EXPERIMENTAL PROCEDURES

Materials and Methods. All reagents were obtained from commercial suppliers and used without further purification unless otherwise indicated. Human hemoglobin A was obtained as a gift from Oxygenix, Inc., or isolated and purified from red cells obtained from the Canadian Blood Services through volunteers with their informed consent. Concentrations of hemoglobin solutions were determined using the cyanomethemoglobin assay, and the purity of hemoglobin was determined through reverse-phase HPLC analysis as described by Jones.[1] Trimesoyl tris(3,5-dibromosalicylate) (TTDS),[2] propynoic acid [4-(4-propynoylamino-benzenesulfonyl)-phenyl]-amide (**3**),[3] propargyl-4,4'-sulfonyldiphenol ether[3] were synthesized according to literature procedures.

*4-Azidomethyl-benzylamine (**2**)*. 4-(Bromomethyl)benzylamine hydrobromide (0.56 g, 2.0 mmol) was suspended in water (5 mL), followed by the addition of sodium azide (0.39 g, 6.0 mmol) in water (7 mL). The mixture was heated to reflux for 16 h under a nitrogen gas flow. The reaction mixture was reduced half of the volume under vacuum. Diethyl ether (20 mL) was added and the mixture was cooled in an ice-water bath. Potassium hydroxide pellets (0.5 g) was added. The organic phase was separated and the aqueous phase was extracted with diethyl ether (20 mL ×3). The organic phase was combined with ether extracts, dried over K_2CO_3. Evaporation of the filtered solution afforded a white solid. Yield: 0.32 g (99.1%).[4] ^{1}H NMR ($CDCl_3$): δ 7.33 (2H, d, J = 8.1 Hz), 7.28 (2H, d, J = 8.1 Hz), 4.31 (2H, s), 3.87 (2H, s), 1.47 (2H, s); ^{13}C NMR: δ 143.7, 134.0, 128.7, 127.8, 54.8, 46.4; LR-ESI: 162.1 $[M]^+$.

Preparation of DBST-Hb. Carbomonoxyhemoglobin (2.0 mL, 1.95 mM) was prepared by passing the solution through a Sephadex G-25 column equilibrated with 0.1 M MOPS (pH 7.2). The resultant solution (~15 mL) was oxygenated and deoxygenated to give deoxyhemoglobin (deoxyHb). TTDS (8.1 mg, 7.8 μmol) was added as a solid with minimal exposure of the deoxyhemoglobin solution to air. The reaction mixture was left for 3 hours under a stream of humidified N_2 at 37 ^{o}C. It was then placed under a stream of humidified CO for 15 min and passed through a Sephadex G-25 column equilibrated with 0.1 M MOPS (pH 8.0) to remove the unreacted cross-linker. The resultant solution was concentrated to ~4.0 mL. It was sealed in an Agilent vial and flushed with CO and frozen.

*Synthesis of hemoglobin azide **1** from the reaction of DBST-Hb with **2***. To a frozen sample of carbonmonoxy DBST-Hb (1.0 mL, 0.975 mM) in a MOPS buffer (0.1 M) with a certain pH was added **2** as a solid. The mixture was sealed in an Agilent vial and flushed with CO and stirred at 4^{o}C for 2 days. The reaction mixture was passed through a MOPS buffer (0.1 M, pH 7.2) column, concentrated, flushed with CO and stored at 4^{o}C.

*Reaction of **1** with a bis-alkyne to afford the bis-tetramer of hemoglobin*. The carbonmonoxy form of hemoglobin azide **1** (0.51 mL, 0.975 mM) was passed through a Sephadex G-25 column equilibrated with a phosphate buffer (0.02 M, pH 7.4). The resultant solution was concentrated to ~1.0 mL. To this solution in an Agilent vial was added bis-alkyne (50 μL of a 0.1 M solution in DMSO), ligand **4** (100 μL of a 20 mM solution in water), $CuSO_4$ (50 μL of a 20 mM solution in water), L-ascorbic acid (200 μL of a 0.1 M solution in water) and a stir bar. The vial was sealed and flushed with CO and wrapped with Al foil. The reaction mixture was stirred at room

temperature. The progress of the reaction was followed by G200 HPLC analysis. Once the reaction was completed (typically within 4 hours), the mixture was filtered and passed through two MOPS buffer (0.1 M, pH 7.2) columns consecutively at 4°C. The mixture was concentrated and stored at 4°C.

Isolation of Modified Hemoglobins. Purification of bistetramers (BT-Hb) was carried out using gel filtration chromatography (Sephadex G-100, 1000 × 35 mm) under slightly dissociating conditions. The eluent used was 37.5 mM Tris, pH 7.4, containing 0.5 M magnesium chloride. The high salt concentration causes partially modified hemoglobin tetramers to dissociate, allowing for separation of larger bis-tetramers from the mixture. Fractions were collected, concentrated, and analyzed using Superdex G-200 size exclusion chromatography, C4 reverse-phase analytical HPLC, and SDS-PAGE gel electrophoresis.

HPLC Analysis of Modified Hemoglobins. Hemoglobin azide **1** and DBST-Hb were analyzed using analytical reverse-phase HPLC with a 330 Å C-4 Vydac column (4.6 × 250 mm) to determine the sites of globin chain modifications.[5] Modified and unmodified globin chains were separated using an eluting solvent containing 0.1% trifluoroacetic acid and a gradient beginning with 20% and ending with 60% acetonitrile (vol%) in water. The effluent was monitored at 220 nm. Hemoglobin bis-tetramers were analyzed using a Superdex G-200 HR (10×300 mm) preparative size exclusion column. Protein samples were eluted under conditions that dissociate the hemoglobin tetramers into dimers (37.5 mM Tris-HCl, pH 7.4, 0.5 M magnesium chloride). The effluent was monitored at 280 nm.

SDS-PAGE Analysis. The molecular weights of constituent proteins were estimated using polyacrylamide gel (12%, Tris-HCl) electrophoresis. Two dimensional Tris-HCl polyacrylamide gels were comprised of 12% separating gel (pH 8.8) and 5% stacking gel (pH 6.8) both with 10% sodium dodecyl sulfate. Protein samples were treated with 2-mercaptoethanol and sodium dodecyl sulfate.[6] Globin chains were further denatured by heating at 95 °C for 10 min before loading onto the gel. Finished gels were stained with Coomassie Brilliant Blue. Further details are described in earlier papers.[7]

Oxygen Binding Analysis. Oxygen binding affinity was measured (28 °C, pH 7.4) with a Hemox analyzer that measures the oxygen pressure for half-saturation (P_{50}) and Hill's coefficient of cooperativity at half-saturation (n_{50}). Hemoglobin bis-tetramer (~1 g/L), in sodium phosphate buffer (I = 0.01M, pH 7.4) were oxygenated before analysis. The oxygenated hemoglobin samples were then deoxygenated by bubbling nitrogen through the sample until the P_{O_2} reached a minimum value. The data were then fitted to the Adair equation to obtain P_{50} and n_{50}.[8]

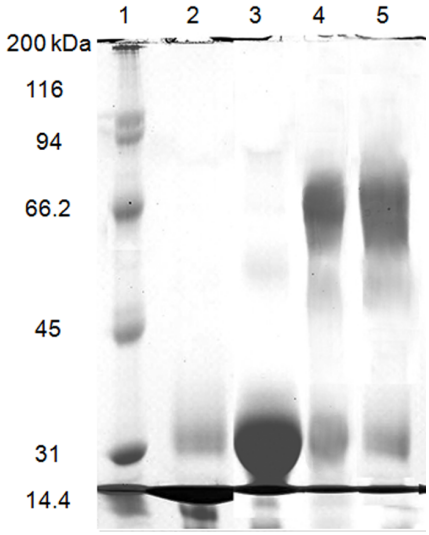

Figure S1: SDS-PAGE analysis: 1. mass standards; 2. native hemoglobin; 3. Hemoglobin azide **1**; cross-linked hemoglobin bis-tetramers; 4. ββ cross-linked hemoglobin bis-tetramer from click reaction; 5. Authentic amide-linked bis-tetramer synthesized by reported methods.[9]

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