of the bis-tetramer is lower ($P_{50} = 9.1$ Torr) than that of native hemoglobin ($P_{50} = 5.0$ Torr) or a typical cross-linked tetramer (Hb-β82-Trimesyl-β'82, $P_{50} = 4.8$ Torr), but greater

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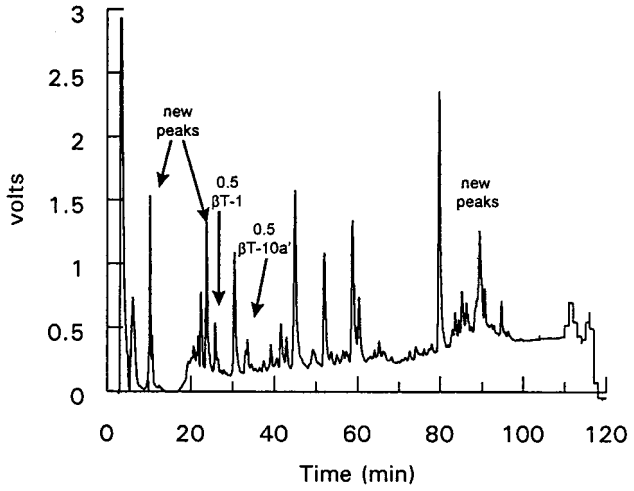

Figure 4. C18 reversed-phase HPLC chromatogram of modified β globin chain digest monitored at 220 nm.

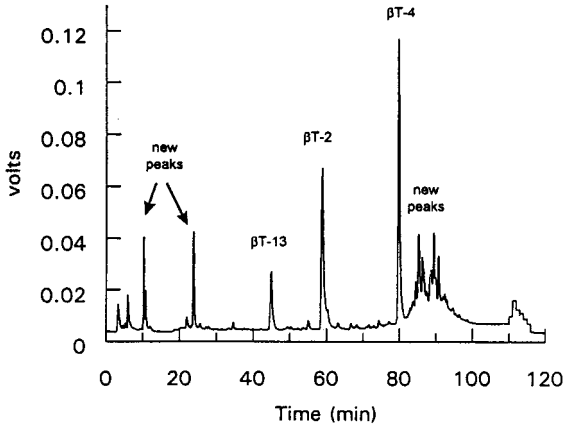

Figure 5. C18 reversed-phase HPLC chromatogram of modified β globin chain digest monitored at 280 nm.

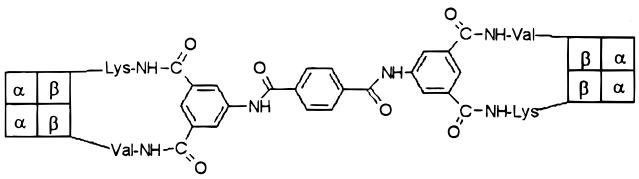

Figure 6. Schematic diagram of the cross-linked bis-tetramer of hemoglobin ("CLBT") from reaction of DBIT with deoxy-Hb.

than that of hemoglobin within red cells (P_{50} = 26.0 Torr). The data indicate there is significantly reduced cooperativity in oxygen binding to the bis-tetramer.

To obtain the necessary parameters for analysis, the data were plotted as log pO_2 vs log(Y/(1 − Y) and fit to a straight line, giving the Hill coefficient index (n_{50}) of cooperativity of 1.3 (=1.29 ± 0.03) (Figure 8). For comparison, unmodified hemoglobin is highly cooperative with n_{50} = 3, a value also seen in the cross-linked tetramers. The loss of cooperativity and decrease in oxygen affinity is also seen in polymerized cross-linked hemoglobin.[32]

Discussion

Reagent Design. Our objective in designing DBIT was to produce a reagent that in one step would produce a defined

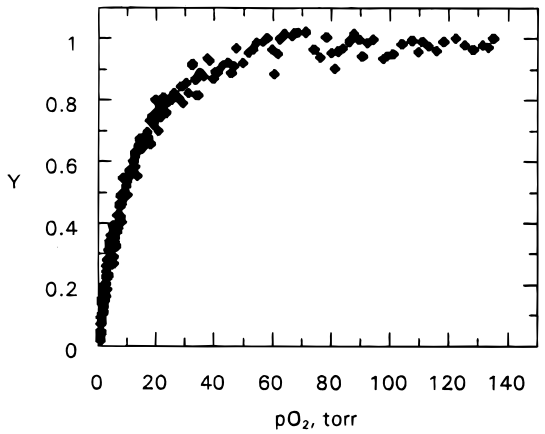

Figure 7. Oxygen binding curve for cross-linked hemoglobin bis-tetramer (CLBT), pH 7, 25 °C. "Y" is the fraction of hemoglobin sites to which oxygen is bound.

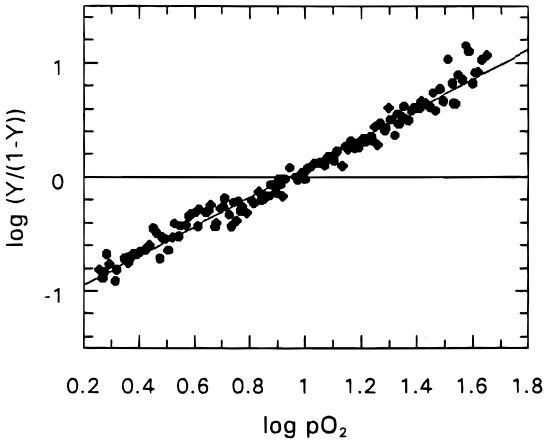

Figure 8. Hill plot for data in the oxygen binding curve for CLBT. The slope at log P_{50} is the Hill coefficient.

cross-linked bis-tetramer of hemoglobin. The goal has been achieved by using a rigid reagent with a moderate span between reaction sites. The material we isolate from the reaction of DBIT with deoxy-Hb is about 50% cross-linked bis-tetramer (CLBT), with the remainder of the altered hemoglobin being cross-linked tetramer, based on the area of peaks in analytical chromatograms.

Previous studies have shown that reagents that are esters of 3,5-dibromosalicylate react selectively with amino groups in the site on hemoglobin that binds the polyanionic effector, 2,3-diphosphoglycerate.[17,18,33] Structural analysis based on the results of the tryptic digest of CLBT indicates that DBIT has the specificity seen with other 3,5-dibromosalicylates.[17,18,33] The production of a cross-linked bis-tetramer in a single reaction process creates new possibilities for controlled chemical alteration of proteins.

An alternative approach to creating connected cross-linked tetramers is a two-stage process in which hemoglobin is first cross-linked. The purified product is then reacted with a reagent that couples proteins to one another. One problem with such an approach, beyond the extra step, is that the specificity of the inter-protein connection reagent must be different than that of the cross-linking reagent, since the primary reaction site on the protein is blocked by the cross-link. The widely used α-99-Lys-fumaryl-α-99-Lys cross-linked hemoglobin tetramer[10] has

(32) Rogers, M. S.; Ryan, B. B.; Cashon, R. E.; Alayash, A. I. *Biochim. Biophys. Acta* **1995**, *1248*, 135−42.

(33) Shibayama, N.; Imai, K.; Hirata, H.; Hiraiwa, H.; Morimoto, H.; Saigo, S. *Biochemistry* **1991**, *30*, 8158−8165.

been oligomerized with bis(maleylglycylamide) polyethylene.[32] The resulting material is polymeric and its overall structure is not known.[32] Physiological studies indicate that polymerized materials may have some functional advantages as a red cell substitute over the corresponding cross-linked tetramer.[14,34] CLBT should be useful in defining the specific physiological effects of linked cross-linked hemoglobins.

Oxygen Affinity of Bis-Tetramers. Specifically cross-linked tetramers bind 4 equiv of oxygen and do so cooperatively.[3] If two cross-linked tetramers are connected, the new entity has eight oxygen-binding sites. If the connection between the two tetramers is long and flexible, it should not perturb the structures. One would expect that such an entity would behave as the cross-linked tetramers do separately. In contrast, with the short, rigid link in CLBT, the tetramers should be strongly influenced by one another. We see that the cooperativity is reduced and oxygen affinity is decreased. This is typical of what is seen in polymerized hemoglobins,[32] indicating that protein coupling is at the heart of the altered behavior.

The binding of oxygen to hemoglobin causes a conformational change that involves substantial movement of the protein and is manifested as cooperativity.[6] The closely coupled proteins in CLBT will have their movements restricted by each other's presence (a combination of steric and mass effects is likely), potentially reducing cooperativity. The decreased oxygen affinity requires that the bound state have a lower thermodynamic barrier to converting to the deoxy state, which is associated with the T state of the protein.[6] This is desirable for a red-cell substitute.

(34) Gould, S. A.; Sehgal, L. R.; Sehgal, H. L.; Moss, G. S. *Crit. Care Clin.* **1992**, *8*, 293−309.

Conclusions

The formation of joined, cross-linked proteins has been achieved by the use of a reagent that combines reaction specificity, appropriate linking span, and rigidity. The resulting cross-linked bis-tetramer has physical properties that indicate the properties of bis-tetramers are considerably different from the corresponding mono-tetramers and similar to polymerized materials. The reagent DBIT presents a model for the design of materials for efficiently connecting proteins in a highly controlled reaction. The availability of CLBT makes it possible to do physical studies that can provide insights into factors controlling protein−protein interactions. Further physical studies on this material and production of other linked hemoglobins is in progress.

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