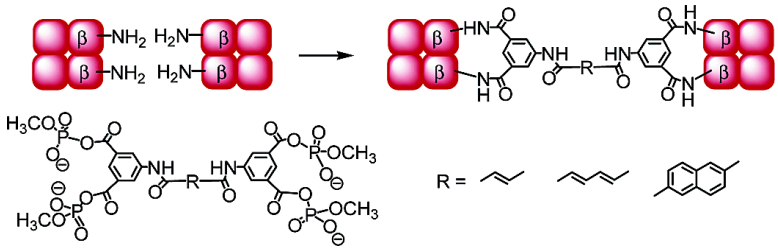
Article

Cross-Linked Bis-hemoglobins: Connections and Oxygen Binding

Nikolai Gourianov, and Ronald Kluger

J. Am. Chem. Soc., **2003**, 125 (36), 10885-10892• DOI: 10.1021/ja036596i • Publication Date (Web): 15 August 2003



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 2 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 08/15/2003

Cross-Linked Bis-hemoglobins: Connections and Oxygen Binding

Nikolai Gourianov and Ronald Kluger*

Contribution from the Davenport Chemical Laboratories, Department of Chemistry, University of Toronto, Toronto, Ontario, Canada M5S 3H6

Received June 10, 2003; E-mail: rkluger@chem.utoronto.ca

Abstract: Covalently linked pairs of cross-linked hemoglobin tetramers ("bis-tetramers", shown schematically as **6**−**8**) were prepared by reacting hemoglobin A with tetrakis acyl phosphate esters (**3**−**5**). The effects of the link between tetramers are observed in the oxygen-binding properties of the bis-tetramers: they bind oxygen cooperatively but with Hill coefficients (n_{50}) lower than that of the native protein and with a high average affinity. The bis-tetramers with longer connections between tetramers show a higher n_{50}, suggesting that steric interactions between the tetramers affect cooperativity. These results correlate to the observed reduced vasoactivity of heterogeneous solutions of oligomeric cross-linked hemoglobin tetramers.

Bifunctional and trifunctional anionic acylating agents have been used to introduce cross-links selectively between amino groups in cationic regions of hemoglobin. The resulting stabilized tetramers bind oxygen reversibly with altered oxygen affinity and with significant cooperativity.[1−3] Systematic variation of the span of these rigid links within the cross-linked tetramers leads to a predictable variation in the free energy of binding of oxygen.[4,5] Crystallographic analysis revealed that the resulting structures are closely related to the transitional structures proposed by Perutz to explain the R to T transition that hemoglobin undergoes upon release of oxygen.[6,7] We have been interested in extending the concept of systematic cross-linking to produce structurally defined connections between two tetramers. Since the tetramers are subject to separation into dimers, we sought reagents that also introduce intratetramer cross-links. Such entities can report changes in physical properties that result from interactions between proteins that are defined by the chemical linkage.

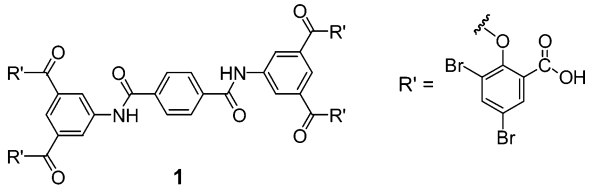
Another motivation for the design of such reagents is the suggestion that connected hemoglobins should have improved characteristics as circulating oxygen carriers (red cell substitutes) compared to cross-linked tetramers, which in clinical studies have shown significant undesirable vasoactivity.[8−12] Many of these results come from studies of heterogeneous cross-linked hemoglobin materials that are thought to contain bis-tetramers and other variously linked oligomers in addition to tetramers.[13−17] It has also been reported that vasoactivity decreases in cross-linked hemoglobin that is connected to poly(ethylene glycol) (PEG) compared to unconjugated cross-linked hemoglobin species, with the size of the material being an important consideration.[10,18,19] Thus, we prepared cross-linked bis-tetramers as structurally defined hemoglobin oligomers to determine the biophysical effects of total size and connecting linkages.

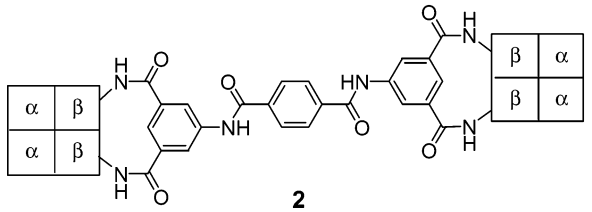
The first example of a specific intramolecular−intermolecular cross-linking reagent was *N*,*N*′-5,5′-bis[bis-(3,5-dibromosalicyl)-isophthalyl]terephthalamide, 1.[20] This tetrafunctional reagent contains two spanned pairs of reaction sites. Reaction with amino groups of lysyl (β-82) residues of human hemoglobin produces a number of separable products, including the cross-linked bis-tetramer of hemoglobin, shown schematically as **2**.

The product (**2**) binds and releases oxygen without the normal cooperative interactions of hemoglobin ($n_{50} = 1.3$). Materials with the same internal cross-link but lacking the intertetramer link bind oxygen with n_{50} close to that of the native protein. Because the span of the bridge between the tetramers in **2** is

(1) Schumacher, M. A.; Dixon, M. M.; Kluger, R.; Jones, R. T.; Brennan, R. G. *Nature (London)* **1995**, *375*, 84−87.
(2) Kluger, R.; Jones, R. T.; Shih, D. T. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **1994**, *22*, 415−428.
(3) Jones, R. T.; Head, C. G.; Fujita, T. S.; Shih, D. T. B.; Wodzinska, J.; Kluger, R. *Biochemistry* **1993**, *32*, 215−223.
(4) Bucci, E.; Gryczynski, Z.; Razynska, A.; Kwansa, H. *Biophys. J.* **1998**, *74*, 2638−2648.
(5) Ji, X.; Braxenthaler, M.; Moult, J.; Fronticelli, C.; Bucci, E.; Gilliland, G. L. *Proteins: Struct., Funct., Genet.* **1998**, *30*, 309−320.
(6) Perutz, M. F. *Q. Rev. Biophys.* **1989**, *22*, 139−237, 138 plates.
(7) Perutz, M. F.; Fermi, G.; Luisi, B.; Shaanan, B.; Liddington, R. C. *Acc. Chem. Res.* **1987**, *20*, 309−321.
(8) Schultz, S. C.; Grady, B.; Cole, F.; Hamilton, I.; Burhop, K.; Malcolm, D. S. *J. Lab. Clin. Med.* **1993**, *122*, 301−308.
(9) Krishnamurti, C.; Alving, B. M. *Blood Sub.* **1996**, 99−111.
(10) Abassi, Z.; Kotob, S.; Pieruzzi, F.; Abouassali, M.; Keiser, H. R.; Fratantoni, J. C.; Alayash, A. I. *J. Lab. Clin. Med.* **1997**, *129*, 603−610.
(11) Erhart, S. M.; Cole, D. J.; Patel, P. M.; Drummond, J. C.; Burhop, K. E. *Artif. Cell Blood Substitutes* **2000**, *28*, 385−396.
(12) Freilich, E.; Freilich, D.; Hacker, M.; Leach, L.; Patel, S.; Hebert, J. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **2002**, *30*, 83−98.
(13) Winslow, R. M. *Vox Sang.* **2000**, *79*, 1−20.
(14) Yu, Z.; Friso, G.; Miranda, J. J.; Patel, M. J.; Lo-Tseng, T.; Moore, E. G.; Burlingame, A. L. *Protein Sci.* **1997**, *6*, 2568−2577.
(15) Kavanaugh, M. P.; Shih, D. T. B.; Jones, R. T. *Acta Haematol.* **1987**, *78*, 99−104.
(16) Looker, D.; Abbott-Brown, D.; Cozart, P.; Durfee, S.; Hoffman, S.; Mathews, A. J.; Miller-Roehrich, J.; Shoemaker, S.; Trimble, S. *Nature (London)* **1992**, *356*, 258−260.
(17) Ogden, J. E. *Trends Biotechnol.* **1992**, *10*, 91−96.
(18) McCarthy, M. R.; Vandegriff, K. D.; Winslow, R. M. *Biophys. Chem.* **2001**, *92*, 103−117.
(19) Winslow, R. M.; Gonzales, A.; Gonzales, M. L.; Magde, M.; McCarthy, M.; Rohlfs, R. J.; Vandegriff, K. D. *J. Appl. Physiol.* **1998**, *85*, 993−1003.
(20) Kluger, R.; Lock-O'Brien, J.; Teytelboym, A. *J. Am. Chem. Soc.* **1999**, *121*, 6780−6785.

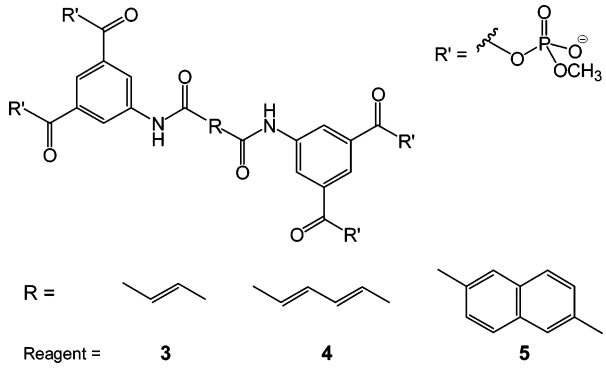
10.1021/ja036596i CCC: $25.00





relatively short, the bis-tetramer will have significant internal interactions that can affect its conformation and solvation, perhaps leading to reduced cooperativity. Thus, we sought to vary the nature of the connection between the tetramers to determine the effects of the chemical nature of the linkage on the protein's oxygen-binding properties.

Reagent **1** utilized Klotz's dibromosalicyl ester group to react selectively with amino groups in Hb.[21,22] However, we found that reagents that combine this leaving group with larger hydrophobic cores were not soluble in water. Since it had previously been demonstrated that acyl phosphate monoesters have similar reaction and site specificity,[23] we reasoned that this ionic functional group would be better at maintaining water solubility. Therefore, we designed reagents that are tetrakis acyl phosphate monoesters, coupled in pairs for cross-linking and connected by a relatively rigid linker. The rigidity of the connection avoids the problem of the reagents folding onto themselves as seen with completely flexible reagents,[24] while defining structural relationships. The acyl phosphate monoesters are hydrophilic enough to induce water solubility of the reagents, even those with highly hydrophobic cores.[15]



We have prepared the tetrafunctional (doubly paired) cross-linking reagents **3**, **4**, and **5** and studied their reaction with hemoglobin. From those reactions we have isolated cross-linked bis-tetramers and assessed the oxygen-binding properties of these novel species.

Experimental Section

Materials and Methods. 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 obtained at 75 or 100.5 MHz. Phosphorus NMR spectra were obtained at 121.5 MHz. Molecular modeling was performed using HyperChem 5 (Hypercube, Inc.). Solutions of human hemoglobin were provided by Hemosol, Inc. or were prepared from donated red cells. Oxygen-binding curves for modified hemoglobins were measured using an apparatus based on that of Imai[25] as modified by Shih and Jones.[26]

***N,N′*-Bis(isophthalyl)fumarate.** 5-Aminoisophthalic acid (7.9 g, 4.4 $\times$ 10^{-2} mol) and 4-(dimethylamino)-pyridine (0.6 g, 5 $\times$ 10^{-3} mol) were dissolved in anhydrous *N*,*N*-dimethylacetamide (100 mL) under argon. Fumaryl chloride (3.06 g, 2.2 mL, 2.0 $\times$ 10^{-2} mol) was added (via syringe). The mixture was stirred for 48 h (dark yellow), then transferred to a 1 L beaker to which 500 mL water was added. Fluffy white crystals were isolated after centrifugation at 9000g for 40 min. The crystals were washed 3 times with water and collected. The crystals were lyophilized (6.0 g, 68% yield). mp: 210 °C (dec). ^{1}H NMR (DMSO-d_6): δ 10.92 (s, 2H, CONH), 8.51 (s, 4H, ArH), 8.18 (s, 2H, ArH), 7.19 (s, 2H, CH−C═O); ^{13}C NMR (DMSO-d_6): δ 165, 162, 135, 132, 123, 124, 120; MS (ESI): 441 (found), 441 (M − H$^-$ calculated for $C_{20}H_{14}N_2O_{10}$).

***N,N′*-Bis[bis(sodium methyl phosphate)isophthalyl]fumarate (3).** *N*,*N*′-Bis(isophthalyl)fumarate (0.50 g, 1.13 $\times$ 10^{-3} mol) was combined with an excess of thionyl chloride (16 mL) under nitrogen and refluxed for 16 h (brown solution). Thionyl chloride was removed by vacuum distillation, leaving the acid chloride as a dark solid (0.50 g, 9.7 $\times$ 10^{-4} mol, 86% yield) that was not purified. This was combined with sodium dimethyl phosphate (0.645 g, 4.36 $\times$ 10^{-3} mol) (prepared in dry acetone from trimethyl phosphate and sodium iodide)[27] in dry THF (50 mL). The mixture was stirred for 20 h (dark brown). THF was evaporated, giving an oil that was pumped under vacuum for 3 h. Sodium iodide (0.46 g, 3.1 $\times$ 10^{-3} mol) was combined with the crude product in dry acetone (50 mL) under nitrogen and the solution was stirred for 24 h. Off-white crystals were collected by vacuum filtration and washed with dry acetone (0.65 g, 74% yield). mp: >250 °C (dec). ^{1}H NMR (DMSO-d_6): δ 11.1 (s, 2H, CONH), 8.5 (s, 4H, ArH), 8.2 (s, 2H, ArH), 7.25 (s, 2H, CH), 3.5 (s, OCH_3); ^{13}C NMR (DMSO-d_6): δ 162, 160, 136, 130, 124, 123, 120, 57; ^{31}P NMR (DMSO-d_6): δ −5.82 (decoupled); MS (ESI): 205 (found), 204 (M − 4H^{4-} calculated as m/z = 814/4 for species without Na^+).

***N,N′*-Bis(isophthalyl)*trans,trans*-muconate**. *trans*,*trans*-Muconic acid (0.30 g, 2.11 $\times$ 10^{-3} mol) was combined with an excess of thionyl chloride (20 mL) in a round-bottom flask containing a stirring bar and a condenser. The mixture was refluxed for 96 h, producing a dark brown solution of the acid chloride. Thionyl chloride was removed by distillation. 5-Amino-isophthalic acid (1.22 g, 6.75 $\times$ 10^{-3} mol) and 4-(dimethylamino)-pyridine (0.11 g, 0.88 $\times$ 10^{-3} mol) were dissolved in anhydrous DMF (20 mL) and cooled to 0 °C. *Trans*,*trans*-muconate chloride was cooled to 0 °C and combined with the solution of 5-amino-isophthalic acid and 4-(dimethylamino)-pyridine (dark brown solution). The mixture was stirred overnight at room temperature. The solvent was removed by vacuum distillation, and water (100 mL) was added to the product, giving a suspension of light brown crystals. The crystals were precipitated by centrifugation (4500g, 30 min), the supernatant was removed, and the crystals were washed with water (3 $\times$ 100 mL). The crystals were lyophilized overnight and rinsed with dry acetone

(21) Walder, J. A.; Zaugg, R. H.; Walder, R. Y.; Steele, J. M.; Klotz, I. M. *Biochemistry* **1979**, *18*, 4265−4270.
(22) Walder, J. A.; Zaugg, R. H.; Iwaoka, R. S.; Watkin, W. G.; Klotz, I. M. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5499−5503.
(23) Kluger, R.; Grant, A. S.; Bearne, S. L.; Trachsel, M. R. *J. Org. Chem.* **1990**, *55*, 2864−2868.
(24) Kluger, R.; Paal, K.; Adamson, J. G. *Can. J. Chem.* **1999**, *77*, 271−279.
(25) Imai, K. *Methods Enzymol.* **1981**, *76*, 438−449.
(26) Shih, D. T.; Jones, R. T. *Methods Enzymol.* **1986**, *15*, 124−141.
(27) Zervas, L.; Dilaris, I. *J. Am. Chem. Soc.* **1953**, *77*, 5354−5365.

(3 × 20 mL), (0.42 g, 42% yield). mp: >200 °C. ^{1}H NMR (DMSO-d_6): δ 13.2 (COOH), 10.64 (s, 2H, CONH), 8.5 (s, 4H, ArH, $C_{6,4}$), 8.2 (s, 2H, ArH, C_2), 7.4 (m, 2H, α-CH), 6.6 (m, 2H, β-CH); ^{13}C NMR (DMSO-d_6): 167, 163, 140, 138, 132, 131, 125, 124.

***N,N′*-Bis[bis(sodium methyl phosphate)isophthalyl]*trans,trans*-muconate (4).** *N,N′*-Bis(isophthalyl)*trans,trans*-muconate (0.18 g, 3.9 × 10^{-4} mol) was combined with thionyl chloride (15 mL) under argon and refluxed for 24 h (red solution). Thionyl chloride was removed by vacuum distillation and the crude solid acid chloride dissolved in dry THF (20 mL, ice-cooled). This was combined with sodium dimethyl phosphate (0.23 g, 1.55 × 10^{-3} mol) that had been freshly prepared in dry acetone from trimethyl phosphate and sodium iodide.[27] The mixture stood at room temperature for 18 h. The precipitate was collected by filtration. The solvent was removed, and the product was dissolved in dry acetone (20 mL) to which sodium iodide (0.26 g, 1.7 × 10^{-3} mol) was added. The mixture was stirred for 24 h at room temperature. Light yellow crystals were collected by vacuum filtration and washed with dry acetone (3 × 10 mL), (0.25 g, 68% yield). mp: 245 °C (dec). ^{1}H NMR (DMSO-d_6): δ 10.8 (s, 2H, CONH), 8.5 (s, 4H, ArH), 8.15 (s, 2H, ArH), 7.4 (s, 2H, α-CH), 6.6 (s, 2H, β-CH), 3.5 (m, 12H, OCH_3); ^{13}C NMR (DMSO-d_6): 163, 161, 146, 128, 125, 122, 121, 119, 59; ^{31}P NMR (DMSO-d_6): δ −5.55 (decoupled); ^{31}P NMR (methyl alcohol-d_4): δ −4.61 (decoupled); MS (ESI): 210 found, 210 (M − $4H^{4-}$ calculated as m/z = 840/4 without sodium).

***N,N′*-Bis(isophthalyl)2,6-naphthalenedicarboxylate.** 2,6-Naphthalenedicarboxylic acid (0.5 g, 2.3 × 10^{-3} mol) was refluxed in excess thionyl chloride (15 mL) overnight under nitrogen. Residual thionyl chloride was removed by vacuum distillation. The dark yellow oil was pumped in vacuo for 2 h. 5-Aminoisophthalic acid (0.92 g, 5.1 × 10^{-3} mol) and 4-(dimethylamino)-pyridine (0.07 g, 5.6 × 10^{-4} mol) were added to the flask containing the acid chloride under nitrogen. Anhydrous *N,N*-dimethylacetamide (40 mL) was added via syringe, and the solution was stirred for 18 h. Water (200 mL) was added to the solution to induce precipitation. The suspension was centrifuged (4500g, 45 min). The supernatant was removed, and the solid pellet was washed with distilled water (3 × 50 mL). The product was separated by centrifugation. The wet crystals were lyophilized to give a white crystalline product (0.93 g, 75% yield). mp: >250 °C (dec). ^{1}H NMR (DMSO-d_6): δ 10.85 (s, 2H, CONH), 8.73 (s, 2H, ArH) 8.71 (s, 4H, ArH), 8.25 (d, 2H, J = 6.8 Hz, ArH), 8.22 (s, 2H, ArH), 8.13 (d, 2H, J = 6.8 Hz, ArH); MS (ESI): 540 (found), 542 (M − H^- calculated for $C_{28}H_{18}N_2O_{10}$).

***N,N′*-Bis[bis(sodium methyl phosphate)isophthalyl]2,6-naphthalenedicarboxylate (5).** *N,N′*-Bis(isophthalyl)2,6-naphthalenedicarboxylate (0.3 g, 5.5 × 10^{-4} mol) was refluxed with excess thionyl chloride (20 mL) for 24 h. Residual thionyl chloride was removed by vacuum distillation. Sodium dimethyl phosphate (0.35 g, 2.3 × 10^{-3} mol) and THF (30 mL) were added to the acid chloride. The reaction was stirred for 3 days at room temperature. The solvent was removed in vacuo leaving a brown oil, which was immediately dissolved in acetone and mixed with sodium iodide (0.20 g, 1.35 × 10^{-3} mol) (brown solution). This solution was stirred for 24 h at room temperature, giving a yellow precipitate. The precipitate was collected by vacuum filtration. The resulting crystals were washed with acetone (3 × 5 mL), (0.47 g, 85% yield). mp: 230 °C (dec). ^{1}H NMR (DMSO-d_6): δ 10.95 (m, 2H, CONH), 8.75 (m, 6H, ArH), 8.25 (d, 2H, J = 6.5 Hz, ArH), 8.20 (d, 2H, J = 6.0 Hz, ArH), 8.15 (d, 2H, J = 6.5 Hz, ArH), 3.5 (m, 12H, OCH_3); ^{31}P NMR (DMSO-d_6): δ −5.77 (decoupled); MS (ESI): 227 found, 228 (M − $4H^{4-}$ calculated as m/z = 914/4 without $4Na^+$).

Cross-Linking. Carbonmonoxyhemoglobin (HbCO) (2.0 mL, 3 × 10^{-6} mol in 0.05 M Bis-Tris, pH 6.5) was passed through a Sephadex G-25 column equilibrated with 0.05 M sodium borate buffer, pH 8.5, at 4 °C. The collected hemoglobin sample (∼1.5 × 10^{-4} M) was oxygenated and photolyzed under a stream of humidified oxygen at 0 °C and adjacent to an illuminated tungsten lamp for 2 h to give oxyhemoglobin (HbO). This was deoxygenated under a stream of humidified nitrogen at 37 °C for 2 h to give deoxyhemoglobin (deoxyHb). One equivalent of cross-linking reagent **3**, **4**, or **5** (3 × 10^{-6} mol) was added to the solution of hemoglobin (3 × 10^{-6} mol). In some cases, the reagent was initially dissolved in a small volume of sodium borate buffer (0.05 M, pH 8.5) and then added to the solution of Hb via syringe. The reaction was allowed to proceed for 18 h at 37 °C under a stream of humidified nitrogen. Carbon monoxide was passed over the mixture for 15 min before the sample was placed into a column of Sephadex G-25 and eluted with 0.1 M MOPS (pH 7.2). The resulting modified HbCO was stored at 4 °C.

HPLC Analysis of Cross-Linked Bis-tetramers. Modified hemoglobins were analyzed according to the procedure of Jones.[28] Analytical reversed-phase HPLC was employed using a 330 Å pore size C-4 Vydac column (4.6 × 250 mm) to monitor globin chain modifications. Modified and unmodified globin chains were separated using developers containing 0.1% trifluoroacetic acid and a gradient beginning with 20% and ending at 60% of 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.

Purification of Bis-tetramers. Modified hemoglobins were separated using preparative size-exclusion FPLC, Superdex G-75 HR (10 × 300 mm). The method allows separation of globular proteins based on their molecular weights. The samples (50−100 μL) were eluted under conditions that dissociate the Hb tetramer into dimers (25 × 10^{-3} M Tris-HCl, 0.5 M $MgCl_2$, pH 7.4).[29] The effluent was monitored at 280 and 414 nm. The first eluting peak was collected separately, and multiple injection fractions were pooled. The collected samples were concentrated in Millipore Centriprep 50 concentrators. The samples were then added into a column Sephadex G-25 and eluted with 0.1 M MOPS (pH 7.2). The collected samples were placed under a stream of humidified carbon monoxide for 10 min and stored at 4 °C. The fraction corresponding to 128 kDa containing a cross-linked bis-tetramer was exchanged for sodium phosphate buffer (I = 0.1 M, pH = 7.4) and oxygenated for 2 h under tungsten light. The sample concentration was adjusted to about 5 × 10^{-3} M of heme, and its oxygen-binding properties were determined (Figure 6).

SDS-PAGE Analysis of Modified Hb. The peaks eluting from the G-75 gel filtration chromatography from the reactions of Hb with cross-linkers **3**, **4**, or **5** were analyzed by SDS-PAGE. Protein standards, modified reaction samples, and native Hb were prepared by mixing a protein sample (2−15 μL) with loading buffer 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 heated to 100 °C for 15 min, and 15−25 μL was loaded onto a polyacrylamide gel (12% Tris-HCl). The gels were processed in a mini-PROTEAN 7 dual-slab cell apparatus at 200 mV in 0.12 M Tris, 1 M glycine, and 0.017 M SDS buffer. The gels were stained with either Coomassie Blue (R-250) or silver nitrate for at least 1 h. Silver nitrate was used for more dilute samples.[30−33]

Stained gels were placed in a series of destaining cycles with 40% methanol and 10% acetic acid solution for 12 h. When gels were stained with silver nitrate, they were fixed in 50% methanol and 10% acetic acid solution, followed by washing in 50% ethanol (3 × 20 min). The gels were then treated with sodium thiosulfate (0.05 g/L) for 1 min and washed with water (3 times). The resulting gels were stained with a solution of silver nitrate (0.5 g/L) and formaldehyde (0.028%). The development of the gels was done using a solution of sodium carbonate (0.57 M), formaldehyde (0.019%), and sodium thiosulfate (0.001 g/L).

(28) Jones, R. T. *Methods Enzymol.* **1994**, *231*, 322−343.
(29) Guidotti, G. *J. Biol. Chem.* **1967**, *242*, 3685−3693.
(30) Switzer, R. C., III.; Merril, C. R.; Shifrin, S. *Anal. Biochem.* **1979**, *98*, 231−237.
(31) Ochs, D. C.; McConkey, E. H.; Sammons, D. W. *Electrophoresis* **1981**, *2*, 304−307.
(32) Oakley, B. R.; Kirsch, D. R.; Morris, N. R. *Anal. Biochem.* **1980**, *105*, 361−363.
(33) Sammons, D. W.; Adams, L. D.; Nishizawa, E. E. *Electrophoresis* **1981**, *2*, 135−141.

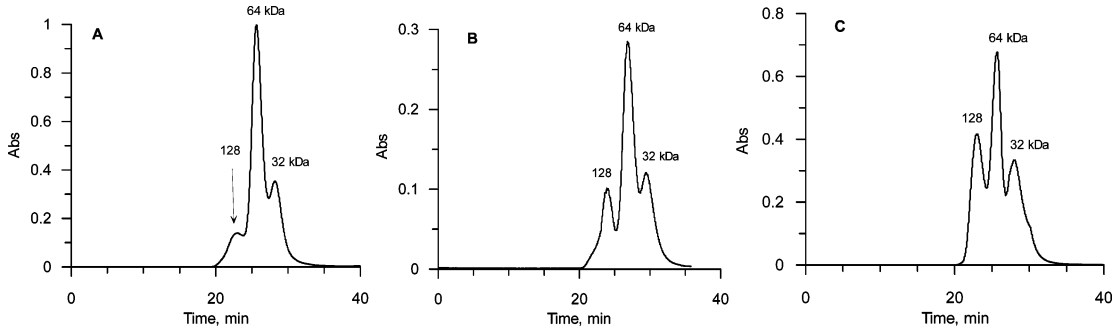
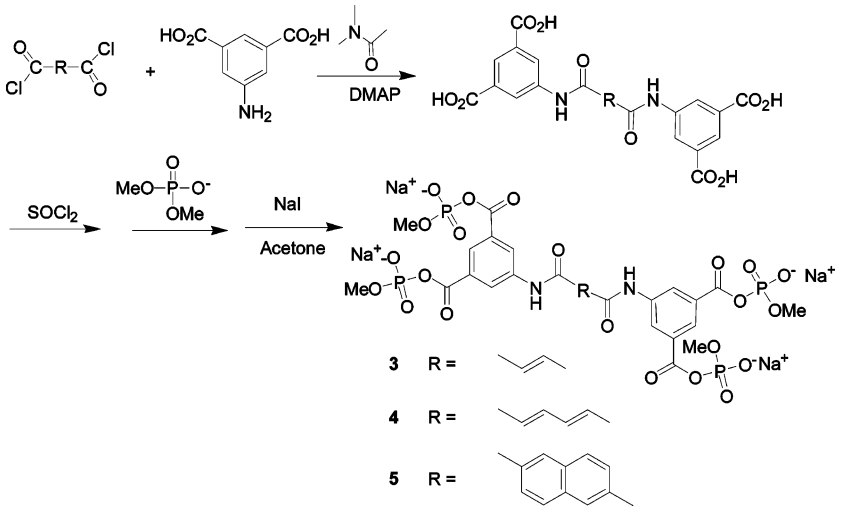


Figure 1. G-75 Superdex size-exclusion HPLC chromatograms of the reaction mixtures of Hb with reagent **3** (A), reagent **4** (B), and reagent **5** (C).

Scheme 1



The stains were fixed with 20% ethanol and 5% glycerol. Stained gels were scanned, and the results digitized using a computer.

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 reacted for 24 h. The tryptic hydrolysate 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 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

Reagents **3**, **4**, and **5** have two pairs of methyl isophthalyl phosphates joined by unsaturated amides. These were prepared from the corresponding dicarboxylic acids as described in the Experimental Section (Scheme 1). The highest yield of cross-linked bis-tetramers resulted from the reaction of 1 equiv of each reagent with 1 equiv of Hb at pH 8.5.

Modified subunits were identified in the reaction mixtures using reversed-phase HPLC. This revealed that modifications occur selectively on the side chains of the β-subunits of hemoglobin. However, the chromatograms did not give resolved separations of the species that had β-chain modifications.

The cross-linked bis-tetramers were identified and isolated using gel filtration chromatography with conditions that facilitate the dissociation of native tetrameric hemoglobin into the 32 kDa α,β-dimers.[29] Under these conditions (high concentrations of magnesium chloride), only dimers that have been covalently linked to other dimers maintain their quaternary structure. Three species (128 kDa, 64 kDa, and 32 kDa) were collected from the reaction mixture of each cross-linking reagent with deoxy Hb and analyzed (Figure 1). Each species was tested for coelution with native Hb and mono-tetrameric cross-linked Hb. Reagent **5**, with the longest span, gave the highest yield of bis-tetramer while reagent **3**, with the shortest span, gave the lowest yield of bis-tetramer. The first eluting peak was collected separately, and fractions from multiple injections were pooled.

SDS-PAGE separates the constituent protein chains by mass. Those that are covalently connected remain combined and have a higher mass. Native Hb produces a single band at about 16 kDa, corresponding to each of its four subunits. Reagents **3**−**5** produced species with masses of 64, 48, 32, and 16 kDa, confirming that each of the cross-linkers reacts with four, three, and two β-chains respectively (Figure 2). The 16 kDa species are unmodified α- or β-chains. Isolation of the 128 kDa peaks from gel chromatography and SDS-PAGE analysis showed that the major products are 64 kDa and 16 kDa species, with trace quantities of 48 kDa species in some cases.

This is consistent with linkage between all four β-subunits (64 kDa) and no modification to the α-subunits. The 48 kDa species comes from reagents that have reacted with three

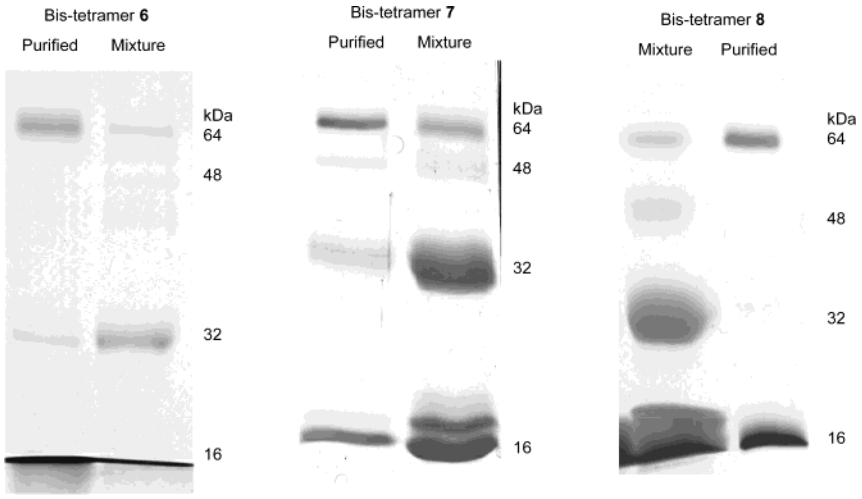


Figure 2. SDS-PAGE analysis of cross-linked bis-tetramers **6**, **7**, and **8**.

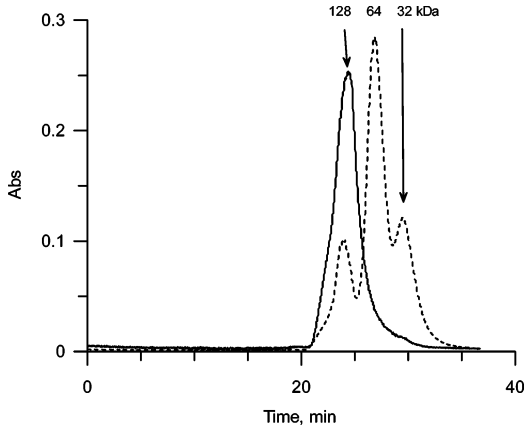
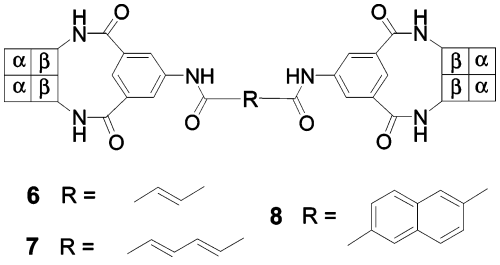


Figure 3. G-75 Superdex size exclusion HPLC of crude mixture (dashed line) and purified bis-tetramer **7** (solid line).

β-subunits (presumably one of the acyl phosphates has hydrolyzed prior to reaction). The schematic structures are shown as **6**−**8**.



The material collected from the bis-tetrameric peaks was passed through the gel filtration column. Only species corresponding to 128 kDa were observed, which is the expected nominal mass of cross-linked bis-tetramers (Figure 3).

The site of the reaction of each reagent with hemoglobin was analyzed by using a tryptic digest and peptide map. The modified β-chains collected from the reversed-phase C4 column were digested. The resulting fragment mixture was assayed employing MALDI-MS. The spectrum from digested β-chains modified with reagent **4** (Figure 5) was compared to unmodified digested β-chains spectrum (Figure 4). The results indicated that molecular fragments corresponding to the T9 peptide chain (m/z = 1670) of Hb and uncut T8 + T9 peptide chains (m/z = 1799) were absent in the modified β-chain digest (Figure 5). The fragment corresponding to T1 (m/z = 953) was substantially reduced in modified β-chain tryptic digest. The absence of T9 and T8 + T9 peptides establishes that **4** reacted with the ϵ-amino group of lysyl residue 82 of β-chains through one of the isophthalic phosphate esters since that is the normal site of hydrolysis between these peptides. The reduced amount of the T1 peptide in the modified β-chain digest indicates that **4** cross-linked Hb through the N-terminal amino group of valyl residue 1 of β'-chains on the second arm of the isophthalic phosphate ester. The presence of these amide linkages prevents trypsin from hydrolyzing the peptide bond between T9 and T10 chains. This is exactly the same pattern seen with acyl phosphate cross-linking reagents in the formation of monotetramers.

The oxygen-binding properties of the bis-tetrameric hemoglobin species are summarized in Table 1. Oxygen affinity (P_{50}) was obtained from fitting the data to the Adair equation[34] (Figure 6). The oxygen affinities of the bis-tetramers (**6**, P_{50} = 5.0; **7**, P_{50} = 4.2; **8**, P_{50} = 4.3) are somewhat higher (lower P_{50}) than those we measured for native Hb itself (P_{50} = 5.0). The Hill coefficient (n_{50}), a measure of cooperativity,[34] was obtained from the slope of a Hill plot, where Y is the saturation factor of oxygen-occupied sites versus the total number of the sites available for oxygen binding (Figure 7). The bis-tetrameric hemoglobins in the present series retain some cooperativity (**6**, n_{50} = 1.8; **7**, n_{50} = 2.1; **8**, n_{50} = 2.0), but it is reduced compared to native Hb (n_{50} = 3.0) or cross-linked tetramers produced from isophthalyl reagents (n_{50} = 2.6, P_{50} = 18.1).[35]

Discussion

Reagents **3**, **4**, and **5** were prepared efficiently and react at concentrations comparable to that of the dissolved protein. The acyl phosphate ester permitted reactions to be carried out in water. Their reaction with Hb introduced the desired internal and external links in a single reaction. The relatively rigid core structures resulting from extended conjugation and the amide linkage to the isophthalyl phosphate esters result in the link having a predictable span while the reagent does not fold onto itself.[24] The isophthalyl relationship of the reacting sites on each

(34) Bell, J. E.; Bell, E. T. *Proteins and Enzymes*; Prentice-Hall: Englewood Cliffs, NJ, 1988.
(35) Kluger, R.; Wodzinska, J.; Jones, R. T.; Head, C.; Fujita, T. S.; Shih, D. T. *Biochemistry* **1992**, *31*, 7551−7559.

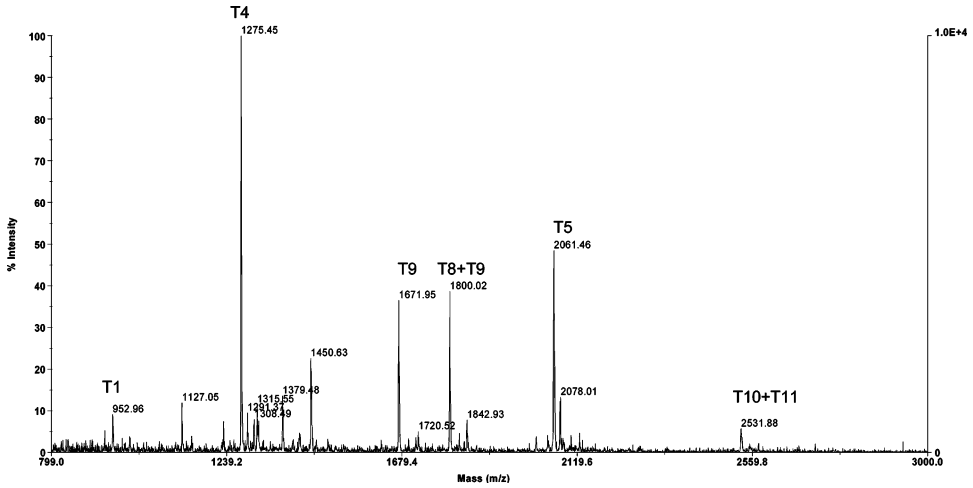


Figure 4. MALDI-MS of tryptic digest mixture from native β-chain of human hemoglobin.

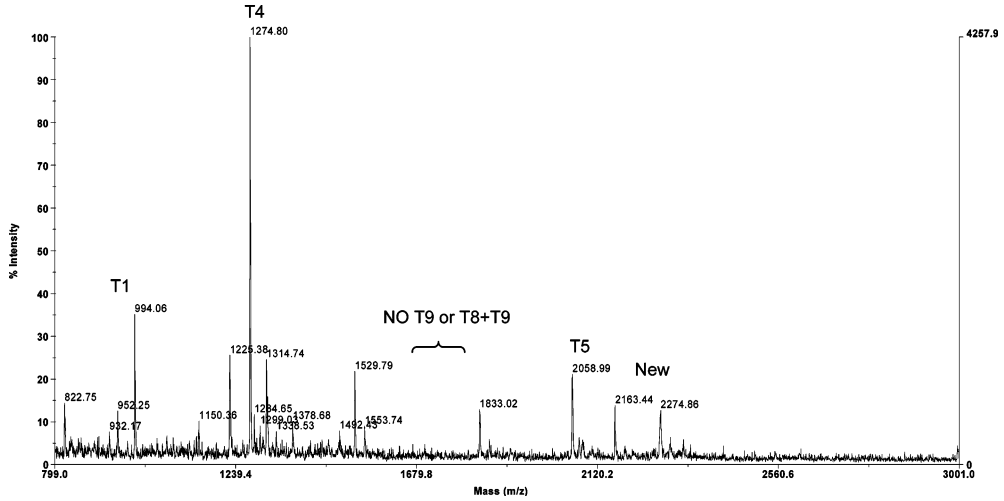


Figure 5. MALDI-MS of tryptic digest mixture from modified β-chain of human hemoglobin with reagent **4**.

Table 1. Oxygen-Binding Properties of Purified Bis-tetrameric Hemoglobin Species

bis-tetramer	P_{50} (torr)	n_{50}	span[a] (Å)
6	5.0 (±0.1)	1.8 (±0.1)	14.5
7	4.2 (±0.1)	2.1 (±0.1)	16.8
8	4.3 (±0.1)	2.0 (±0.1)	18.4
native Hb	5.0 (±0.1)	3.0 (±0.1)	n/a
2^{20}	9.1	1.29 (±0.03)	16.2

[a] Distance is measured between two C═O groups on opposite ends of the link between tetramers.

end facilitates intratetramer cross-linking of two β-subunits at ϵ-amino groups of β-lys-82 and the amino group of the N-terminal β'-Val-1 residues.[3,35−37] When two of the isophthalyl moieties are separated by a sufficient distance, the bis-tetrameric hemoglobin species is generated.

Our results clearly show that linking the cross-linked tetramers affects the oxygen-binding properties of the constituent heme sites. These materials retain cooperative properties, unlike the previously reported cross-linked bis-tetramer (**2**), whose binding oxygen-binding curve is hyperbolic.[20]

Acyl phosphate ester groups undergo hydrolysis in competition with protein modification. This leads to the yield of cross-linked bis-tetramer being less than that of smaller species that result from reaction with reagent that has undergone partial hydrolysis. In producing bis-tetramers, simply increasing the concentration of the reagent will not improve the yield because the coupling process requires a combination of the two protein molecules and one tetrafunctional reagent molecule. However,

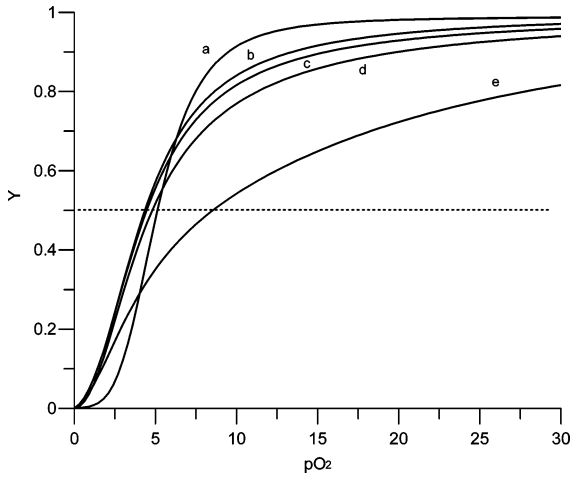


Figure 6. Oxygenation of native Hb (a) and cross-linked bis-tetramers: **8** (b), **7** (c), **6** (d), and calculated **2**^{20} (e). Oxygen affinity (P_{50}) is derived at $Y = 0.5$.

(36) Kluger, R.; Shen, L.; Xiao, H.; Jones, R. T. *J. Am. Chem. Soc.* **1996**, *118*, 8782−8786.
(37) Paal, K.; Jones, R. T.; Kluger, R. *J. Am. Chem. Soc.* **1996**, *118*, 10380−10383.

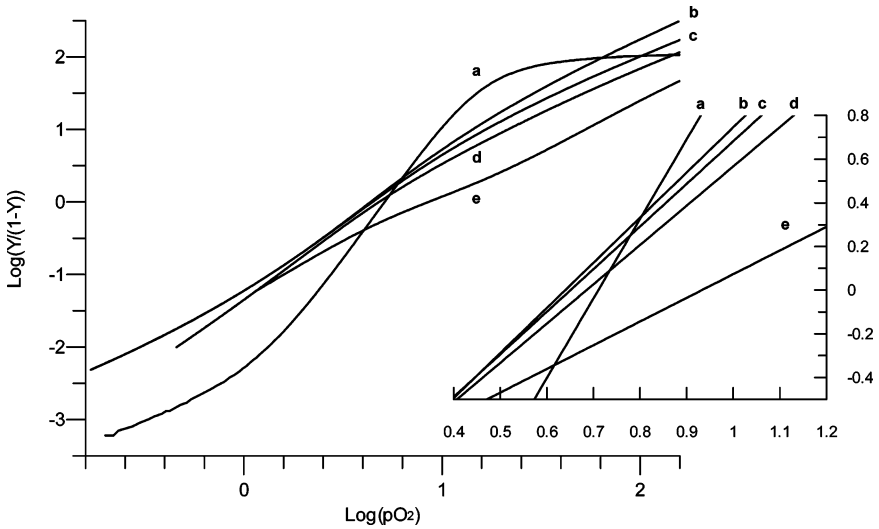


Figure 7. Hill plot of oxygen binding of native hemoglobin (a), bis-tetramer **8** (b), bis-tetramer **7** (c), and bis-tetramer **6** (d). For comparison, a calculated curve for bis-tetramer **2**[20] is also shown. The slopes at $Y = 0.5$ are the Hill coefficients (n_{50}) and are shown on the inset.

yields can be improved by the procedures used in producing hemoglobin−dendrimer conjugates,[38] especially variation in the timing of addition of the reagent to the protein solution. In the present study, we were interested in establishing the existence and properties of cross-linked bis-tetramers rather than developing a practical route for their production.

We varied the span of the intertetramer link to test the specific effect of length on cooperativity. Reagents **3** and **4** contain amides with linear conjugated hydrocarbon cores. There is a small increase in cooperativity ($\Delta n_{50} = 0.2$) where the separation involves an additional allylic unit (2.3 Å), suggesting that interactions of the tetramers decrease cooperative interactions within tetramers that lead to higher Hill coefficients.[39]

The separation between constituent tetramers in **2**, which has a terephthalyl core, is about the same as in the linear dienyl core (*trans*,*trans*-muconate) of **7**: about 16 Å (estimated from molecular modeling). Thus, the difference in cooperativity must be due to the chemical properties of the linking structure. To investigate the separation−cooperativity relationship, we prepared **5**, a reagent with a larger aromatic core, producing **8**, which can be compared to **2**. Here $n_{50} = 2.0$, indicating that cooperativity is retained with the longer link, even with a more hydrophobic core. This suggests that cooperativity requires movements that cannot be accommodated in the shorter, more rigid structure of the terephthalyl linkage while hydrophobicity in the link itself does not prevent cooperative interactions.

The oxygenation of the bis-tetramers in principle could involve interactions caused by the binding of up to eight molecules of oxygen on the eight hemes of the connected tetramers. If there were positive cooperativity between units in different tetramers, we would expect an *increase* in the observed Hill coefficient, but this is not the case. Thus, cooperativity remains the result of interactions within each tetramer, following the Perutz model.

The intertetramer links should also generate interfacial interactions. The close interaction leads to reduction of the exposure to water of each tetramer, resulting in the change of the hydration level within each connected tetramer.[18] Studies of osmotic stress indicate that 25−72 water molecules bind a tetramer in the course of oxygenation.[40] A decrease in accessibility of the connected tetramers to the solvent will result in a change in hydration of the protein. Such a change in hydration level has been observed to be associated with cooperative binding of oxygen to Hb.[41−44] Thus, reduction of cooperativity of the bis-tetramers may result from alteration of the hydration level as well as from restricted allosteric movement.

The bis-tetramers are possible alternatives to nonspecifically linked oligomers in red cell substitutes. Research with PEG-Hb's has shown that an increase in the overall size of the species results in prolonged circulation life and minimizes increases in mean arterial blood pressure.[18,19] In addition, the bis-tetramers are good candidates for studying the relationship of structure to function in oxygen carriers.[20,45] The idea that cross-linked bis-tetramers might be less vasoactive in circulation than cross-linked tetramers derives from analyses of heterogeneously cross-linked hemoglobins that have been used in clinical trials.[8−12] The oxygen-binding properties of the individual species in such mixtures have not been determined, whereas those of the purified cross-linked tetramers in other trials have been measured.[3] Our studies reveal that cross-linked bis-tetramers have higher oxygen affinities and decreased cooperativity compared to the clinically tested mono-tetramers. It has been assumed that vasoactivity is associated with scavenging of nitric oxide from endothelia by the hemes in these species.[46,47] Our results show oxygen will bind more tightly to cross-linked bis-tetramers and produce a high net occupancy in these species relative to cross-linked tetramers. To the extent that the site is occupied by oxygen there is less of an opportunity to acquire nitric oxide, which could cause vasoconstriction and increased blood pressure.

(38) Kluger, R.; Zhang, J. *J. Am. Chem. Soc.* **2003**, *125*, 6070−6071.
(39) Jayaraman, V.; Rodgers, K. R.; Mukerji, I.; Spiro, T. G. *Science (Washington, D.C.)* **1995**, *269*, 1843−1848.
(40) Zhou, Y.; Zhou, H.; Karplus, M. *J. Mol. Biol.* **2003**, *326*, 593−606.
(41) Salvay, A. G.; Grigera, J. R.; Colombo, M. F. *Biophys. J.* **2003**, *84*, 564−570.
(42) Colombo, M. F.; Seixas, F. A. V. *Biochemistry* **1999**, *38*, 11741−11748.
(43) Colombo, M. F.; Sanches, R. *Biophys. Chem.* **1990**, *36*, 33−39.
(44) Colombo, M. F.; Rau, D. C.; Parsegian, V. A. *Science (Washington, D.C.)* **1992**, *256*, 655−659.
(45) Kluger, R. *Can. J. Chem.* **2002**, *80*, 217−221.
(46) Wicks, D.; Wong, L. T.; Sandhu, R.; Stewart, R. K.; Biro, G. P. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* **2003**, *31*, 1−17.
(47) Baldwin, A. L.; Wiley, E. B.; Alayash, A. I. *Am. J. Physiol.* **2002**, *283*, H1292−H1301.

In cases where oxygen-binding properties of red cell substitutes had been rationally produced, cooperative, low oxygen affinity species were sought to maximize oxygen delivery.[45] However, such species would also be most able to acquire nitric oxide. Clinical trials have been conducted for situations where a small fraction of the total blood volume is replaced. In such situations, oxygen comes from the normal red cells: the altered hemoglobin serves as a sink of additional oxygen. Where the added oxygen carrier has a high oxygen affinity, it would serve as a source when the red cells are largely depleted but would probably not be vasoactive, since it would not selectively bind nitric oxide.

Conclusions

Cross-linked bis-tetramers with a variety of connecting cores can be generated from reagents that contain paired reaction sites. These materials bind oxygen reversibly and cooperatively with smaller Hill coefficients than do similarly cross-linked tetramers. These results suggest that there are significant interactions between the connected tetramers.

Acknowledgment. We thank the Natural Sciences and Engineering Research Council of Canada for support through a Strategic Project.

JA036596I