Conjoined Hemoglobins. Loss of Cooperativity and Protein−Protein Interactions†

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ABSTRACT: Hemoglobin cross-linked as a bis(isophthalamide) of the ϵ-amino groups of lysine 82 of each β-subunit binds and releases oxygen with a Hill coefficient indicative of cooperative oxygen binding (typically ~2.0). However, connecting two such cross-linked tetramers with a relatively short covalent linkage produces cross-linked bis-tetramers that bind oxygen with Hill coefficients near unity. To separate the effect of the linkages from the effects of protein−protein interactions in the conjoined proteins, reagents (**1** and **2**) were used to produce bis-tetramers (**A** and **B**). These have a considerably greater distance between cross-linked tetramers than earlier examples. Yet, the bis-tetramers (**A** and **B**) bind oxygen with minimal cooperativity ($n_{50} = 1.4$, 1.2). To assess the effect of the linkage itself, cross-linked tetramers (**C** and **D**) were prepared from reactions with the same reagents. These bind oxygen with cooperativity similar to that of cross-linked tetramers without the extended chain (**C**, $n_{50} = 2.0$; **D**, $n_{50} = 1.8$). Other tetramers (**E** and **F**) with flexible, saturated hydrocarbon appendages were also prepared. These also showed cooperativity in oxygen binding (**E**, $n_{50} = 1.7$; **F**, $n_{50} = 1.8$) despite their high degree of hydrophobicity. Thus, the intertetrameric linkages themselves do not induce the loss of cooperativity, leading to the conclusion that solution effects of the tetramers upon one another are the source of the decline in cooperativity: protein−protein interactions are most significant in disrupting the cooperativity of the bis-tetramers, regardless of the span or composition of the linker. This suggests that effects of oligomerization of hemoglobin within red cell substitutes should be considered in terms of such interactions.

The search for safe and effective alternatives to red cells as circulating oxygen carriers has led to widespread interest in the properties of cross-linked hemoglobins. Clinical results associated vasoactivity with cross-linked tetramers (*1*). Materials consisting of mixtures of oligomers of cross-linked tetramers included examples with lower vasoactivity (*2*). Since vasoactivity is a highly undesirable clinical attribute, finding the source of the problem could be the basis for designing and producing acceptable oxygen carriers. The origins of this difference have been the subject of considerable speculation, but the lack of structurally defined cross-linked hemoglobin oligomers has limited experimental characterization (*3*). Thus, we have been engaged in developing methods to produce structurally defined cross-linked hemoglobin oligomers. The first examples showed a loss of cooperativity in oxygen binding and release (*4*, *5*). In this paper we report the synthesis and properties of conjoined cross-linked hemoglobins: bis-tetramers with extended linkages between tetramers as well as related cross-linked tetramers that test the effect of the intertetramer link on the material's oxygen-binding properties.

Reactions of hemoglobin with bifunctional and trifunctional acylating reagents produce internally cross-linked tetrameric species that exhibit significant cooperativity in oxygen binding (*6*−*8*). Systematic variation of the span of bifunctional cross-linking reagents as bisamides between β-subunits led to the observation of a linear free energy relationship between the average oxygen affinity of the altered hemoglobin and the length of the cross-link (*6*, *9*). The cross-linked hemoglobins in that series all showed cooperativity in oxygen binding that was similar to that of the native protein (*10*−*12*). That work established that the restrictions to motion imposed by the span of the linker affect the free energy of oxygen binding without altering its cooperativity.

We have been developing methods to extend the systematic alteration of hemoglobin to methods that introduce a cross-link while connecting two hemoglobin tetramers to one another (*4*). Continuing that approach, we became interested in the effects of connecting proteins, as such species have become common approaches in the development of hemoglobin-based oxygen carriers (*2*, *13*, *14*). The general approach is based on using anionic bis-bifunctional reagents that can introduce defined cross-links within and *between* two hemoglobin tetramers (*5*). If all four sites of the reagents react with hemoglobin, the product is a cross-linked bis-tetramer (*5*). This general approach to creating reagents with the ability to cross-link and connect tetramers permits us to create materials that reveal the effects of connections *between* tetramers as reflected in the functional properties of the bis-tetramers.

The first examples of cross-linked bis-tetramers had properties that differ markedly from those of the related cross-linked tetramers (*4*, *5*). In particular, those bis-tetramers have increased oxygen affinity and reduced cooperativity, which would logically be the result of protein−protein interactions. An alternative explanation for these observations is that the linker itself causes disruption of essential solvation. To find the origin of these altered properties, we have now produced soluble site-directed reagents **1** and **2** to create an

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increased span between tetramers. In addition, we used these and related reagents (**3** and **4**) to produce cross-linked tetramers. Modeling suggests that the resulting bis-tetramers would have a smaller extent of direct interaction but the proteins remain well within the solvation sphere of one another on a permanent basis. The larger intertetramer link itself should create increased hydrophobic disruption of the solvation sphere, providing a clear distinction between the consequences expected from these alternative sources of altered function.

EXPERIMENTAL PROCEDURES

Materials and Methods. All newly synthesized materials were characterized using a combination of NMR spectroscopy, mass spectroscopy, and infrared spectroscopy. Proton NMR spectra were recorded at 300 or 400 MHz; carbon NMR spectra were recorded at 75 or 100.5 MHz. Molecular modeling was performed using Spartan '04 (Windows). Solutions of human hemoglobin were prepared from erythrocytes that were separated from freshly drawn red blood cells. Oxygen-binding properties for native and modified were obtained using a modified UV−vis spectrometer based on the method of Imai (*15*) with modifications of Shih and Jones (*16*).

N,N′-Bis(isophthalyl)-2,2′-bipyridinyl-5,5′-dicarboxylate. 2,2′-Bipyridinyl-5,5′-dicarboxylic acid (0.50 g, 2.05×10^{-3} mol) was combined with excess thionyl chloride (25 mL) in a round-bottom flask containing a stirring bar and a condenser. The mixture was refluxed for 72 h, producing a yellow solution. Thionyl chloride was removed by distillation. 5-Aminoisophthalic acid (0.74 g, 4.10×10^{-3} mol) and a small amount of 4-(*N*,*N*-dimethylamino)pyridine (0.05 g, 4.1×10^{-4} mol) were dissolved in anhydrous dimethylacetamide (25 mL). 2,2′-Bipyridinyl-5,5′-dicarboxylate chloride was combined with a solution of 5-aminoisophthalic acid and 4-(dimethylamino)pyridine and stirred for 72 h at room temperature. The product precipitated upon addition of 250 mL of water. The crystals were separated by centrifugation (5000*g*, 30 min). The supernatant was decanted, and the crystals were washed with additional water and separated three times by further centrifugation. The resulting wet crystals were lyophilized and rinsed with dry acetone (0.89 g, 76% yield): mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 8.27 (s, 2H, ArH, C_2), 8.60 (d, 2H, PyrH, C_4), 8.66 (d, 2H, PyrH, C_3), 8.72 (s, 4H, ArH, $C_{4,6}$), 9.33 (s, 2H, PyrH, C_6), 10.92 (s, 2H, NH), 11.3 (s, 4H, COOH); ^{13}C NMR (DMSO-d_6) 121, 125, 126, 131, 132,137, 140, 150, 157, 165, 167; MS (ESI) *m*/*z* 569.1 (M − H).

*N,N′-Bis[bis(sodium methyl phosphate)isophthalyl]-2,2′-bipyridinyl-5,5′-dicarboxylate (**1**).* *N,N′*-Bis(isophthalyl)-2,2′-bipyridinyl-5,5′-dicarboxylate (0.2 g, 3.5×10^{-4} mol) was combined with thionyl chloride (15 mL) and refluxed for 8 h, giving a brown solution. Thionyl chloride was removed by vacuum distillation, and the crude acid chloride was dissolved in dry THF (20 mL). This was combined with sodium dimethyl phosphate (0.23 g, 1.58×10^{-3} mol) that had been freshly prepared in dry acetone from trimethyl phosphate and sodium iodide (*17*). The mixture was stirred for 12 h at room temperature. The newly formed precipitate was removed by filtration, and the solvent was evaporated from the supernatant under vacuum. Crude product (oil) was dissolved in dry acetone (25 mL) containing sodium iodide (0.24 g, 1.58×10^{-3} mol). The mixture was stirred for 24 h under nitrogen in the dark. The resulting pale white crystals were collected by vacuum filtration and washed with dry acetone (0.27 g, 75% yield): mp >250 °C dec; ^{1}H NMR (DMSO-d_6) δ 3.72 (m, 12H, OCH_3), 8.35 (s, 2H, ArH, C_2), 8.65 (d, 2H, PyrH, C_4), 8.71 (d, 2H, PyrH, C_3), 8.88 (s, 4H, ArH, $C_{4,6}$), 9.30 (s, 2H, PyrH, C_6), 10.85 (s, 2H, NH); ^{13}C NMR (DMSO-d_6) 62, 122, 128, 130, 131, 134, 137, 141, 153, 158, 166, 170; ^{31}P NMR (DMSO-d_6) δ −6.32 (decoupled); MS (ESI) *m*/*z* 236 (M − 4H/4).

trans-Stilbene-4,4′-dicarboxylic Acid. p-Toluic acid (50 g, 0.37 mol) was combined with sulfur (5.9 g, 0.18 mol) in a round-bottom flask with a condenser under flowing nitrogen. The exiting gas was bubbled though a trap containing sodium hydroxide to remove hydrogen sulfide. The mixture was kept at 270 °C for 2 h and then cooled to room temperature. Xylene (150 mL, mixed isomers) was added, and the solution was refluxed for 30 min. The resulting crystals were collected using hot filtration and washed with additional hot xylene. The solid was boiled in 1,4-dioxane (50 mL) for 30 min and filtered hot followed by washings with hot 1,4-dioxane. The solvent was removed, leaving crystals that were dissolved in boiling aqueous potassium hydroxide (150 mL of 30 g/L) and boiled for 1 h. This reduced the volume by about one-third. Yellow crystals were obtained upon cooling (ice bath). The crude crystals were redissolved in hot water (100 mL), and the solution was acidified with 10 mL of concentrated HCl. Cooling produced crystals that were filtered and lyophilized to give the stilbenedicarboxylic acid (14.7 g, 30% yield): ^{1}H NMR (DMSO-d_6) δ 7.49 (s, 2H), 7.76 (d, J = 8.2 Hz, ArH), 7.96 (d, J = 8.2 Hz, ArH) 11.5 (s, 2H, COOH); ^{13}C NMR (DMSO-d_6) 126.9, 129.8, 129.9, 130.0, 141.0, 167.1; MS (ESI) *m*/*z* 267.3 (M − H).

N,N′-Bis(isophthalyl)-trans-stilbene-4,4′-dicarboxylate. trans-Stilbene-4,4′-dicarboxylic acid (2 g, 7.5 × 10−3 mol) was refluxed in excess thionyl chloride (30 mL) for 5 h. Residual thionyl chloride was removed under vacuum. 5-Aminoisophthalic acid (2.72 g, 1.5×10^{-2} mol) and 4-(dimethylamino)pyridine (0.24 g, 2×10^{-3} mol) were dissolved in anhydrous dimethylacetamide (75 mL). This solution was combined with the acid chloride at 0 °C and stirred for 20 h at room temperature. Slightly acidic water (500 mL) was added to the solution to induce precipitation, giving a crystalline suspension. The crystals were collected using centrifugation (5000*g*, 40 min), washed with water, and air-dried overnight. Crystals were washed with hot ethanol (2.68 g, 60% yield): ^{1}H NMR (DMSO-d_6) δ 7.55 (s, 2H, CH), 7.83 (d, J = 8.4 Hz, 4H, ArH), 8.06 (d, J = 8.4 Hz, 4H, ArH), 8.22 (s, 2H, ArH), 8.70 (s, 4H, ArH), 10.60 (s, 2H, NH), 13.2 (s, 4H, COOH); ^{13}C NMR (DMSO-d_6) 125.2, 125.4, 127, 129, 130, 132, 134, 140.4, 140.7, 166, 167; MS (ESI) *m*/*z* 593.5 (M − H).

*N,N′-Bis[bis(sodium methyl phosphate)isophthalyl]-trans-stilbene-4,4′-dicarboxylate (**2**).* *N,N′*-Bis(isophthalyl)-*trans*-stilbene-4,4′-dicarboxylate (0.2 g, 3.4×10^{-4} mol) was refluxed in excess thionyl chloride (20 mL) for 72 h. Thionyl chloride was removed under vacuum, leaving a yellow solid. Sodium dimethyl phosphate (0.22 g, 1.5×10^{-3} was suspended in THF (10 mL) and added to acid chloride under nitrogen environment. The reaction mixture was stirred for 24 h. The solvent was evaporated under reduced pressure

leaving a yellow oil. This was immediately dissolved in dry acetone followed by addition of sodium iodide (0.22 g, 1.5 $\times$ 10^{-3} mol). The mixture was stirred under nitrogen for 72 h at room temperature. The resulting crystals were collected by vacuum filtration and washed with dry acetone (0.21 g, 65%): mp >250 °C dec; ^{1}H NMR (DMSO-d_6) δ 3.65 (m, 12H, OCH_3), 7.48 (s, 2H, CH), 7.77 (d, 4H, ArH), 7.99 (d, 4H, ArH), 8.15 (s, 2H, ArH), 8.72 (s, 4H, ArH), 10.8 (s, 2H, NH); ^{13}C NMR (DMSO-d_6) 59, 124, 125, 127, 129, 131, 133, 136, 140, 141, 168, 169; ^{31}P NMR δ −7.32; MS (ESI) *m*/*z* 241 (M − 4H/4).

N,N′-Bis(isophthalyl)glutarate. 5-Aminoisophthalic acid (9.1 g, 5 $\times$ 10^{-2} mol) and 4-(dimethylamino)pyridine (5 $\times$ 10^{-3} mol) were dissolved in anhydrous *N,N*-dimethylacetamide (100 mL) under nitrogen. Glutaryl chloride (4.2 g, 2.5 $\times$ 10^{-2} mol) was added (via syringe). The mixture was stirred for 48 h and then transferred to a 500 mL beaker followed by addition of water (250 mL). White crystals were isolated after precipitation with the assistance of centrifugation at 5000*g* for 40 min. The crystals were washed (water) and precipitated three times. The final crystals were lyophilized (8.3 g, 73% yield): ^{1}H NMR (DMSO-d_6) δ 1.95 (q, J = 7.2 Hz, 2H, CH_2), 2.44 (t, J = 7.2 Hz, 4H, CH_2), 8.14 (s, 2H, ArH), 8.45 (s, 4H, ArH), 10.30 (s, 2H, NH), 13.2 (s, 4H, COOH); ^{13}C NMR (DMSO-d_6) 21.3, 36.1, 124.2, 125.1, 132.3, 140.5, 167.2, 172.0; MS (ESI) *m*/*z* 457.1 (M − H).

*N,N′-Bis[bis(sodium methyl phosphate)isophthalyl]glutarate (**3**).* *N,N′*-Bis(isophthalyl)glutarate (0.5 g, 1.1 $\times$ 10^{-3} mol) was refluxed in excess thionyl chloride (20 mL) for 72 h. Thionyl chloride was removed under vacuum, leaving a brown oil. Sodium dimethyl phosphate (0.68 g, 4.5 $\times$ 10^{-3} mol) was suspended in THF (10 mL) and added to acid chloride under nitrogen environment. The reaction mixture was stirred for 24 h. The solvent was evaporated under reduced pressure, leaving a yellow oil. This was immediately dissolved in dry acetone followed by addition of sodium iodide (0.68 g, 4.5 $\times$ 10^{-3} mol). The mixture was stirred under nitrogen for 72 h at room temperature. The resulting crystals were collected by vacuum filtration and washed with dry acetone (0.46 g, 45%): mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 1.89 (q, J = 7.3 Hz, 2H, CH_2), 2.51 (t, J = 7.3 Hz, 4H, CH_2), 3.38 (s, 12H, OCH_3), 8.08 (s, 2H, ArH), 8.40 (s, 4H, ArH), 10.25 (s, 2H, NH); ^{13}C NMR (DMSO-d_6) 21.5, 35.9, 48.7, 124.9, 126.1, 131.4, 139.3, 169.1, 173.0; ^{31}P NMR δ −2.85; MS (ESI) *m*/*z* 206 (M − 4H/4).

N,N′-Bis(isophthalyl)dodecanedioic Acid. Dodecanedioic acid (3 g, 1.30 $\times$ 10^{-2} mol) was refluxed in excess thionyl chloride (40 mL) for 10 h. Residual thionyl chloride was removed under vacuum. 5-Aminoisophthalic acid (4.73 g, 2.61 $\times$ 10^{-2} mol) and 4-(dimethylamino)pyridine (0.32 g, 2.6 $\times$ 10^{-3} mol) were dissolved in anhydrous dimethylacetamide (60 mL). This solution was combined with the acid chloride at 0 °C and stirred for 48 h at room temperature. Slightly acidic water (300 mL) was added to the solution to induce precipitation, giving a crystalline suspension. The crystals were precipitated using centrifugation (5000*g*, 90 min), washed with water three times, and air-dried (under a steam of air) overnight (5.83 g, 81% yield): ^{1}H NMR (DMSO-d_6) δ 1.32 (m, 12H, CH_2), 1.62 (q, J = 9.6 Hz, 4H, CH_2), 2.36 (t, J = 9.6 Hz, 4H, CH_2CO), 8.17 (s, 2H, ArH), 8.48 (s, 4H, ArH), 10.3 (s, 2H, NH), 12.5 (s, 4H, COOH); ^{13}C NMR (DMSO-d_6) 25.0, 28.5, 29.0, 30.0, 36.0, 124.1, 125.8, 131.5, 139.7, 168.4, 172.3; MS (ESI) *m*/*z* 555.5 (M − H).

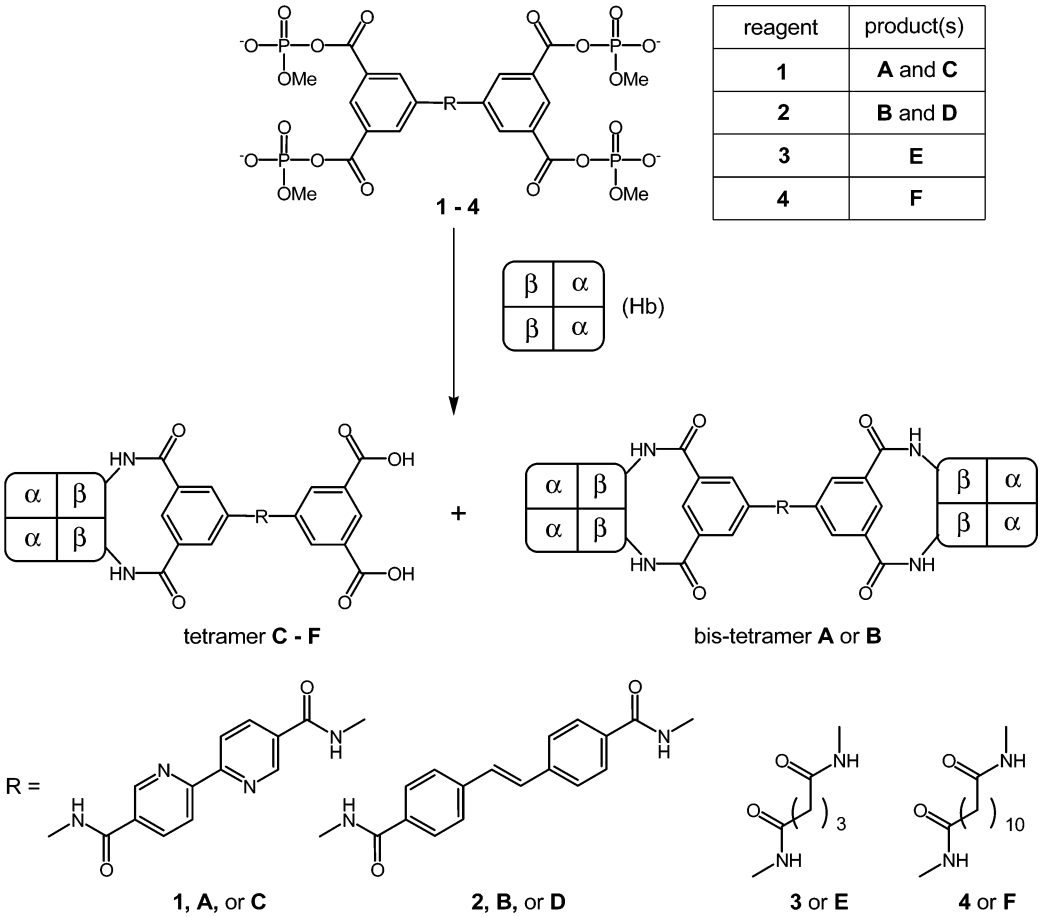
*N,N′-Bis[bis(sodium methyl phosphate)isophthalyl]dodecanedioic Acid (**4**).* *N,N′*-Bis(isophthalyl)dodecanedioic acid (0.6 g, 1.1 $\times$ 10^{-3} mol) was refluxed in excess thionyl chloride (20 mL) for 12 h. Thionyl chloride was removed under vacuum, leaving a yellow oil. Sodium dimethyl phosphate (0.96 g, 6.5 $\times$ 10^{-3} mol) was suspended in THF (30 mL) and added to acid chloride under nitrogen environment. The reaction mixture was stirred for 48 h. The mixture was vacuum filtered though a sintered glass funnel, and the filtrate was collected. The solvent was evaporated under reduced pressure, leaving a yellow oil. This was immediately dissolved in dry acetone followed by addition of sodium iodide (0.65 g, 4.3 $\times$ 10^{-3} mol). The mixture was stirred under nitrogen for 24 h at room temperature. The resulting crystals were collected by vacuum filtration and washed with dry acetone (0.71 g, 63%): mp >250 °C dec; ^{1}H NMR (DMSO-d_6) δ 1.31 (m, 12H, CH_2), 1.65 (q, J = 9.5 Hz, 4H, CH_2), 2.26 (t, J = 9.5 Hz, 4H, CH_2CO), 3.45 (s, 12H, OCH_3), 8.27 (s, 2H, ArH), 8.70 (s, 4H, ArH), 10.0 (s, 2H, NH); ^{13}C NMR (DMSO-d_6) 25.6, 29.1, 29.6, 29.9, 36.8, 52.1, 124.1, 124.6, 129.3, 138.7, 169.6, 175.1; ^{31}P NMR δ 0.82; MS (ESI) *m*/*z* 231 (M − 4H/4).

Cross-Linking. Carbonmonoxyhemoglobin (2.0 mL, 2.8 $\times$ 10^{-6} mol) was passed though a Sephadex G-25 column and eluted with 0.05 M sodium borate buffer (pH 8.5) at 4 °C. The collected hemoglobin sample was oxygenated under a stream of humidified oxygen in a rotating flask in an ice bath under illumination with a tungsten lamp for 2 h to give oxyhemoglobin. This sample was then deoxygenated under a stream of humidified nitrogen at 37 °C for 2 h to obtain deoxyhemoglobin. One equivalent of **1**, **2**, **3**, or **4** (2.8 $\times$ 10^{-6} mol) was added to the solution of deoxyhemoglobin (2.8 $\times$ 10^{-6} mol). The mixture was reacted for 24 h at 37 °C under a stream of humidified nitrogen. At the end of the reaction, carbon monoxide was blown over the reaction mixture for 20 min, followed by Sephadex G-25 chromatography, eluting with water. The resulting modified hemoglobin solution was stored at 4 °C.

HPLC Analysis of Cross-Linked Hemoglobin. Modified hemoglobins were analyzed according to the procedure developed by Jones (*18*). Analytical reversed-phase HPLC was employed using a 330 Å C-4 Vydac column (4.6 $\times$ 250 mm) to determine which globin chain of hemoglobin was modified. 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 in water. The effluent was monitored at 220 nm. The α-chains, β-chains, and modified β-chains were collected and recovered by lyophilization if necessary.

Purification of Bis-tetramers and Tetramers. Modified hemoglobins were isolated using a preparative size-exclusion column, Sephadex G-100 (superfine, 25 $\times$ 1500 mm). The samples were eluted with pH 7.4 Tris-HCl (0.025 M) containing magnesium chloride (0.5 M). The latter induces dissociation of non-cross-linked tetramers into $\alpha\beta$ dimers. The effluent was monitored at 420 nm. This method allows separation of tetrameric hemoglobin from the bis-tetramer. The collected samples were concentrated in Millipore Centriprep 50 concentrators and passed through Sephadex G-25 in a sodium phosphate buffer (0.1 M, pH 7.4). The collected

Scheme 1: General Reactions of Hemoglobin with Reagents



samples were placed under a stream of humidified carbon monoxide for 20 min and stored at 4 °C. Each fraction was analyzed using the size-exclusion FPLC, Superdex G-75 HR (10 × 300 mm) column. The samples were eluted with Tris-HCl (0.025 M) and magnesium chloride (0.5 M) buffer (pH 7.4), monitored at 280 nm. The fraction corresponding to 128 kDa (containing a cross-linked bis-tetramer) was oxygenated for 2 h under tungsten light and assayed for its oxygen-binding properties.

SDS−PAGE Analysis of Bis-tetramers and Tetramers. The reaction mixture and purified bis-tetramers were analyzed by SDS−PAGE. Protein standards, reaction samples, and native Hb were prepared by mixing a protein sample (2−15 μL) with loading buffer (15−25 μL) consisting of 0.0625 M Tris-HCl (pH 6.8), 1.3 M glycerol, 2% SDS, 0.0125 (w/v) bromophenol blue, and 0.7 M β-mercaptoethanol. The samples were denatured at 95 °C for 15 min, and 10−15 μL was loaded onto a polyacrylamide gel (12% Tris-HCl). The gel was run in a dual-slab cell apparatus at 200 mV in 0.12 M Tris-HCl, 1 M glycine, and 0.014 M SDS running buffer. The gels were stained with Coomassie Brilliant Blue R-250 followed by destaining with 30% methanol and 10% acetic acid solution.

Tryptic Peptide Digest Followed by MALDI-MS Analysis. Modified β-chains from cross-linked Hb and unmodified β-chains from native Hb were separately collected from a reversed-phase C4 preparative column using the HPLC technique described above. The samples were lyophilized, leaving the dry denatured protein chains. The chains were dissolved in 8 M urea to which fresh trypsin solution was added (4% of total mass of Hb). The solution was diluted with 0.080 M ammonium bicarbonate (pH = 8.5) to give a final urea concentration of 2 M and allowed to react for 24 h. The tryptic hydrolase then was heated to 95 °C for 10 min and stored at −10 °C before MALDI mass spectroscopy analysis.

The molecular weight fragments from the digest were analyzed by MALDI-TOF mass spectroscopy (*19*) using a 2 μL sample of the above mixture by evaporation on an ionization tray after addition of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) to each sample.

RESULTS

Tetrafunctional reagents **1**, **2**, **3**, and **4** were prepared following the general procedure we reported previously based on coupling of two *m*-aminoisophthalic acids with a diacid chloride followed by conversion to acyl phosphate monoesters (*5*). Thus, reagents **1** and **2** contain an amide linked to an aromatic core with two pairs of acyl phosphate monoester reaction sites. This design yields a predictable span between reacting pairs of methyl isophthaloyl phosphates with a core structure that resists folding, due to its constituent hybridization (*20*). The distances between the two most widely separated acyl groups of **1** and **2** are 20 and 23 Å, respectively, determined by molecular mechanics energy minimization. Reagents **3** and **4** contain a flexible hydrocarbon chain between the reactive ends. While derivatives of **3** and **4** have the potential to extend as far as 15 and 24 Å (respectively), the chain structure is flexible and dynamic.

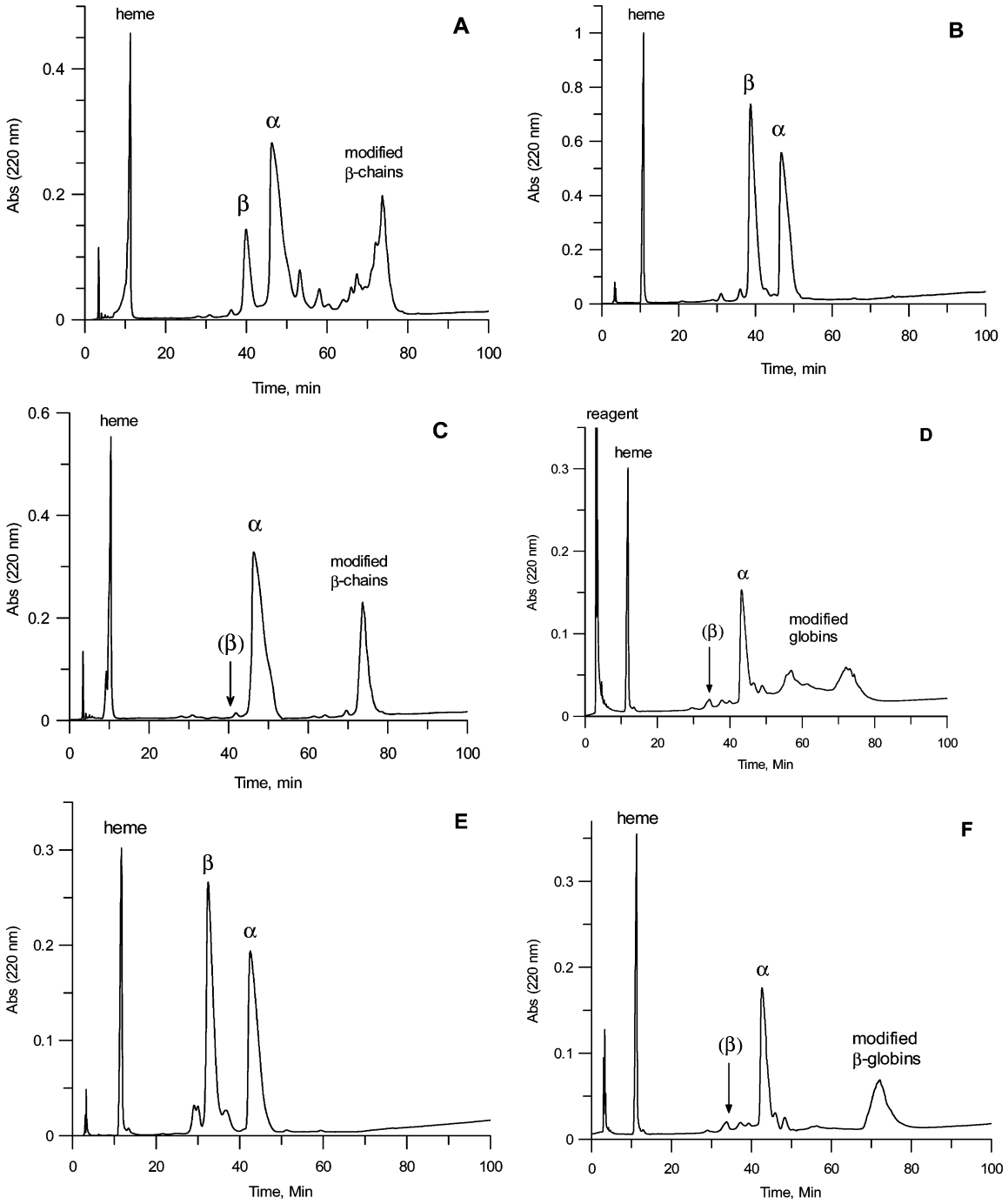


FIGURE 1: C-4 reversed-phase HPLC of the reaction mixture of Hb with reagent **1** (A), unmodified Hb control (B), and purified bis-tetramer **A** (C). Reaction mixture of Hb with reagent **3** (D), unmodified Hb control (E), and purified tetramer **E** (F).

Reaction of hemoglobin with reagents **1** and **2** produced bis-tetrameric hemoglobins, comprised of two cross-linked tetramers, as well as tetrameric hemoglobin, comprised of a single cross-linked tetramer (Scheme 1), while the flexible hydrocarbon reagents gave cross-linked tetramers. The highest yield of bis-tetramers was achieved using equimolar reagent (**1** or **2**) and hemoglobin, while cross-linked tetramers were obtained with excess reagent.

The β-subunit of hemoglobin was identified as the site of modification through reversed-phase HPLC chromatographic analysis (Figure 1) of the reaction product. The modified hemoglobin product was eluted after the α and β globin chains.

Cross-linked bis-tetramers and tetramers were isolated by gel filtration chromatography using conditions that facilitate dissociation of hemoglobin into $\alpha\beta$ dimers (*21*). At high magnesium chloride concentrations, only species that have a covalent cross-link between $\alpha\beta$ dimers maintain their quaternary structure and can be separated from individual $\alpha\beta$ dimers.

Three species were separated and collected: 128, 64, and 32 kDa, corresponding to bis-tetramers, tetramers, and $\alpha\beta$ dimers, respectively (Figures 2 and 3, left). Purified bis-tetramers (**A** and **B)** and tetramers (**C** and **D**) were compared to native protein and the original reaction mixture for molecular species content (Figures 2 and 3, right). When

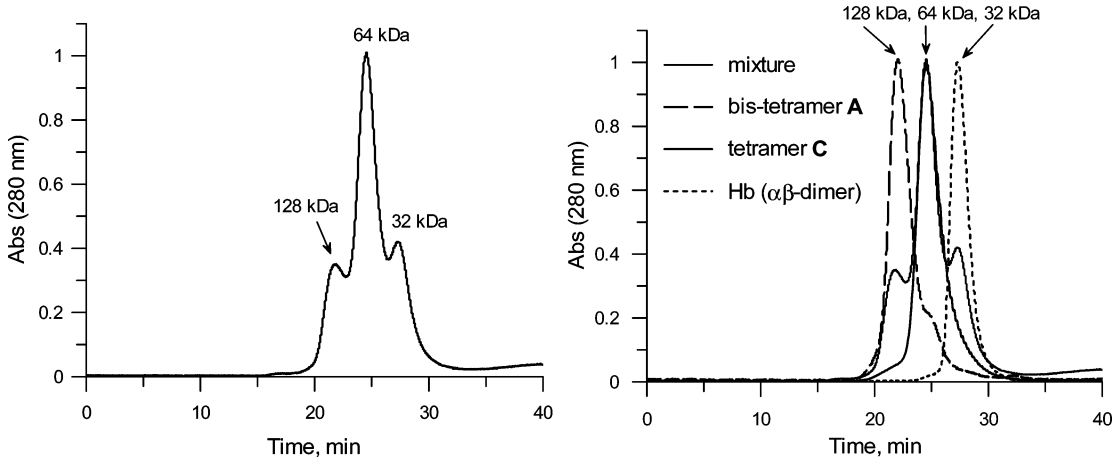


FIGURE 2: Superdex G-75 gel filtration chromatograms of Hb reacted with reagent **1** (left) compared to purified bis-tetramer **A**, tetramer **C**, and native Hb (right).

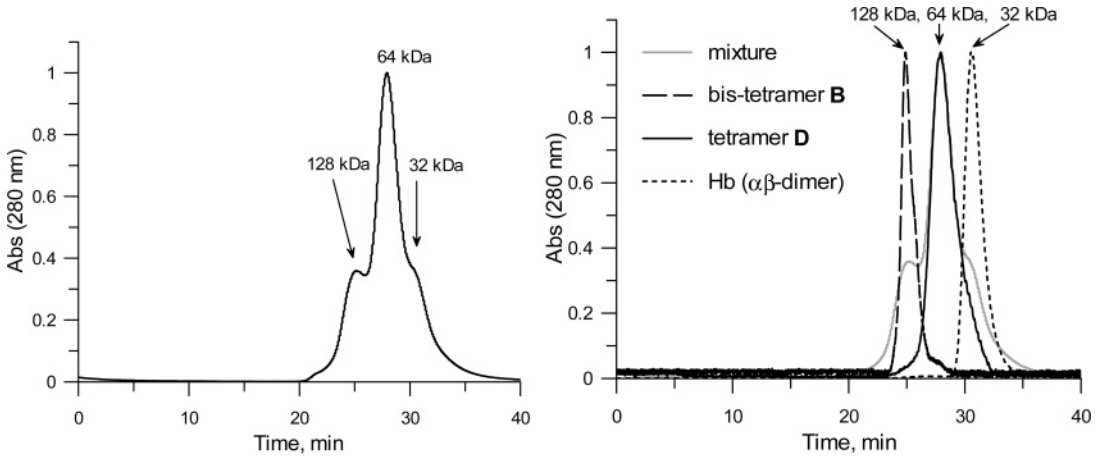


FIGURE 3: Superdex G-75 gel filtration chromatograms of Hb reacted with reagent **2** (left) compared to purified bis-tetramer **B**, tetramer **D**, and native Hb (right).

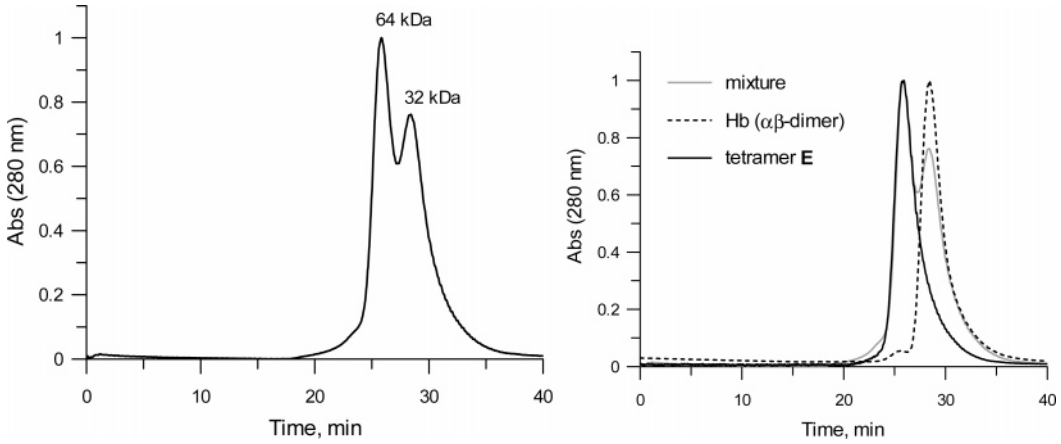


FIGURE 4: Superdex G-75 gel filtration chromatograms of Hb reacted with reagent **3** (left) compared to purified tetramer **E** and native Hb (right).

reaction mixtures of Hb with reagent **3** and **4** were analyzed by gel filtration chromatography, a single new species (64 kDa) was observed corresponding to cross-linked tetramers **E** and **F** (Figures 4 and 5, left). Fractions containing cross-linked tetramers were separated (Figures 4 and 5, right) and used for oxygen-binding measurements.

SDS−PAGE analysis allows separation and detection of the constituent protein chains based on molecular mass. Covalently cross-linked chains appear as species with higher mass and can therefore be detected. Native hemoglobin contains α-chains and β-chains (16 kDa) that produce a single band on the gel (Figure 6, lane 2). When native hemoglobin was reacted with reagent **1** or **2**, two distinct bands, 32 and 64 kDa, appear in addition to the 16 kDa band (Figure 6, lane 3). Reaction of reagents **3** and **4** with hemoglobin gave only one new band corresponding to 32

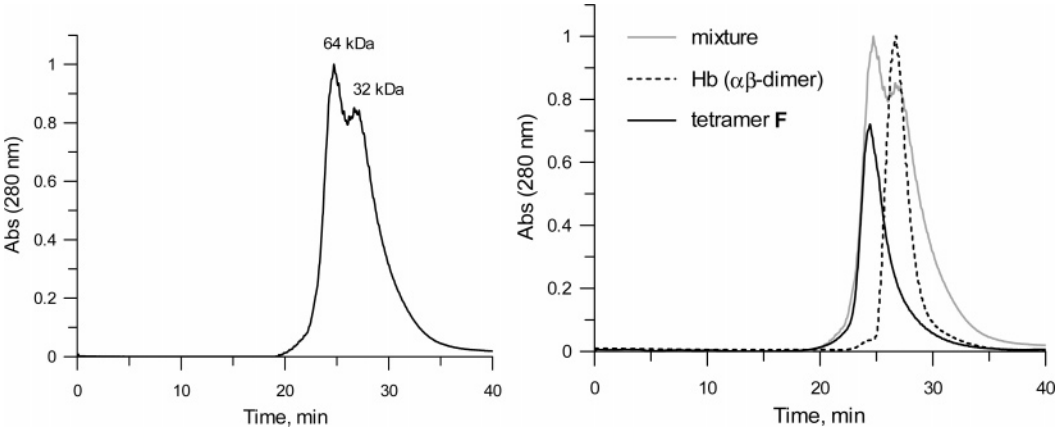


FIGURE 5: Superdex G-75 gel filtration chromatograms of Hb reacted with reagent **4** (left) compared to purified tetramer **F** and native Hb (right).

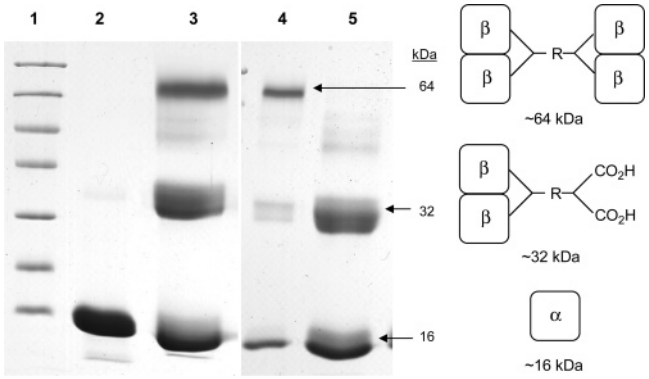


FIGURE 6: SDS−PAGE analysis of the product after reaction with reagent **2** (lane 3). Purified bis-tetramer **B** (lane 4) and separated tetramer **D** (lane 5) are compared to native hemoglobin (lane 2) and protein standards (lane 1: 14, 18, 25, 35, 45, 66, and 116 kDa).

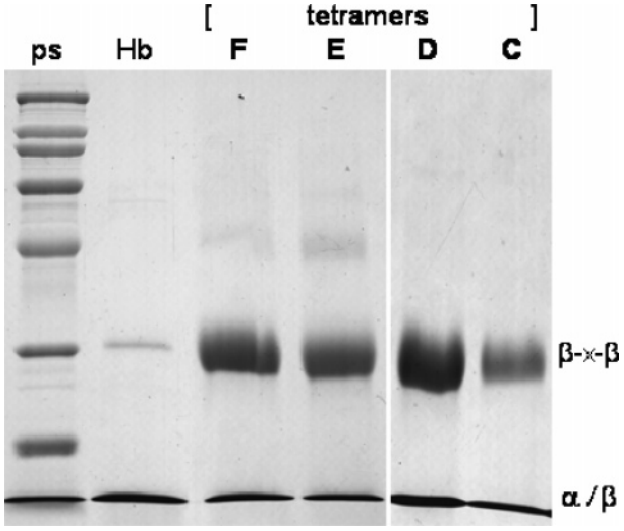


FIGURE 7: SDS−PAGE analysis of purified tetramers **C**−**F** compared to native protein (Hb) and calibration standards (14, 21, 31, 45, 66, 97, 116, and 200 kDa).

kDa. This confirms that reagents **1** and **2** can simultaneously connect two and four β-chains, respectively, and reagents **3** and **4** connect only two β-chains.

Bis-tetramers **A** and **B** were subjected to gel electrophoresis, which showed two fragments, 64 kDa (bis-tetramers) and 16 kDa (α-chains) (Figure 6, lane 4). The 64 kDa fragment is consistent with four β-subunits being connected by the reagent while α-subunits are unattached and separate on the gel as the 16 kDa band. Cross-linked tetramer **D** was successfully separated from bis-tetramer **B** (shown in lane 5 of Figure 6).

The isolated tetramers **C**−**F** were subjected to electrophoresis to confirm the covalent connection between two β-chains (Figure 7).

The site of chemical modification was identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The modified β-chains of bis-tetramer **B** and tetramer **E** were isolated using reversed-phase chromatography and subjected to tryptic digest followed by MALDI-TOF MS analysis. These gave the digest maps shown in Figures 8 and 9, respectively. The results indicate that molecular fragments corresponding to the T9 peptide ($m/z = 1670$) and uncut T9 + T10 peptide ($m/z = 1799$) are missing in the modified β-chain digests compared to the unmodified β-chains (Figure 10). The presence of amide linkages prevents trypsin from hydrolyzing the peptide bond between the T9 and T10 chains. This confirms that the acyl phosphate reagents **2** and **3** react with ϵ-amino groups of lysyl residue 82 of the β-chains.

The oxygen-binding properties of bis-tetrameric hemoglobins (**A** and **B**) and cross-linked tetramers (**C**−**F**) are summarized in Table 1. Oxygen affinity (P_{50}) was determined from fitting to the Adair equation. Bis-tetramers **A** and **B** have slightly higher oxygen affinities (**A**, $P_{50} = 4.2$; **B**, $P_{50} = 4.5$) compared to native Hb ($P_{50} = 5.0$). The oxygen affinities of tetramers **C** and **D** (**C**, $P_{50} = 4.0$; **D**, $P_{50} = 4.8$) are also similar. Thus, the R-T state energy difference is not affected by the modifications. Tetramer **E** has the same oxygen affinity ($P_{50} = 4.8$), while tetramer **F** has an increased affinity ($P_{50} = 3.8$). The cooperativity index was obtained from a Hill plot. The bis-tetramers **A** and **B** show little cooperativity (**A**, $n_{50} = 1.4$; **B**, $n_{50} = 1.2$) while native hemoglobin is highly cooperative. Tetramers **C**−**F** retain moderate cooperativity (**C**, $n_{50} = 2.0$; **D**, $n_{50} = 1.8$; **E**, $n_{50} = 1.7$; **F**, $n_{50} = 1.8$). Typical curves for the various classes are shown in Figures 11 and 12.

DISCUSSION

Cross-linked bis-tetrameric hemoglobins were successfully prepared from reagents **1** and **2**. These form along with considerable amounts of cross-linked tetramers. The reaction

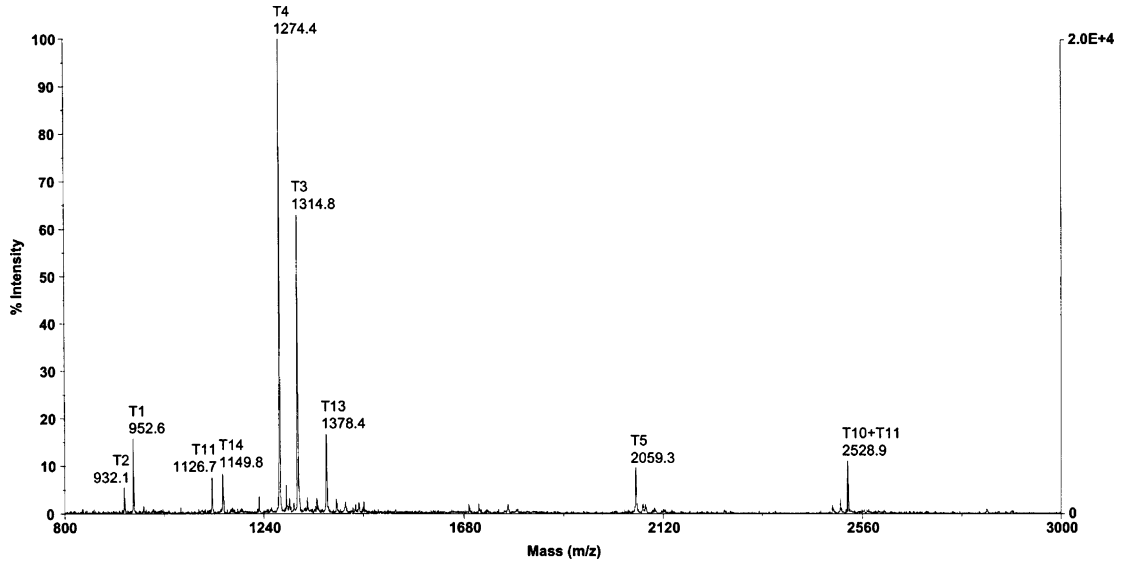


FIGURE 8: MALDI-TOF MS of the tryptic digest from modified β-chains with reagent **2**.

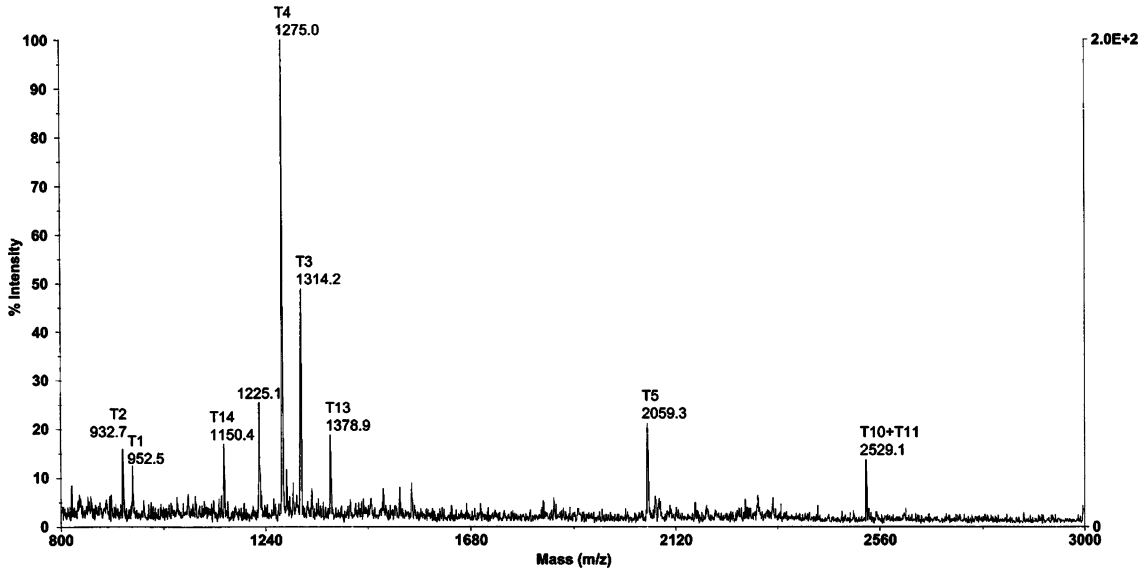


FIGURE 9: MALDI-TOF MS of the tryptic digest from modified β-chains with reagent **3**.

Table 1: Oxygen-Binding Properties of Purified Cross-Linked Bis-tetramers and Tetramers of Hemoglobin

sample	P_{50} (Torr)	n_{50}	span (Å)[a]
bis-tetramer **A**	4.2 (±0.1)	1.4 (±0.1)	20
bis-tetramer **B**	4.5 (±0.1)	1.2 (±0.1)	23
tetramer **C**	4.0 (±0.1)	2.0 (±0.1)	n/a
tetramer **D**	4.8 (±0.1)	1.8 (±0.1)	n/a
tetramer **E**	4.6 (±0.1)	1.7 (±0.1)	n/a
tetramer **F**	3.8 (±0.1)	1.8 (±0.1)	n/a
native Hb	5.0 (±0.1)	3.0 (±0.1)	n/a

[a] Distance is measured between two newly formed CONH linkages on opposite ends of the bis-tetramers in models.

with free amines is faster than hydrolysis, but concentrations are intentionally maintained low to avoid over-reaction. The complication from hydrolysis has been noted in previous cases of hemoglobin modification with multifunctional reagents (*4*, *5*, *12*). However, the bis-tetrameric species were readily purified to provide sufficient quantities for full characterization.

One goal of this study was to determine if there exists an empirical relationship between a property of the cross-linker's core structure and oxygen-binding properties of the corresponding bis-tetramers. In our earlier study we reported bis-tetramers that have relatively low cooperativity in oxygen binding, which might be related to interactions between the two tetramers (*5*). In the present study, the bis-tetramers (**A** and **B**) are separated by a longer span. Yet, these species exhibit little or no cooperativity in oxygen binding and have similar oxygen affinities to the species with shorter linkages, showing that protein−protein interactions are still maintained.

Cross-linking hemoglobin with a small trifunctional reagent gives species with high cooperativity in oxygen binding ($n_{50} = 2.6$) (*8*). Since extending the span between tetramers did not lead to a product with significant cooperativity, we investigated the properties of cross-linked tetramers (**C** and **D**) containing the extended link without a second protein moiety. These cross-linked tetramers have reduced, but significant, cooperativity. The solvation of Hb by $\alpha\beta$ dimer interfacial water molecules has been suggested by the

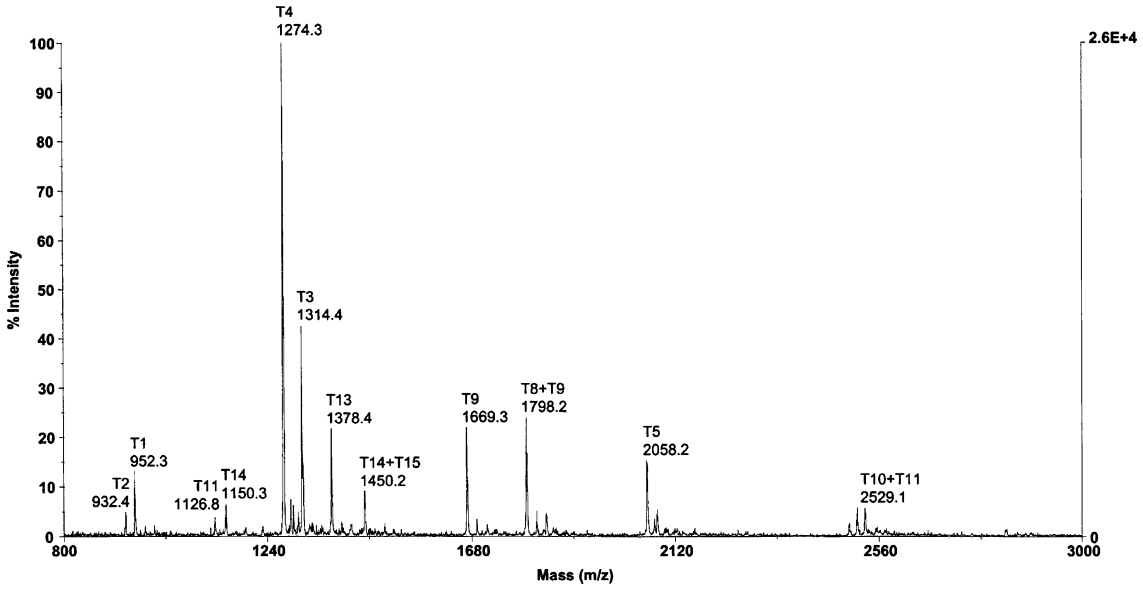


FIGURE 10: MALDI-TOF MS of the tryptic digest from β-chains of native Hb.

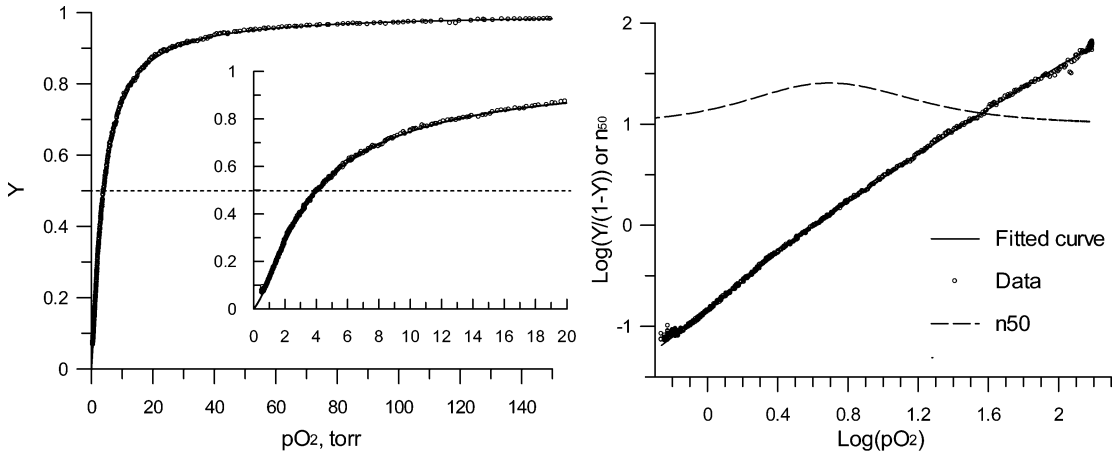


FIGURE 11: Oxygen saturation curve (left) and Hill plot (right) of bis-tetramer **A**. The inset shows enlargement for clarity.

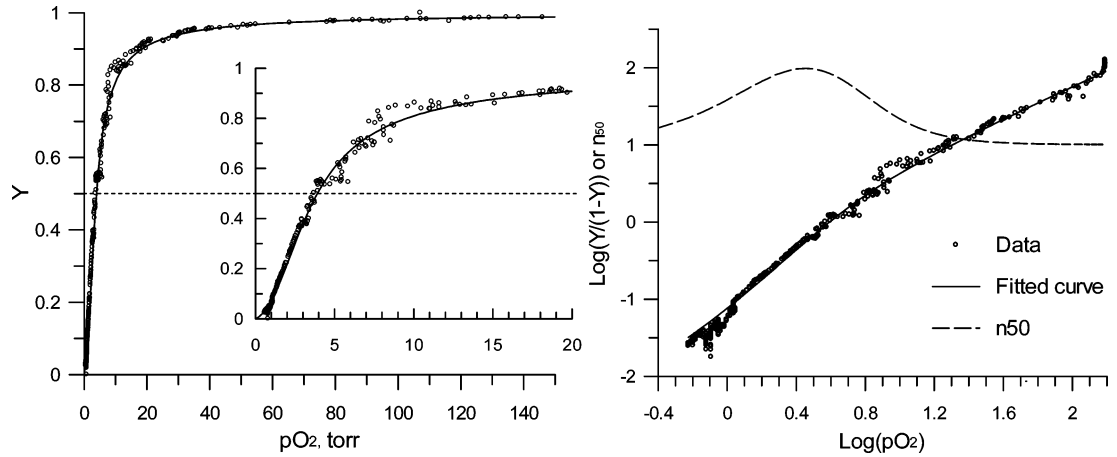


FIGURE 12: Oxygen saturation curve (left) and Hill plot (right) of tetramer **C**. The inset shows enlargement at $Y = 0.5$ for clarity.

calculations of Karplus and co-workers to be essential for cooperative oxygen binding and release (*22*). The disruption of solvation by a larger reagent may be more significant here.

Extending the cross-linking reagents with a flexible saturated hydrocarbon chain (**3** and **4)** predictably produces cross-linked tetramers (**E** and **F**) rather than bis-tetramers. The properties of these tetramers are similar to those of **C** and **D**, indicating that neither the specific nature of the chain nor its length controls the properties of the protein.

Our results give insights to the changes seen in heterogeneously linked hemoglobins. Palmer's analysis of materials separated on the basis of aggregate size indicates the overall

properties of the mixture (*23*). A decrease in cooperativity ($n_{50} = 1.2-1.5$) (*23*) is typically observed in hemoglobin that has been cross-linked and oligomerized with glutaraldehyde and is consistent with the effect of the proteins being closely associated.

Recently, Su and co-workers reported bis-tetrameric bovine hemoglobin that was prepared selectively using glutaraldehyde in combination with column-adsorbed bovine hemoglobin. The material has an increased oxygen affinity and significant cooperativity ($n_{50} = 1.72$) (*24*). The Hill coefficient is larger than we see in the structurally defined human hemoglobin bis-tetramers (*4*, *5*). While much of the evidence for the production of the material in Su's paper is convincing, it is difficult to know if they actually obtained bis-tetrameric species according to their results: the reported PAGE labeled for the bis-tetramer does not show the band that would correspond to four cross-linked subunits from two tetramers held by covalent bonds, which would be essential in establishing that structure.

Our results suggest that the cooperativity is reduced severely in cross-linked bis-tetramers relative to cross-linked tetramers in general. The oxygen affinity is increased in cross-linked hemoglobins as seen in β82-β'82 cross-linked hemoglobins in general. The resulting hyperbolic oxygen-binding and release curve in the bis-tetramer means that oxygen release occurs over a narrow oxygen saturation range at low oxygen pressures. The bis-tetramers will acquire oxygen more readily and hold it more tightly in circulation, only being near 50% saturation under very hypoxic conditions. Such a material might well be a safe and useful clinical entity (*25*). It would allow the materials to function as an oxygen source of last resort compared to red cells, which might be a successful strategy for emergency medicine. High oxygen affinity can also explain the low vasoactivity of the glutaraldehyde-derived products. The heme binding sites would be occupied by oxygen in circulation, reducing potential competition with nitric oxide. These materials would not cause a rapid release of oxygen in the larger blood vessels, avoiding vasoactivity that may be the result of a homeostatic response (although they would not limit diffusive loss). These results suggest that the low vasoactivity of the oligomeric Hb species may be the direct result of low oxygen-binding cooperativity and high oxygen affinity of each of the constituent entities. Thus, loss of cooperativity accompanies oligomerization, even with a greater separation between tetramers. Detailed thermodynamic analysis (*10*, *26*) of these bis-tetrameric arrays should provide important insights into the precise origins of the changes we observe.

Our results demonstrate that the most important factor in reducing the cooperativity of hemoglobin in these materials is the presence of a second hemoglobin, which is held by covalent attachment. The nature of the link between tetramers does not lead to a change in the properties of the tetramers as it does in the case of the link within tetramers. Furthermore, the link itself without the second tetramer has relatively little effect upon the properties of the modified hemoglobin. This is consistent with linked proteins interacting to reduce the cooperative properties of one another.

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