A Novel Site-Directed Affinity Reagent for Cross-Linking Human Hemoglobin: Bis[2-(4-phosphonooxyphenoxy)carbonylethyl]phosphinic Acid

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Bis[2-(4-phosphonooxyphenoxy)carbonylethyl]phosphinc acid (BPPCEP) was prepared and evaluated as a site-directed affinity reagent for cross-linking human hemoglobin. It was synthesized in four steps starting from 4-benzyloxyphenol and was converted to its pentasodium salt so as to afford efficient cross-linking in an aqueous medium. The reagent was found to specifically cross-link human hemoglobin A_0 in the β-cleft chains under oxygenated reaction conditions at neutral pH. The amino acid residues involved in the cross-linking were determined by mass spectral analyses of tryptic digest fragments of cross-linked hemoglobin, employing a MALDI-TOF mass spectrometer. The MS analyses suggested that the most likely amino acids involved in the cross-links are Val-1 or Lys-82 present on one of the β subunits and Lys-82 or Lys-144 on the other. Molecular modeling studies performed on the reagent−HbA_0 complex corroborated the conclusions reached by MALDI-MS analyses. The oxygen equilibrium measurements of the three major BPPCEP-cross-linked Hb products, isolated and purified by preparative cation exchange chromatography, exhibited oxygen affinity (P_{50}) values of 14.5, 12.1, and 15.5 Torr as compared with the P_{50} of 13.1 Torr for cell-free hemoglobin. The oxygen-binding cooperativity of the modified products, as determined by the Hill coefficient generated from the Hill plots of the respective P_{50} values, coupled with the absence of sigmoidal shape of the O_2 equilibrium curves, was considerably lower than that of the native hemoglobin.

Introduction

The need to develop a red cell blood substitute is well documented and has been the focus of the scientific community for well over 60 years.[1−6] Hemoglobin (Hb) being the natural oxygen carrier inside the red blood cell (RBC) has been the preferred choice for developing such a substitute. Unfortunately, when used outside of the RBC, Hb oxygen affinity is increased to an extent that may impair oxygen delivery from lungs to tissues. Furthermore, it suffers from short circulation times in the blood stream (1 to 4 h) due mainly to its breakdown from a large tetrameric protein, $\alpha_1\alpha_2\beta_1\beta_2$, into two smaller dimeric units, $\alpha_1\beta_1$ and $\alpha_2\beta_2$, consequently facilitating its rapid renal elimination and afflicting considerable renal toxicity.[2,3] These drawbacks have now been attributed to the loss of the natural allosteric effector of Hb, called 2,3-bisphosphoglycerate (BPG), upon isolation of pure stroma-free Hb from RBC. The BPG, equipped with five negative charges at physiological pH, acts like an affinity reagent within RBC to bring together the two dimers containing a cluster of positively charged amino acid residues, forming a stable, tetrameric Hb structure with a central cavity called the BPG pocket or the β-cleft[7] (see Figure 1). In addition to its role in stabilizing the tetrameric Hb structure, BPG is responsible for maintaining the proper equilibrium between the oxy and deoxy forms of Hb for adequate oxygen delivery from lungs (predominantly oxy form) to the tissues (predominantly deoxy form). BPG, which favors the deoxy form, tunes or lowers the oxygen affinity of Hb to the desired level so as to deliver the oxygen acquired from lungs to the tissues. The formation of such a stable tetramer outside the red blood cells is believed to alleviate a large part of the two major problems associated with using cell-free Hb as a blood substitute: short intravascular circulation time and high oxygen affinity. This would necessitate covalent cross-linking of the two Hb dimers with a BPG mimic, preferably within the β-cleft site, to produce an undissociable tetramer with adequately tuned or lowered oxygen affinity characteristics. To this end, many labs, including ours, have designed and synthesized a wide variety of bifunctional and polyfunctional cross-linking

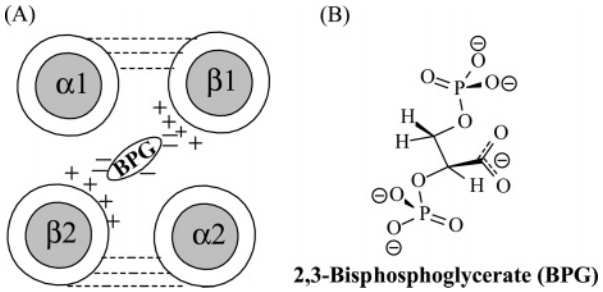

Figure 1. (A) The schematic representation of the tetrameric structure of hemoglobin formed by electrostatic interaction between the anionic charges of 2,3-bisphosphoglycerate (BPG) and the cationic charges of amino acid residues present on the β-subunits of hemoglobin, and (B) the molecular structure of 2,3-bisphosphoglycerate.

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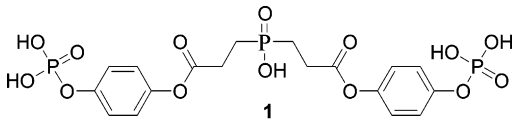
agents to chemically modify cell-free Hb.[3,8−21] Different degrees of success, as measured by the favorable functional properties of the cross-linked Hb, have been achieved, and a few have even made to clinical trials.[16,22] Nevertheless, the challenge still remains to synthesize an ideal reagent which so closely mimics BPG that it is highly specific to be attracted to the β-cleft and causes the least conformational change in the native structure of hemoglobin upon subsequent cross-linking. Such a reagent would render the desired tetrameric stability and oxygen affinity to the modified hemoglobins.

We report herein the synthesis of a novel cross-linking reagent that mimics BPG in physicochemical characteristics, including the possession of two phosphate groups and the same number (five) of anionic charges as BPG. The reagent bis[2-(4-phosphonooxyphenoxy)-carbonylethyl]phosphinc acid (BPPCEP, **1**) was designed



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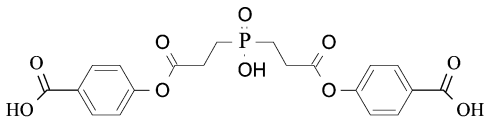
Reagent BPPCEP

by extensive molecular modeling studies and synthesized in four easy, efficient steps. The reagent is a white solid that can be stored for indefinite periods of time with the customary care for protection from the atmospheric moisture. The synthesis uses the common, commercially available, inexpensive starting materials and can be easily scaled up. This is important considering the fact that a number of otherwise promising reagents were rejected by industry because of perceived synthetic difficulty, poor yields, and high costs of production.[16,23] The reagent BPPCEP has one other advantage in that it can be employed to cross-link hemoglobin under convenient, ambient, natural oxygenated conditions without having to resort to rigorous deoxygenated media that is usually required for many, if not all,[24,25] of the leading Hb cross-linking reagents.[16,23] It is to be noted, however, that the reagent can also be used for cross-linking both the deoxy- and carbonmonoxy forms of hemoglobin, although that is not the focus of the present investigation.

Results and Discussion

In our earlier studies, we designed and synthesized a few activated aromatic esters of phosphinic acids as anionic Hb cross-linking reagents.[18,20,21] They were successfully employed to introduce specific cross-links of defined dimensions with the amino acid residues lining the periphery of the β-cleft (BPG pocket) of hemoglobin to produce modified hemoglobins with many desired features. The best reagent in the series, bis[2-(4-carboxyphenoxy)carbonylethyl]phosphinic acid (BCCEP), produced the modified Hb whose oxygen affinity



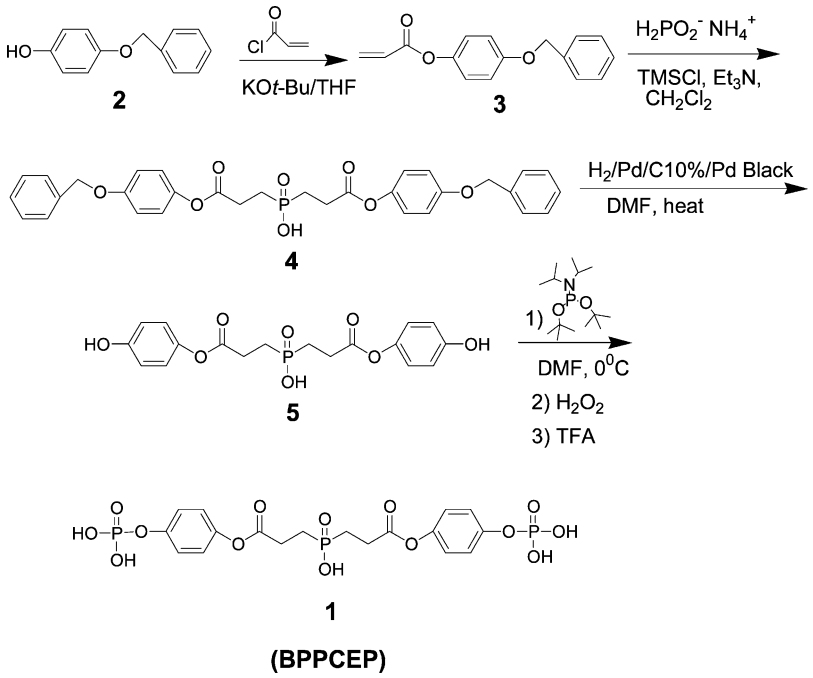
Reagent BCCEP

(P_{50} = 31.3 Torr) characteristics was very similar to that of the whole blood (P_{50} = 27.0 Torr).[18] Nevertheless, the reagent BCCEP was by no means perfect. It suffered from a couple of conspicuous drawbacks, including its low cross-linking efficiency (15−20%) and the production of a multitude of side products. Realistically, a value of 50% or more cross-linked hemoglobin would be necessary for useful commercial applications, with as few side products as possible. As a matter of fact, even after an extended reaction period of 4 h or more and/or with a large excess of BCCEP, there was substantially more unreacted Hb than there was product. In addition, there were many Hb side products that were modified by the reagent, but possessed no cross-link between β chains, including adducts that had only reacted with the reagent on one β chain before being hydrolyzed. From these results it was clearly evident that the reagent needed to be more specific for the BPG pocket and that the carboxyphenoxy esters needed to be more activated in order to facilitate nucleophilic attack by the free amino groups located in this active site. Therefore, it was hypothesized that the cross-linking activity could be greatly enhanced if the reagent more closely mimicked the hemoglobin's natural allosteric effector BPG.

A simple structural comparison of BCCEP with BPG suggested that, at biological pH, BCCEP would have only three anionic charges as contrasted with BPG, which is known to exist as a penta-anion. A way to fix this charge discrepancy was to substitute the two terminal carboxy groups of BCCEP with two phosphate groups. This would not only adjust the number of charges but also the number of phosphate groups to become equal to those of BPG. In addition, the phenoxycarbonyl esters will be further activated by electron-withdrawing inductive effects of the phosphate moieties attached to the benzene ring. Thus, the reagent BPPCEP was conceived.

Synthesis of the Reagent. The reagent BPPCEP was synthesized in four steps (Scheme 1) starting from 4-benzyloxyphenol. The latter was reacted with acryloyl chloride in the presence of potassium *tert*-butoxide in tetrahydrofuran to obtain 4-benzyoxyphenyl acrylate (**3**) in 99% yield. The condensation of 2 equiv of **3** with ammonium phosphinate in the presence of chlorotrimethylsilane and triethylamine in methylene chloride at 0 °C for 24 h, followed by aqueous acid workup, yielded bis[2-(4-benzyloxyphenoxy)carbonylethyl]phosphinic acid (**4**) in 73% yield. The debenzylation of the latter was accomplished by catalytic hydrogenation over palladium/charcoal to give bis[2-(4-hydroxyphenoxy)-carbonylethyl]phosphinic acid (**5**) in 81% yield. As **4** was insoluble in almost all organic solvents other than hot DMSO or near-boiling DMF, the hydrogenation had to be carried out under unusual reaction conditions that required heating in DMF at 135 °C with a mixture of 10% Pd/C and Pd black at 80 psi on a Parr shaker. Compound **5** was phosphorylated by sequential reactions in one-pot using (a) 2 equiv of *O*,*O*-di-*tert*-butyl-*N*,*N*-diisopropylphosphoramidite with an excess of 1*H*-tetrazole in DMF, (b) 30% aqueous hydrogen peroxide, and (c) trifluoroacetic acid to yield the target reagent bis[2-(4-phosphonooxyphenoxy)carbonylethyl]phosphinic acid (BPPCEP, **1**) in 71% yield.

Scheme 1



Although the overall yield (~42%) for the above four-step synthesis of BPPCEP was satisfactory, the synthesis suffered from one major drawback. As mentioned earlier, the debenzylation procedure using catalytic hydrogenation in step 3 required unusually harsh reaction conditions to the point of risking a violent explosion, which indeed occurred during one of the many attempted runs. Therefore, an alternative, safe synthetic procedure was sought focusing on alleviation of the described solubility problem associated with the dibenzyl precursor **4**, which necessitated the use of such vigorous reaction conditions. This procedure, outlined in Scheme 2, employed 4-*tert*-butoxyphenol (**6**) in place of 4-benzyloxyphenol as the starting material. Deprotonation of **6** with potassium *tert*-butoxide in THF, followed by addition of acryloyl chloride, gave 4-*tert*-butoxyphenyl acrylate (**7**) in 100% yield. As above, the ammonium salt of hypophosphorous acid was then reacted with 2 equiv of **7** in dichlormethane with an excess of chlorotrimethylsilane and triethylamine at 0 °C for 24 h. The acidic aqueous work up produced bis-[2-(4-*tert*-butoxyphenoxy)carbonylethyl]phosphinic acid (**8**) in 66% yield. The replacement of the benzyl groups of **4** with the *tert*-butyl groups of **8** dramatically changed the solubility properties in that **8** is soluble in all common organic solvents. In addition, the subsequent deprotection step was simplified to refluxing with TFA in dichloromethane, followed by evaporation of the solvent to yield the analytically pure **5** in a 100% yield. The procedure for further phosphorylation of **5** to obtain the target reagent BPPCEP (**1**) is the same as described in Scheme 1.

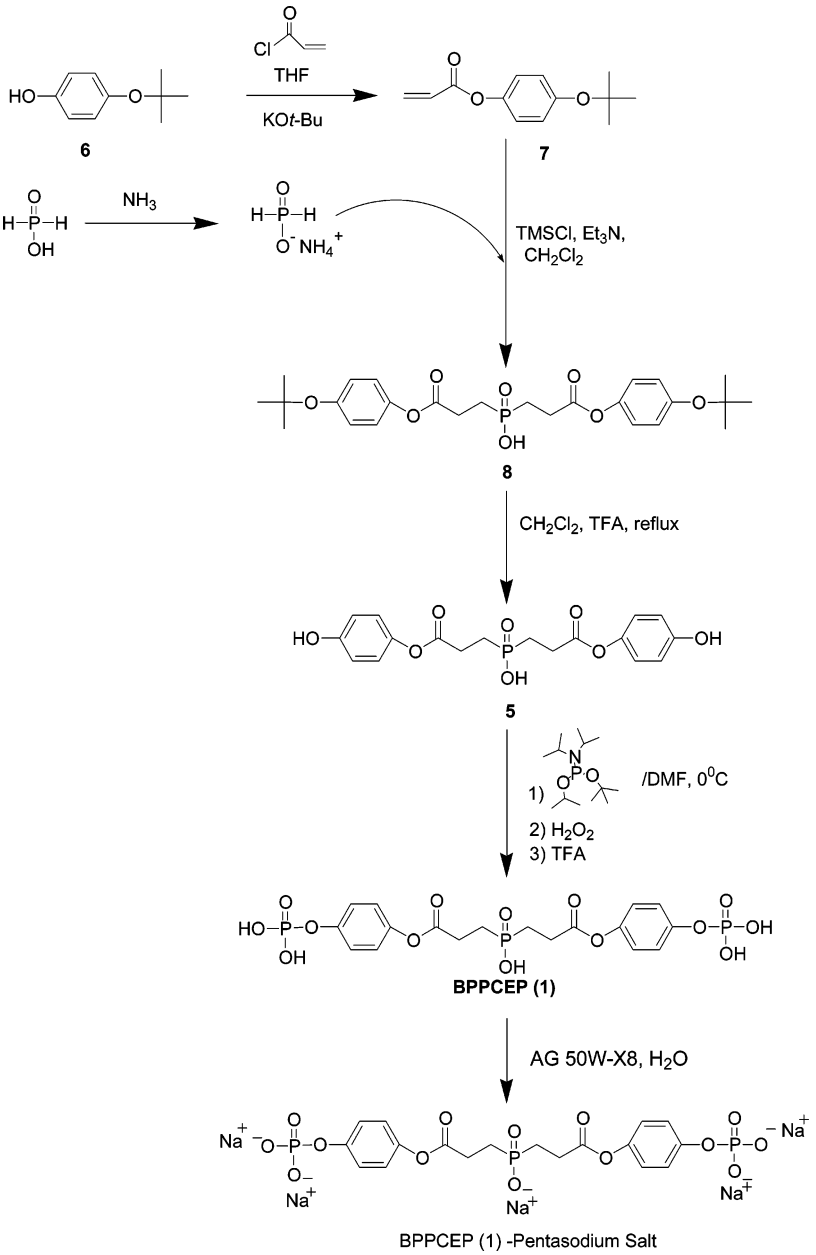
The reagent BPPCEP (**1**) is a fine white powder, albeit somewhat hygroscopic and becomes sticky upon exposure to air for long periods of time. The activated ester groups of the reagent undergo gradual hydrolysis as confirmed by periodic ^{1}H NMR spectral runs of the reagent in D_2O, and after 15+ h, approximately 50% hydrolysis was observed.

Hemoglobin Cross-Linking Studies. The first attempt at cross-linking hemoglobin with the reagent BPPCEP (**1**) was carried out under oxygenated conditions in a phosphate buffer (pH 8.5) at 24 °C using a 0.5 mM human stroma-free hemoglobin (SFHb) stock solution. The basic reaction conditions employed were anticipated to deprotonate the acidic protons on the reagent to form a polyanionic species, which should then be easily drawn into the polycationic BPG pocket. Ironically, under these conditions the reagent had a great difficulty going into the buffer solution and instead became an unmanageable slimy mass. Analysis of the reaction mixture by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE) and reverse phase high performance liquid chromatography (HPLC) showed only minute amounts of the cross-linked product. It was then concluded that the reagent had to be fully soluble in the buffer solution in order to assess the optimum cross-linking conditions. Therefore, BPPCEP was converted into its pentasodium salt form (see Scheme 2) by ion exchange chromatography over 50W−X8 resin (Na^+ form), followed by lyophilization of the collected fractions. In the salt form, the reagent appears as globular crystals and is completely soluble in water and any aqueous buffer solutions.

The cross-linking experiment was repeated using the above pentasodium salt of BPPCEP. Various concentrations of the reagent (2×, 5×, 10×, 25×, 50×, 100×, and 200×) were attempted using a phosphate buffer (pH 7.4) at different temperatures. Under these conditions, the cross-linking was found to be considerably more efficient. The best results, as revealed by SDS−PAGE, were obtained when the reagent concentration (25 mM) was 50× excess to that of the protein (0.5 mM) and when the reaction was carried out at 37 °C.

To remove the excess reagent as well as to dissociate any un-cross-linked Hb, the reaction mixture was passed through a size exclusion Sephadex G-100 column equilibrated with 0.25 M Tris buffer (pH 7.4) containing

Scheme 2



1 M $MgCl_2$ and eluted with the same solvent.[26,27] The fastest eluting fractions were pooled, concentrated, desalted, and buffer-exchanged (0.1 M Tris, pH 7.4) using Millipore Biomax 10 concentrators. The reverse phase HPLC on a VYDAC C4 column, monitored at a wavelength of 214 nm, was employed to determine if the cross-linking occurred between the α−α or $\beta-\beta$ subunits. The chromatogram of the reaction mixture (Figure 2A), when contrasted against that of native Hb (Figure 2B) in conjunction with that of the former spiked with additional amounts of Hb (Figure 2C), clearly indicated that the β chains were selectively being modified while the α chains were being practically left alone.

HPLC analysis of the above fractions using a preparative cation exchange column (Synchropak CM 300) revealed the presence of multiple products (labeled A−F, in Figure 3). The products were collected and further analyzed by SDS−PAGE (see Figure 4). Lanes 2 (peak A), 3 (peak B), and 4 (peak C) clearly show a strong band at 32 kDa representing a cross-linked Hb. Lanes 7 (peak D), 8 (peak E), and 9 (peak F) show bands at only 16 kDa representing monomeric globin chains. Lanes 1 and 10 are molecular weight markers. As the two cross-linked α/β dimers would dissociate into one $\beta-\beta$ dimer and two α monomers under gel electrophoretic conditions, the observed two bands in lanes 2, 3, and 4, corresponding to molecular weights of 32 kDa and 16 kDa, are consistent with what is expected. Also, since peaks D, E, and F all exhibit MW of 16 kDa, but have inherently different cation exchange retention times when contrasted with that of the native Hb (see Figure 5), it is reasonable to speculate that these modified monomeric hemoglobin subunits contain surface modifications. This can be expected considering that there are 42 reactive lysines and 2 other reactive amino groups per hemoglobin excluding the ones in the BPG pocket.[3]

Tryptic Digestion and Analyses by MALDI-MS. The tryptic digestion of the β-chain of normal human

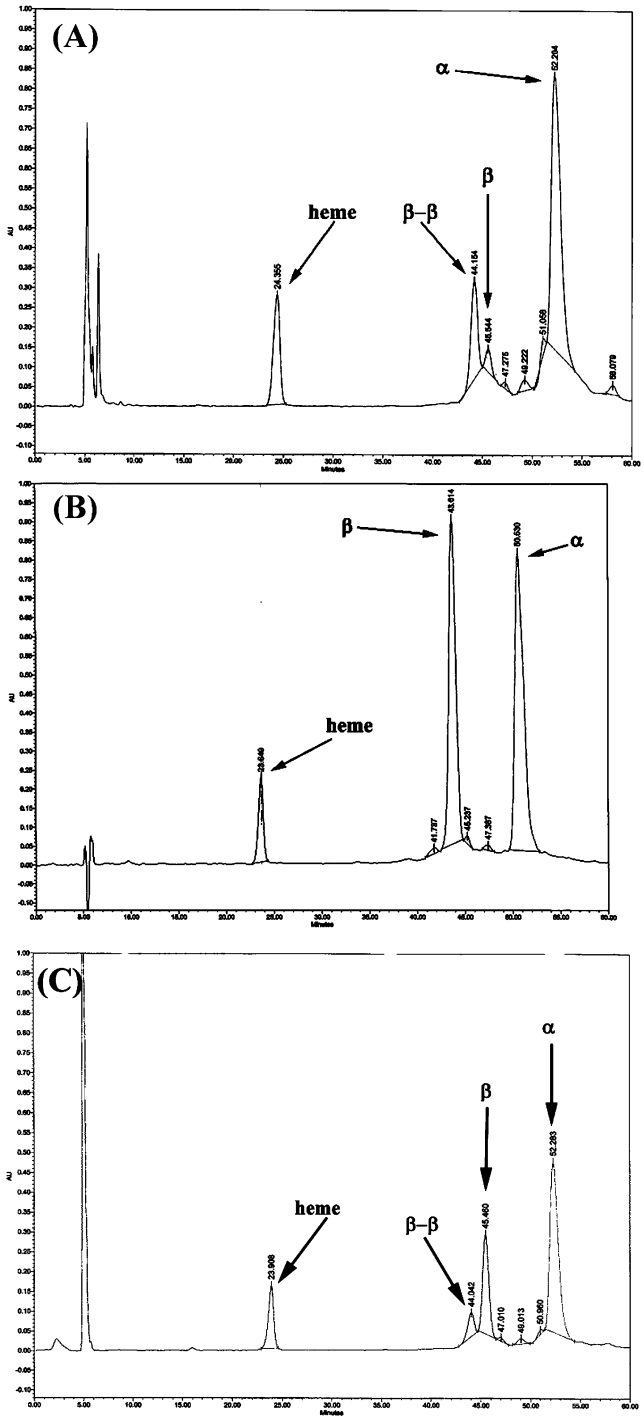


Figure 2. (A) C4 chromatogram of stoma-free hemoglobin (SFHb), cross-linked by the reagent BPPCEP showing modification in the β globin chains monitored at 214 nm. (B) C4 chromatogram of SFHb ran under the same conditions as Figure 2A, but without any BPPCEP monitored at 214 nm. (C) C4 chromatogram of stroma-free hemoglobin (SFHb), cross-linked by the reagent BPPCEP, as in Figure 2A spiked with additional SFHb monitored at 214 nm. Note that the unmodified β globin chain intensity increases.

hemoglobin (containing 11 lysine and 3 arginine residues) is expected to produce 15 peptides.[18,28] The complete tryptic digestion will result in all peptides having a mass of less than 3000 Da. Six of the 14 peptides will have masses of less than 1000 Da and will be difficult to detect because of interfering low-mass

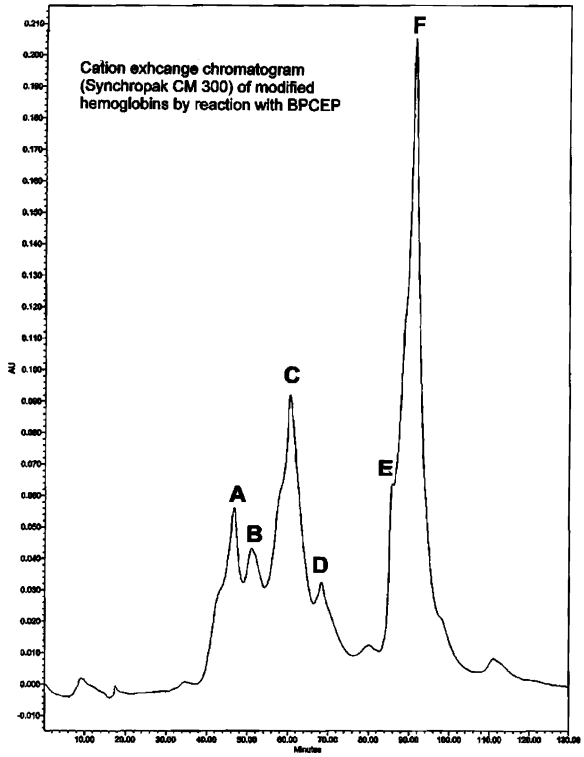


Figure 3. Cation exchange chromatogram showing modified hemoglobins after reaction with BPPCEP monitored at 415 nm.

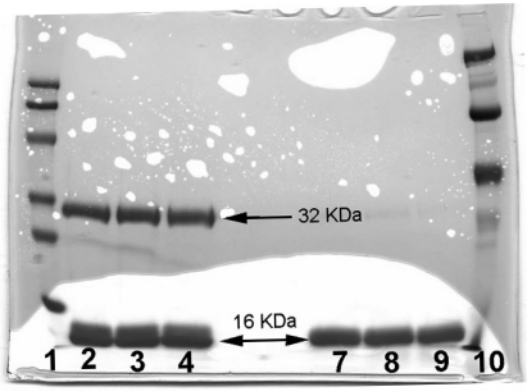


Figure 4. SDS−PAGE of peaks collected from Figure 3. Lanes 1 and 10 MW standards; Lane 2, peak A; Lane 3, peak B; Lane 4, peak C; Lane 7, peak D; Lane 8, peak E; Lane 9, peak F.

matrix ions.[28] The rest of the tryptic peptides will have $[M + H]^+$ ions between 1000 and 3000 Da and thus should be observable by the matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). Tables **1** and **2** show the MALDI mass spectral fragments of typtic peptides generated from samples of uncross-linked Hb and BPPCEP cross-linked Hb, respectively. The presence of Na^+ and K^+ ions in a number of the observed fragments is believed to be from the buffer solution used during the cross-linking, and is consistent with the literature precedent.[18] Also listed in Tables **1** and **2** are disulfide-linked peptides in the native as well as the cross-linked hemoglobins. Since the native human hemoglobin does not normally possess any disulfide bridge, it is assumed that they are the result of air oxidation during the prolonged tryptic digestion and/or cross-linking reaction conditions.

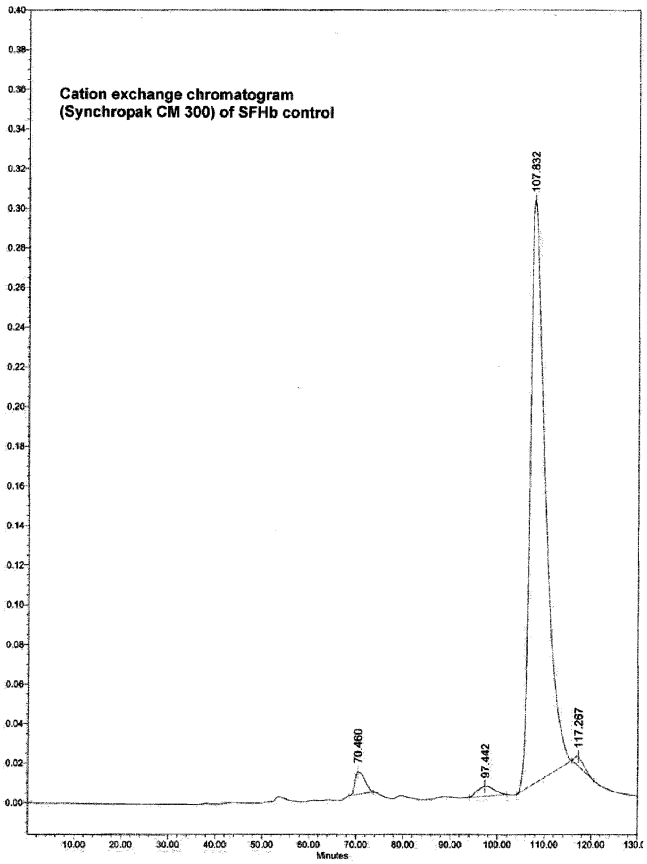


Figure 5. Cation exchange chromatogram of stroma-free hemoglobin (control) monitored at 415 nm.

The exact sites involved in the cross-linking were determined by identification of ions whose mass was equal to the sum of two tryptic fragments plus an additional mass equal to that of the cross-linking reagent minus the leaving group moieties (in this case, a mass of 176.1 corresponds to the BBPCEP tether). The observed ions listed in Table 2 suggest that there are three intersubunit cross-links in the hemoglobin modified by BPPCEP (See Figure 6). They are (a) β_1Val 1 to β_2 Lys 82, as corroborated by ions at *m/z* 4350.0, 4373.3, 4416.9, 5552.8, and 5546.6, (b) β_1Val 1 to β_2 Lys 144, supported by ions at *m/z* 2659.4 and 6156.3, and (c) β_1-Lys 82 to β_2 Lys 144, validated by ions at *m/z* 6157.8, 4773.5, and 4803.1. As the cross-linking is anticipated to block the action of trypsin at the lysine (or arginine) residue involved in the cross-link, all the cross-linked peptide fragments other than those involving T_1 with a terminal valine residue would contain at least one additional peptide unit beyond the point of cross-link. Thus, fragments T_9 or T_{14} containing cross-linked lysine 82 or lysine 144 also had T10 or T15 attached to it.

The computation of molecular weight of each cross-linked peptide species is based on (a) adding the molecular weights of the concerned peptides, (b) subtraction of 2 mass units and addition of 176.1 mass units to account for the addition of the reagent tether to two peptides, (c) subtraction of 18 mass units corresponding to a molecule of water for each pair of peptides present, for example, 36 (2 H_2O) or 54 (3 H_2O) mass units would be deducted from a fragment containing 3 or 4 peptide units, respectively, and (d) one mass unit is deducted for each sodium or potassium ion added. An exception, however, was noted with two ions at *m/z* 2779.9 and *m/z* 2258.8. As an example, an ion at *m/z* 2658.8, representing a cross-link between β_1 valine 1 and β_2 lysine 144, is computationally reconciled as follows: [(β_1 $T_1 = 952.1$) + (β_2 T_{14} + β_2 T_{15} − H_2O = 1449.7) + (XL = 176.1) − (peptide amino hydrogens = 2) + (2 Na + 1 K − 3 = 82) + (protonation of the cross-linked peptide during the ionization process = 1) = total *m/z* 2658.8].

Table 1. Comparison of the Observed and Calculated Average Masses of Tryptic Peptides of Human Hb β-Chain[a]

fragment residues	calcd avg mass $[M + H]^+$	obsd avg mass $[M + H]^+$	tentative structural analysis of tryptic fragment
(1−8)	953.0	952.9	T_1
(9−17)	933.0	n.o.[b]	n.o.[b] (T_2)
(18−30)	1315.4	1315.5	T_3
	1337.4	1337.6	(T_3 − 1) + Na
(31−40)	1275.5	1275.7	T_4
(41−59)	2060.3	2060.8	T_5
	2082.3	2083.1	(T_5 −1) + Na
	2098.4	2099.4	(T_5 −1) + K
(60−61)	246.3	n.a.a.[b]	n.a.a.[b] (T_6)
(62−65)	412.5	n.a.a.[b]	n.a.a.[b] (T_7)
(66−66)	147.2	n.a.a.[b]	n.a.a.[b] (T_8)
(66−82)	1799.1	1798.7	(T_8 + T_9) − H_2O
(67−82)	1670.9	1671.5	T_9
	1692.8	1692.8	(T_9 − 1)+ Na
(83−95)	1422.6	n.o.[b]	n.o.[b] (T_{10})
(83−104)	2530.8	2529.2	(T_{10} + T_{11}) − H_2O
(83−95)Cys_{93}−S−S−Cys_{93}-β(83−104)	3950.4	3952.5	(T_{10})Cys_{93}−SS−Cys_{93}(T_{10} + T_{11})
(96−104)	1127.2	1127.4	T_{11}
(105−120)	1721.1	n.o.[b]	n.o.[b] (T_{12})
(121−132)	1379.6	n.o.[b]	n.o.[b] (T_{13})
	1401.6	1403.7	(βT_{13} − 1) + Na
(133−144)	1150.4	1150.6	T_{14}
(145−146)	319.3	n.a.a.[b]	n.a.a.[b] (T_{15})

[a] The average mass of peptide fragments was calculated using the computer program, GPMAW (General Protein Mass Analyzer for Windows), Ver. 2.0, available from Light House Data, Engvej 35, DK-5230, Odense M, Denmark. The following are the calculated masses for the peptide fragments resulting from tryptic digestion of the β-chain of hemoglobin, and were employed in all subsequent computations involving these fragments: T_1 (1−8), 952.08; T_2 (9−17), 932.09; T_3 (18−30). 1314.42; T_4 (31−40), 1274.53; T_5 (41−59), 2059.28; T_6 (60−61), 245.33; T_7 (62−65), 411.46; T_8 (66−66), 146.19; T_9 (67−82), 1669.90; T_{10} (83−95), 1421.59; T_{11} (96−104), 1126.24; T_{12} (105−120), 1720.11; T_{13} (121−132), 1378.55; T_{14} (133−144), 1149.36; T_{15} (145−146), 318.34. [b] The following are the abbreviations used in the Table: n.a.a., n.o. assignment attempted; n.o., not observed; T, tryptic peptide.

Table 2. Comparison of the Observed and Calculated Average Masses of Tryptic Peptides of Human Hb β-Chain Cross-Linked with the Reagent BPPCEP[a]

fragment residues	calcd avg mass $[M + H]^+$	obsd avg mass $[M + H]^+$	tentative structural analysis of tryptic fragment
(1−8)	953.0	954.1	T_1
(1−8)	975.1	976.3	$(T_1 - 1) + Na$
(1−8) + reagent	1145.2	1145.6	$(T_1 - 1) + R^b$
(1−8) XL (66−95)	4350.8	4350.0	$(T_1 - 1)$ XL $(T_8 + T_9 + T_{10} - 2\ H_2O - 1) + (-1 + Na)$
(1−8) XL (66−95)	4372.8	4373.3	$(T_1 - 1)$ XL $(T_8 + T_9 + T_{10} - 2\ H_2O - 1) + (-2 + 2Na)$
(1−8) XL (66−95)	4416.8	4416.9	$(T_1 - 1)$ XL $(T_8 + T_9 + T_{10} - 2\ H_2O - 1) + (-4 + 4\ Na)$
(1−8) XL (66−104)	5551.1	5552.8	$(T_1 - 1)$ XL $(T_8 + T_9 + T_{10} + T_{11} - 3\ H_2O - 1) + (-3 + 3\ K)$
(1−8) XL (66−104)	5547.1	5546.6	$(T_1 - 1)$ XL $(T_8 + T_9 + T_{10} + T_{11} - 3\ H_2O - 1) + (-5 + 5\ Na)$
(1−8) XL (133−146)	2658.8	2659.4	$(T_1 - 1)$ XL $(T_{14} + T_{15} - H_2O - 1) + (-3 + 2\ Na + 1\ K)$
(1−40)	4495.1	4495.8	$(T_1 + T_2 + T_3 + T_4 - 3\ H_2O) + (-2 + 2\ K)$
(1−40) XL (133−146)	6156.8	6156.3	$(T_1 + T_2 + T_3 + T_4 - 3\ H_2O - 1)$ XL $(T_{14} + T_{15} - H_2O - 1) + (-3 + 3\ K)$
(9−17)	933.0	933.4	T_2
(9−17)	971.1	971.5	$(T_2 - 1) + K$
(18−30)	1315.4	1315.7	T_3
	1337.4	1338.4	$(T_3 - 1) + Na$
	1353.4	1354.0	$(T_3 - 1) + K$
(31−40)	1275.5	1275.7	T_4
(31−40)	1297.5	1298.2	$(T_4 - 1) + Na$
(41−59)	2060.3	2060.5	T_5
	2082.3	2082.9	$(T_5 - 1) + Na$
	2098.3	2098.4	$(T_5 - 1) + K$
(41−65)	2763.1	2763.1	$(T_5 + T_6 + T_7 - 2\ H_2O) + (-3 + 1\ K + 2\ Na)$
(41−65)	2779.1	2779.9	$(T_5 + T_6 + T_7 - 2\ H_2O) + (-3 + 2\ K + 1\ Na)$
(60−61)	246.3	n.a.a.[b]	n.a.a.[b] (T_6)
(60−95) XL (133−146)	5527.3	5530.8	$(T_6 + T_7 + T_8 + T_9 + T_{10} - 4\ H_2O - 1)$ XL $(T_{14} + T_{15} - H_2O - 1) + (-2 + K + Na)$
(62−82)	2258.6	2258.5	$(T_7 + T_8 + T_9 - 2\ H_2O) + (-3 + 3\ Na)$
(62−65)	412.5	n.a.a.[b]	n.a.a.[b] (T_7)
(66−66)	147.2	n.a.a.[b]	n.a.a.[b] (T_8)
(66−82)	1821.1	1823.8	$(T_8 + T_9 - H_2O) + Na$
(66−104)	4310.9	4311.4	$(T_8 + T_9 + T_{10} + T_{11} - 3\ H_2O)$
(67−82)	1670.9	1671.9	T_9
(67−95) XL (121−146)	6156.9	6157.8	$(T_9 + T_{10} - H_2O - 1)$ XL $(T_{13} + T_{14} + T_{15} - 2\ H_2O - 1) + (-3 + 3\ K)$
(67−95) XL (133−146)	4773.3	4773.5	$(T_9 + T_{10} - H_2O - 1)$ XL $(T_{14} + T_{15} - H_2O - 1) + (-2 + 2\ K)$
(67−95) XL (133−146)	4802.3	4803.1	$(T_9 + T_{10} - H_2O - 1)$ XL $(T_{14} + T_{15} - H_2O - 1) + (-4 + 3\ Na + 1\ K)$
(83−95)	1422.6	n.o.[b]	n.o.[b] (T_{10})
(83−104)	2530.8	2532.9	$(T_{10} + T_{11} - H_2O)$
(83−95)Cys_{93}−S−S−Cys_{93}- (83−104)	3951.4	3951.1	$(T_{10})Cys_{93}$−SS−$Cys_{93}(T_{10} + T_{11})$
(96−104)	1127.2	1127.1	T_{11}
(105−120)	1721.1	1722.4	T_{12}
(121−132)	1379.6	1380.2	T_{13}
(121−146) + Reagent	3085.3	3084.1	$(T_{13} + T_{14} + T_{15} - 2H_2O - 1) + R^b + (-3 + 1\ K + 2\ Na)$
(121−132)	1401.6	1402.2	$(T_{13} - 1) + Na$
(133−144)	1150.4	1150.6	T_{14}
(133−144)	1172.4	1173.4	$(T_{14} - 1) + Na$
(133−146) + Reagent	1642.8	1643.4	$(T_{14} + T_{15} - H_2O - 1) + R^b$
(145−146)	319.3	n.a.a.[b]	n.a.a.[b] (T_{15})

[a] The average mass of peptide fragments was calculated using the computer program, GPMAW (General Protein Mass Analyzer for Windows), Ver. 2.0, available from Light House Data, Engvej 35, DK-5230, Odense M, Denmark. The following are the calculated masses for the peptide fragments resulting from tryptic digestion of the β-chain of hemoglobin and were employed in all subsequent computations involving these fragments: T_1 (1−8), 952.08; T_2 (9−17), 932.09; T_3 (18−30). 1314.42; T_4 (31−40), 1274.53; T_5 (41−59), 2059.28; T_6 (60−61), 245.33; T_7 (62−65), 411.46; T_8 (66−66), 146.19; T_9 (67−82), 1669.90; T_{10} (83−95), 1421.59; T_{11} (96−104), 1126.24; T_{12} (105−120), 1720.11; T_{13} (121−132), 1378.55; T_{14} (133−144), 1149.36; T_{15} (145−146), 318.34. [b] The following are the abbreviations used in the Table: n.a.a., no assignment attempted; n.o., not observed; T, tryptic peptide; XL = $C(O)(CH_2)_2P(O)(OH)(CH_2)_2C(O)$ = cross-linked reagent with a mass = 176.1; *R* = $C(O)(CH_2)_2P(O)(OH)(CH_2)_2CO_2H$ = reagent reacted on only one side while the other side is hydrolyzed, with a mass = 193.12.

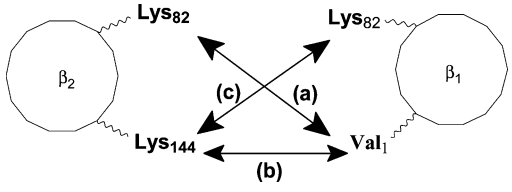


Figure 6. The three intersubunit cross-links present in the BPPCEP-modified Hb.

All other ions listed in Table 2 can be similarly accounted for as shown in the last column of the table. Some peptide fragments listed in Table 2 also suggest that the peptides contained the reagent BPPCEP linked only to one β chain, but was subsequently hydrolyzed before being able to react with another amino group on the other β chain. These include ions at *m/z* 1145.6, representing linkage at T_1 that contains the terminal valine 1, and at *m/z* 1643.4 and 3084.1, representing linkage at fragments T_{14}−T_{15} and T_{13}−T_{15}, both of which contain lysine 144.

Molecular Modeling Studies. The above conclusions reached by biochemical methods concerning the cross-linking sites of BPPCEP on hemoglobin were corroborated by molecular modeling studies. The modeling was performed on a Silicon Graphics workstation, using the X-ray coordinates of human HbA_0,[29] imported from the Brookhaven National Laboratory, Upton, New York. The reagent BPPCEP was built and energy-minimized employing the molecular modeling software Insight/Discover (Molecular Simulations, Inc., San Di-

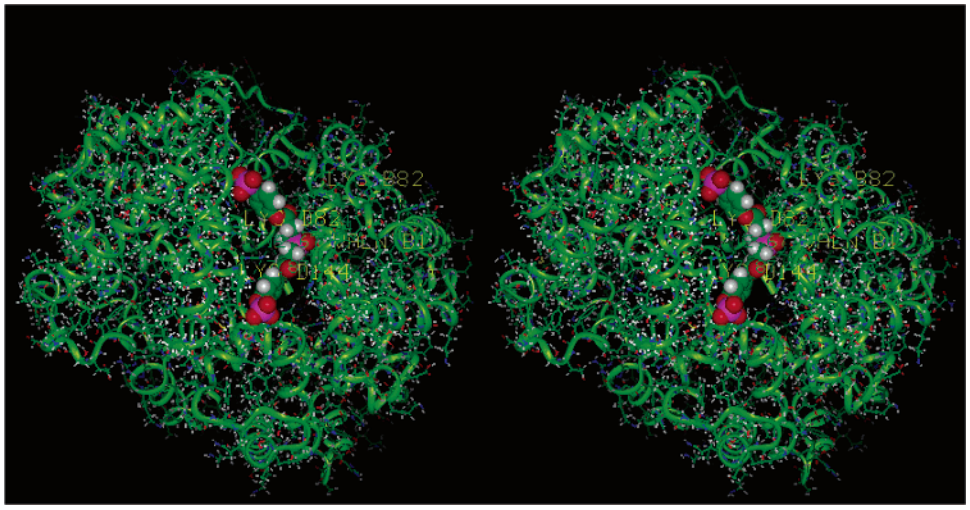


Figure 7. A stereoview of the energy-minimized BPPCEP−HbA_0 complex.

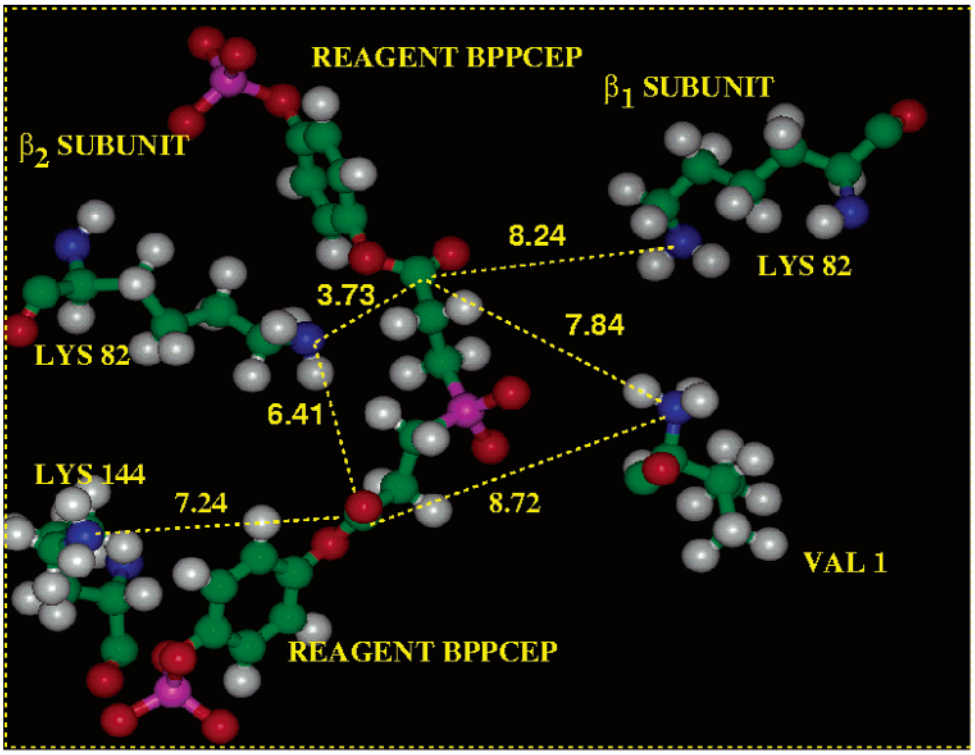


Figure 8. A close-up view of the energy-minimized BPPCEP−HbA_0 complex shown in Figure 7, showing the amino acid residues (lysines or α-terminus valine) whose appropriate (ϵ or α) amino groups fall within a 9 Å distance from the two cross-linking sites of the reagent.

ego, CA) and docked into the β-cleft of Hb. All atoms that were 15 Å or farther from the ligand were fixed with a temperature constant of 300 K. No constraints were applied to the remaining residues in and around the ligand site. The BPPCEP−Hb complex was then energy-minimized to convergence using consecutive Steepest Descent, Conjugate Gradient, VAO9A, and Newton energy minimization protocols. The final result gave a value for the Insight energy = 82.78 kcal/mol with an average absolute derivative = 0.00019 (standard deviation = 0.00017) and an average RMS derivative = 0.00025. A stereoview of the energy-minimized BPPCEP−HbA_0 complex is shown in Figure 7, while a close-up view of the same is given as a monoview in Figure 8, highlighting the concerned amino acid residues (lysines or α-terminus valine) whose appropriate (ϵ or α) amino groups fall within a 9 Å distance from the two cross-linking sites of the reagent.

The stereoview in Figure 7 clearly shows that the reagent BPPCEP fits snugly into the β-cleft (BPG pocket) of hemoglobin. The close-up in Figure 8 suggests that there are a total of four amino acid residues whose potentially cross-linkable amino groups lie within a 9 Å distance from the two activated ester carbonyl groups of the reagent, the potential cross-linking sites. Taking into account the sum of the van der Waals radii of the two concerned atoms involved in cross-linking [(C = 2.0 + N = 1.5) = 3.5 Å total], coupled with the fact that the amino acids lining the periphery of the β-cleft are known to extend as much as 3−5 Å into the cleft to effect cross-

links,[30] the consideration of all amino acids lying within a 9.0 Å radius from the reagent sites as potential candidates for cross-linking is reasonable. A careful analysis of the distance data suggest there are four possibilities for the formation of cross-links between the reagent and the mentioned amino acid residues: (a) β_1-Val 1 to β_2 Lys 82 [(NH-7.84-CO and NH-6.41-CO) or (NH-8.72-CO and NH-3.73-CO)], (b) β_1Val 1 to β_2 Lys 144 [(NH-7.84-CO and NH-7.24-CO)], (c) β_1Lys 82 to β_2 Lys 144 [(NH-8.24-CO and NH-7.24-CO)], and (d) β_1-Lys 82 to β_2 Lys 82 [(NH-8.24-CO and NH-6.41-CO)]. Out of the four possible cross-links listed, we have experimentally observed the presence of the first three as described above under Tryptic Digestion and MALDI MS Analyses. It is not clear at the present time why the fourth cross-link (β_1Lys 82 to β_2 Lys 82) predicted by the molecular modeling studies has not been experimentally realized. A possible explanation is that the ϵ-amino group of β_2 Lys 82 is significantly closer (3.73 Å) to one of the reagent carbonyl groups as compared to β_1Lys 82 (8.24 Å) to the same carbonyl group. This means that β_2Lys 82 will preferentially react with this carbonyl group over β_1Lys 82. However, the measured distance between the ϵ-amino group of β_1 Lys 82 and the other carbonyl group of the reagent is too far (13.24 Å) to effect any cross-link. In this regard, it is also possible that out of the two possible modes of cross-link listed above for the cross-link between β_1Val 1 and β_2 Lys 82, the first mode with a 7.84 Å tether is more likely than the second with 8.72 Å.

Oxygen Equilibrium Studies. Oxygen-binding studies were performed using rapid enzymatic oxygen depletion in a Hemox-Analyzer spectral cuvette. The three BPPCEP-modified hemoglobins, corresponding to peaks A, B, and C from Figure 3, were collected using preparative cation exchange HPLC and further analyzed for oxygen affinity (P_{50})[31] characteristics at 37 °C. For the sake of comparison, the P_{50} values were also measured for the α−α cross-linked Hb[32] under identical conditions. The graphs of log PO_2 vs fraction of Hb saturation for peaks A, B, and C of the BPPCEP-modified Hb, as contrasted with *a*-α cross-linked Hb,[32] are shown in Figures 9A, 9B, and 9C, respectively. The computed values for P_{50},[31] generated from the above oxygen equilibrium curves (OEC's) and the Hill coefficient (an indicator of oxygen-binding cooperativity),[33] generated from the Hill plots of the respective P_{50} values of the modified hemoglobins, are collected in Table 3. As can be seen from the table, the three BPPCEP-modified Hb's showed decreased P_{50} values (increased oxygen affinity) when compared with that of the α−α cross-linked Hb.[32] These Hb's exhibited P_{50} values close to the value obtained with unmodified HbA_0 (13.1 Torr) using this reaction system but without the observed cooperativity as indicated by a Hill coefficient of 2.5. The P_{50} of the control α−α cross-linked Hb (33.6 Torr) is close to that of the whole blood, which has a P_{50} of 26−27 Torr.[13,23,34]

The listed Hill coefficients for the three BPPCEP-modified hemoglobins in Table 3 suggest that there is a significant loss of oxygen binding cooperativity. This lack of cooperativity is also evident from the lack of sigmoidal shape of OEC's of peaks A, B, and C (Figures 9A, 9B, and 9C). Nevertheless, while the Hill Coefficient would normally be a quantitative indicator of oxygen binding cooperativity, the computed values of *n* from the Hill plots of the BPPCEP-modified Hb is somewhat less meaningful in view of the heterogeneous nature of the cross-linking. Furthermore, all of the current red blood cell substitutes that have been approved by the

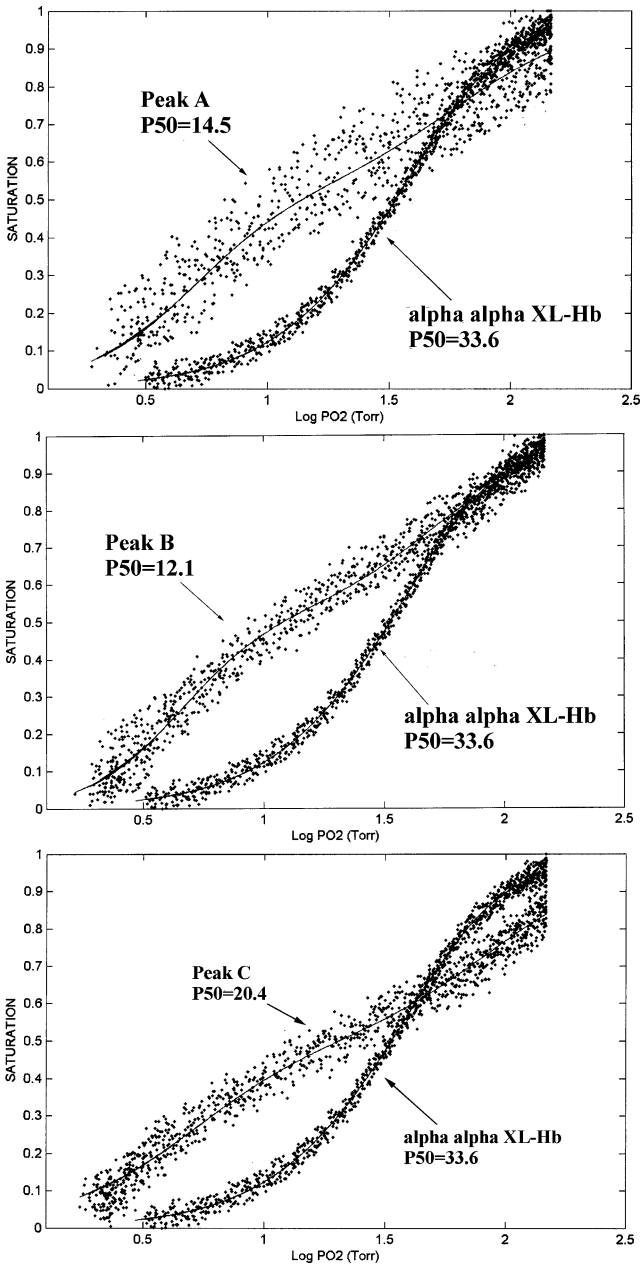


Figure 9. A. The oxygen equilibrium curves (OEC) for Peak A of Figure 3, as contrasted with those for the α−α cross-linked Hb. B. The OECs for Peak B of Figure 3, as contrasted with those for the α−α cross-linked Hb. C. The OECs for Peak C of Figure 3, as contrasted with those for the α−α cross-linked Hb.

Table 3. Results of Oxygen Equilibrium Measurement Studies for Peaks A, B, and C of the BPPCEP−Modified Hemoglobins, along with Those for Stroma-Free Hb (SFHb) and the α−α Cross-Linked Hb

property	HbA_0	α−α cross-linked Hb	Peak A	Peak B	Peak C
P_{50} (mM/Hg)	13.1	33.6	14.5	12.1	15.5
Hill coefficient (*n*)	2.5	1.9	0.9	0.5	0.9

FDA or those that are presently in phase III clinical trials demonstrate little or no cooperativity at all.[4,6,35,36]

To verify if the yield of the BPPCEP-modified hemoglobins could be further improved by changing experimental conditions, the cross-linking reactions were carried out under deoxygenated and carbonmonoxy conditions. Hemoglobin was converted into the deoxy form by adding SFHb (25 mL) to a 50 mL-vacuum vial, and the partial pressure of oxygen in the vial was continuously lowered to reach 10^{-3} Torr, while flushing with argon at 37 °C. The reactions with BPPCEP were then carried out as before but in a glovebox under an inert atmosphere (argon). The carbonmonoxy hemoglobin was prepared in the same manner except that the flask was continually flushed with carbon monoxide (CO) and that the reactions did not need to be carried out in the glovebox because carbonmonoxy hemoglobin is known to be very stable under ambient conditions. Neither the deoxy nor the carbonmonoxy form of hemoglobin had any effect in increasing the overall yield of the cross-linked product. It should be noted, however, that none of the previous cross-linking reagents synthesized in our lab succeeded in cross-linking with hemoglobin in these states. Increasing the temperature on the other hand did show slightly improved results especially at 37 °C. However, noticeable amounts of precipitate could be observed after about 2 h, indicating that hemoglobin was being denatured at this temperature.[37]

Conclusion

We have synthesized a novel, water-soluble, site-specific hemoglobin cross-linking reagent in four easy, convenient, and efficient steps, which can be easily scaled- up to kilogram quantities. The reagent mimics BPG in the number of phosphate groups as well as in the number of anionic charges present in an aqueous medium and, like BPG, is very specific for the β-cleft of Hb. Once in the BPG pocket, it covalently cross-links the two β-subunits. Another convenient and beneficial feature of the reagent is that the cross-linking can be performed at neutral pH and ambient, oxygenated reaction conditions. The tryptic digestion and MALDI mass spectral analyses of the modified β-chain fragments suggest that the reagent BPPCEP forms three specific intersubunit cross-links between the following amino acid residues: (a) β_1Val 1 to β_2 Lys 82, (b) β_1Val 1 to β_2 Lys 144, and (c) β_1Lys144 to β_2 Lys 82. These cross-linking sites are remarkably consistent with those predicted by molecular modeling studies. The three isolated BPPCEP-cross-linked Hb products exhibited oxygen affinities close to that of unmodified Hb (P_{50} = 13.1 Torr), which is higher than that of whole blood (P_{50} = 26−27 Torr) but is still in a range capable of delivering oxygen.[38] A new generation of chemically modified hemoglobins with even lower P_{50}s (higher oxygen affinities) and decreased cooperativity are under development as oxygen therapeutics based on the idea that they may enhance targeted delivery of oxgyen to the tissues that need it most.[39−41] However, the reagent suffered from one major drawback in that its oxygen-binding cooperativity characteristics, as measured by the Hill Coefficient n, was significantly lower ($n < 1.0$) than that of the whole blood ($n = 2.3-2.5$). Upon further scrutiny, it became apparent that in view of the observed multiple cross-linking sites and the consequent heterogeneity of the BPPCEP-modified Hb, the n value obtained from the Hill plot was less reliable and meaningful. Therefore, it appears necessary to further enhance BPPCEP's specificity by judicious structural modifications, which would minimize the multiple cross-linked products. The modified reagent should not only be specific to the β-cleft but also to the amino acid residues lying within the β-cleft.

Finally, the discovery of an ideal Hb cross-linking agent is only a first step in a long journey toward an optimal, physiologically feasible, and clinically compatible blood substitute. As with SFHb and all the known modified hemoglobin products containing a single cross-link and weighing 64 kDa, even the most ideal reagent-modified Hb products will undoubtedly suffer from the historical problems of short circulation times in the blood stream (half-lives), a colloidal osmotic pressure (COP) that limits the amount of oxygen carrying heme that can be loaded, and vasoconstriction.[13,16,23,34,36] To alleviate some of these problems, it may be necessary to further join two or more intramolecularly cross-linked Hb tetramers by an intermolecular bridge, forming uniform high molecular weight dendrimeric Hb products. Such products would not only further prevent renal and reticulo-endothelial elimination from the blood stream, but also would allow loading of more oxygen carrier as well as reduction of the vasoconstriction (and the consequent hypertension) that has been attributed to the nitric oxide scavenging capability of the cross-linked Hb tetramers.[42]

Experimental Section

Organic Synthesis. Commercial reagents and chemicals were used without further purification from Aldrich. Solvents were dried prior to use. Reagents for the preparation of buffers were of analytical grade or better. Highly purified stroma-free hemoglobin A in the oxygenated state was obtained from Walter Reed Army Institute of Research, Washington, D.C. ^{1}H and ^{13}CNMR spectra were obtained using a 300 MHz General Electric QE-300 instrument. Mass spectrometric data were obtained by Dr. Susanne Hoffmann-Benning at the Michigan State University Mass Spectrometry Facility. Elemental Microanalyses were performed by either (a) Atlantic Microlab, Inc, Norcross, GA, or (b) Galbraith Laboratory, Inc, Knoxville, TN.

4-Benzyloxyphenyl Acrylate (3). To a flame-dried, 50 mL round-bottom flask equipped with a stir bar were added 35 mL of anhydrous THF and commercially available 4-benzyloxyphenol (**1**) (1.00 g, 4.99 mmol). The solution was brought to 0 °C, potassium *tert*-butoxide (0.59 g, 4.99 mmol) added, and the mixture stirred for 20 min. Acryloyl chloride (0.45 g, 4.99 mmol) was then added by a syringe at 0 °C and the solution stirred at room temperature for 24 h. The solvent was removed under reduced pressure to leave a clear yellowish solid. The solid was then dissolved in diethyl ether, the precipitated salt filtered, and the filtrate thoroughly washed with water. The organic and aqueous layers were then separated, and the organic layer was dried over anhydrous $MgSO_4$. Filtration followed by solvent removal under reduced pressure yielded 1.22 g (99%) of pure (**3**) as a yellowish powder. This solid was used for the next step without further purification. Mp 85−88 ° C. ^{1}H NMR ($CDCl_3$): δ 7.41 (m, 5H, aromatic *H*), 7.03 (d, J = 9 Hz, 2H, aromatic *H*), 6.98 (d, J = 9 Hz, 2H, aromatic *H*), 6.62−6.56 (dd, J = 17.2 Hz, J = 1.5 Hz, 1H, CH=C*H*$_2$), 6.35−6.26 (dd, J = 17.6 Hz, J = 10.6 Hz, 1H, C*H*=CH$_2$), 6.01−5.97 (dd, J = 10.6 Hz, J = 1.5 Hz, 1H, CH=C*H*$_2$), 5.05 (s, 2H, OC*H*$_2$-aromatic). Anal. ($C_{16}H_{14}O_3$) C, H.

Bis[2-(4-benzyloxyphenoxy)carbonylethyl]phosphinic Acid (4). To an ice-cold solution of hypophosphorus acid (50%, 20 mL, 0.15 mol) was added dropwise, via a coldfinger, concentrated ammonia (15 mL, 0.15 mol), and the mixture was allowed to stir for 2 h. The reaction mixture was then made anhydrous by repeated coevaporations with anhydrous toluene (10 × 100 mL) under reduced pressure using a rotary evaporator, resulting in ammonium phosphinate, as a white crystalline solid. The solid was dried overnight with phosphorus pentoxide, and the salt was used without further purification. To a stirred solution of ammonium phosphinate (1.19 g, 14.3 mmol) in 250 mL anhydrous CH_2Cl_2 at 0 °C were added chlorotrimethylsilane (5.45 g, 50.1 mmol) and triethylamine (5.07 g, 50.1 mmol) while under argon, and the mixture was allowed to come to room temperature. After 2 h of stirring, the solution was again brought to 0 °C, 4-benzyloxyphenyl acrylate (7.30 g, 28.7 mmol) dissolved in dry CH_2Cl_2 was added, and the mixture was stirred for an additional 24 h. The reaction was worked up by the addition of 600 mL of 0.1 N HCl with continuous stirring causing the instant precipitation of a white sticky solid. Filtration of the white solid followed by continuous washing with water, hexanes, and diethyl ether and then drying while on a Buchner funnel under vacuum by water aspiration provided 6.0 g (73%) of analytically pure (**4**) as a fine white powder. It was dried with phosphorus pentoxide in a drying piston at 81 °C for 24 h before using in further reactions. This compound is insoluble in all solvents except hot DMF and hot DMSO. Mp: 224−227 °C, ^{1}H NMR (DMSO): δ 2.04−1.94 (m, 4H, O=PCH_2CH$_2$COO), 2.80−2.71 (m, 4H, O=PCH$_2$CH_2COO), 3.50−3.05 (bs, 1H, O=PO*H*), 5.07 (s, 4H, benzyl-CH_2), 5.07 (2 × d, J = 9 Hz, 8H, aromatic *H*), 5.07 (apparent m, 10H, aromatic *H*); HRMS (FAB) cacld for $C_{32}H_{33}O_8P$ (MH^+) = 575.1835, found: 575.1833. Anal. ($C_{32}H_{32}O_8P$) C, H, P.

Bis[2-(4-hydroxyphenoxy)carbonylethyl]phosphinic Acid (5). Method A. Bis[2-(4-benzyloxyphenoxy)carbonylethyl]phosphinic acid (**4**) (769 mg, 1.33 mmol) was dissolved into almost boiling DMF and then poured into a 250 mL hydrogenation flask. A mixture of catalytic amounts of 10% Pd/C and palladium black was added to the hydrogenation flask, the flask sealed and connected to the Parr shaker, and the system purged a few times with hydrogen gas to rid the closed system of any residual air. The hydrogenation flask was then brought to 90 psi with hydrogen gas while heating to approximately 145 °C with continuous shaking. (***Caution***: *There is a steady increase in pressure as the temperature rises, and the pressure must never be allowed to exceed 90 psi due to the risk of explosion!*) The heating was discontinued after 15 min and allowed to cool to room temperature after which time the system was again heated to the same temperature as above and allowed to cool again. This sequence was repeated three times, and then the flask was left under hydrogen (70 psi) on the Parr shaker for 24 h at room temperature. The reaction mixture was then filtered through a bed of Celite equilibrated with DMF and the solvent removed under reduced pressure to leave a colorless oil. Trituration of this oil with acetone and diethyl ether precipitated a white powder. Filtration followed by washing with hexanes and diethyl ether while on the Buchner funnel provided 0.43 g (81%) of pure **5** as a white light fine powder. Mp: 193−196 °C; ^{1}H NMR (CD_3OD): δ 2.16−2.06 (m, 4H, O=PCH_2CH$_2$COO), 2.84−2.75 (m, 4H, O=PCH$_2$$CH_2$COO), 3.45−3.08 (bs, 1H, O=POH, only in DMSO), 6.70−6.67 (d, J = 9 Hz, 4H, aromatic *H*), 6.86−6.83(d, J = 9 Hz, 4H, aromatic *H*), [9.4 (s, 2H, phenol OH, only in DMSO)]; HRMS (FAB) cacld for $C_{18}H_{20}O_8P$ (MH^+) = 395.0911, found: 395.0910. Anal. ($C_{18}H_{19}O_8P$·0.5 H_2O) C, H, P.

Method B. Bis[2-(4-*tert*-butoxyphenoxy)carbonylethyl]phosphinic acid (**8**) (900 mg, 1.77 mmol) was dissolved into 35 mL of CH_2Cl_2, and trifluoroacetic acid (5 mL) was added. The mixture was then refluxed for 4 h and cooled to room temperature, and the solvent was removed under reduced pressure to leave a crude powder. To this power was added 100 mL of diethyl ether and the unndissolved powder filtered and washed with more diethyl ether to provide 700 mg (100%) of pure **5** as a white light fine powder. The spectral and analytical data of this compound were identical to those of **5** obtained by Method A above.

4-*tert*-Butoxyphenyl Acrylate (7). To a flame-dried 50-mL round-bottom flask equipped with a stir bar were added 35 mL of anhydrous THF and commercially available 4-(*tert*-butoxy)phenol (**6**) (6.00 g, 36.1 mmol). The solution was brought to 0 °C in an ice bath, triethylamine (6.03 mL, 43.3 mmol) added, and the mixture stirred for 1 h. Acryloyl chloride (3.27 g, 36.1 mmol) was then added by a syringe at 0 °C and the solution stirred for 48 h. The solution was then filtered to get rid of triethylamine hydrochloride that precipitated during this time. The solvent was then removed under reduced pressure to leave dark brown oil that was dissolved in diethyl ether and thoroughly washed 1 M HCl (5 × 300 mL). The organic and aqueous layers were then separated and the organic layers dried over anhydrous $MgSO_4$. Filtration followed by solvent removal under reduced pressure yielded 7.70 g (97%) of analytically pure **7** as a dark brown oil that solidified upon standing leaving clear light orange colored crystals. These crystals were used without further purification. ^{1}H NMR ($CDCl_3$): δ 7.01 (d, 4H, Ar), 6.61−6.55 (dd, J = 17.2 Hz, J = 1.5 Hz, 1H, CH=CH_2), 6.35−6.26 (dd, J = 17.6 Hz, J = 10.3 Hz, 1H, C*H*=CH$_2$), 6.01−5.97 (dd, J = 11.7 Hz, J = 1.5 Hz, 1H, CH=CH_2), 1.33 (s, 9H, OC(CH_3)$_3$). Anal. ($C_{13}H_{16}O_3$) C, H.

Bis[2-(4-*tert*-butoxyphenoxy)carbonylethyl]phosphinic Acid (8). To an ice-cold solution of hypophosphorus acid (50%, 20 mL, 0.15 mol) was added dropwise, via a coldfinger, concentrated ammonia (15 mL, 0.15 mol), and the mixture was allowed to stir for 2 h. The reaction mixture was then made anhydrous by repeated coevaporations with anhydrous toluene (10 × 100 mL) under reduced pressure using a rotary evaporator, resuting in ammonium phosphinate, as a white crystalline solid. The solid was dried overnight with phosphorus pentoxide and the salt used without further purification. To a stirred solution of ammonium phosphinate (600 mg, 7.23 mmol) in 35 mL anhydrous CH_2Cl_2 at 0 °C were added chlorotrimethylsilane (3.22 mL, 25.3 mmol) and triethylamine (3.53 mL, 25.3 mmol) while under argon, and the mixture was allowed to come to room temperature. After 2 h of stirring, the solution was again brought to 0 °C, 4-*tert*-butoxyphenyl acrylate (3.34 g, 15.2 mmol) dissolved in dry CH_2Cl_2 was added, and the mixture was stirred for an additional 24 h. The reaction was worked up by the washing with 1 N HCl (4 × 250 mL). The organic and aqueous layers were separated and the organic layers dried over anhydrous $MgSO_4$. Filtration followed by solvent removal under reduced pressure left the crude acid that was further washed with diethyl ether and filtered to yield 3.66 g (66%) of pure bis[2-(4-*tert*-butoxyphenoxy)carbonylethyl]-phosphinic acid (**8**) as a fine brownish white powder. Mp: 107−109 °C, ^{1}H NMR ($CDCl_3$): δ 1.32 (s, 18H, OC(CH_3)$_3$, 2.17−2.27 (m, 4H, O=PCH_2CH$_2$COO), 2.88−2.98 (m, 4H, O=PCH$_2$CH_2COO), 6.95 (s, 8H, aromatic *H*); HRMS (FAB) cacld for $C_{26}H_{36}O_8P$ (MH^+) = 507.2150, found: 507.2150. Anal. ($C_{26}H_{35}O_8P$) C, H, P.

Bis[2-(4-phosphonooxyphenoxy)carbonylethyl]-phosphinc Acid (BPPCEP, 1). Di-*tert*-butyl diisopropylphosphoramidite (756 mg, 2.72 mmol) was added to a solution of bis[2-(4-benzyloxyphenoxy)carbonylethyl]phosphinic acid (**5**) (489 mg, 1.24 mmol) and 1*H*-tetrazole (364.9 mg, 5.20 mmol) in 50 mL of anhydrous DMF. After 3 h of stirring at room temperature, the temperature was dropped to −76 °C, and 30% hydrogen peroxide (4 mL) was added and stirred for an additional 2 h. The reaction was then diluted with 300 mL of ethyl acetate and the excess hydrogen peroxide quenched with saturated aqueous sodium metabisulfite (5 × 100 mL) followed by washing with distilled water (3 × 100 mL). The aqueous and organic layers were separated, the organic layers were dried with anhydrous $MgSO_4$ and filtered, and the solvent was removed under reduced pressure to yield a colorless oil. The oil was dissolved into a minimum amount of CH_2Cl_2, TFA (10 mL) was added, and the solution was stirred for approximately 15 min after which time some precipitation was noticeable. Addition of diethyl ether caused a white precipitate to crash

out of solution. Filtration and continuous washing with diethyl ether yielded 0.49 g (71%) of analytically pure (BPPCEP, **1**) as a fine hygroscopic white powder. Mp: 155−160 °C; ^{1}H NMR (D_2O): δ 2.85−2.74 (m, 4H, O=PCH_2CH_2COO), 2.13−2.04 (m, 4H, O=PCH_2CH_2COO), 6.99−6.96 (d, J = 8.7 Hz, 4H, aromatic H), 7.10−7.07 (d, J = 9 Hz, 4H, aromatic H); HRMS (FAB) cacld for $C_{18}H_{22}O_{14}P_3$ (MH^+) = 555.0223, found: 555.0221. Anal. ($C_{18}H_{21}O_{14}P_3$) C, H, P.

Pentasodium Salt of Bis[2-(4-phosphonooxyphenoxy)-carbonylethyl]phosphinic Acid (BPPCEP Salt). A glass column (30 cm × 100 cm) was packed with the ion-exchange resin AG 50W−X8 (H^+ form) (Bio-Rad). The resin was converted to its Na^+ form by equilibration with 3 N NaOH until the pH was basic (pH 14 or more). The column was then thoroughly washed with deionized water until the pH of the eluent was neutral (or equal to the pH of the water). Reagent **1** (220 mg, 0.396 mmol) was then dissolved into deionized water (25 mL) loaded onto the top of the resin, and the column was eluted with an additional 350 mL of water. The combined fractions were frozen using liquid nitrogen and lyophilized overnight to yield 0.26 g (100%) of a yellowish globular salt. This salt was used for hemoglobin modification without further purification. ^{1}H NMR (D_2O): δ 1.85−1.81 (m, 4H, O=PCH_2-CH_2COO), 2.73−2.69 (m, 4H, O=PCH_2CH_2COO), 6.98−6.95 (d, J = 9 Hz, 4H, aromatic *H*), 7.10−7.07 (d, J = 9 Hz, 4H, aromatic *H*).

Reaction of BPPCEP with Hemoglobin. A stock solution (2 mM) of human hemoglobin A_0 was made by dilution of 2.80 mL of concentrated hemoglobin (13.7 g/dL) in the oxygenated state at room temperature with 0.20 mL of 0.1 M phosphate buffer, pH 7.4. Next, into six 2 mL Eppendorf tubes was added 500 μL of the stock (2 mM) Hb solution and 1.16 mL of 0.1 M phosphate buffer, pH 7.4. A 150 mM stock reagent solution of BPPCEP was prepared by dissolving 0.18 g of BPPCEP (pentasodium salt form) into 1.8 mL of 0.1 M phosphate buffer, pH, 7.4) and used immediately in the cross-linking reactions by adding 333 μL to five of the above Eppendorf tubes for a total volume of 2 mL per tube. The final concentration was 25 mM (50-fold excess) for the reagent and 0.5 mM for Hb. The sixth tube was used for control purposes and was brought to a volume of 2 mL with additional buffer. The reactions were then kept at 24 °C and monitored by HPLC using a C4 column (conditions listed below) for 3−4 h. After that time, no further modifications were detected in the chromatograms, and the solutions were frozen and stored at −81 °C until further analyses could be performed.

Analysis and Purification of Modified Hemoglobins. The above cross-linking solutions were passed through a Sephadex G-100 column (2.5 × 45 cm) equilibrated with 0.25 mM Tris buffer, pH 7.4, 1 M $MgCl_2$, and eluted with the same solvent to denature any un-cross-linked Hb. The fractions were combined and then simultaneously desalted, and the buffer was exchanged (0.1 M Tris, pH 7.4) using Millipore Biomax 10 concentrators. The modified and native heme and globin chains were separated and analyzed by reverse-phase HPLC using a C-4 Vydac 214TP1010 column (10 × 250 mm) with developer A (CH_3CN, 0.1% TFA), and developer B (H_2O, 0.1%TFA). The column was equilibrated with 35% developer A and 65% developer B and a gradient employed to give a final composition of 50% developer A and 50% developer B over a period of 90 min at 3 mL per minute while monitoring at 214 nm.

Intact cross-linked hemoglobins were separated and analyzed by HPLC cation exchange chromatography using two semipreparative Synchropak CM 300 columns (10 × 250 mm) in series. The solvent system consisted of developer A (0.03 M bis-Tris buffer, 0.15 M sodium acetate, 0.0015 M potassium cyanide, pH adjusted to 6.40 with acetic acid) and developer B (0.03 M bis-Tris buffer, 0.0015 M potassium cyanide, pH adjusted to 6.4 with acetic acid). A linear gradient was employed using 80% developer B and 20% developer A to 20% developer B and 80% developer A over a period of 90 min with a flow rate of 3 mL per minute while monitoring at 415 nm. Fractions were collected and then concentrated using Millipore Biomax 30 concentrators. The concentrated Hb was then added to 0.1 M Tris buffer, pH 7.4, and frozen at −81 degrees °C until further use.

SDS−PAGE Analysis of Hemoglobins. The extent of the cross-linking of the globin chains was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE). The protocols for the Mini-PROTEAN II Electrophoresis Cell (Bio-Rad, Hercules, CA) were followed accordingly. Approximately 40 μg of hemoglobin isolated from the HPLC run using the Synchropak CM 300 columns was loaded onto 12% polyacrylamide gel, 0.375 M Tris-HCl, pH 8.8, and ran at 180 V for 45 min. The protein bands on the gel were then stained for 45 min with Coomassie brilliant blue and then destained using Bio-Rad destain.

Analyses of Globin Chains by Tryptic Digestion. The native and modified globin chains were purified for tryptic digestion by preparative reverse-phase HPLC using a C-4 Vydac 214TP1010 column (22 × 250 mm). The column was equilibrated with 35% developer A (CH_3CN, 0.1% TFA) and 65% developer B (H_2O, 0.1%TFA) and a linear gradient employed to give a final composition of 50% developer A and 50% developer B over a period of 95 min at 13 mL per minute while monitoring at 214 nm. The appropriate fractions were then pooled and frozen in a liquid nitrogen bath followed by lyophilization to a powder. The isolated β and XL-β chains (0.15 mg) were then dissolved into 2 mL of 0.2 M NH_4HCO_3 buffer, pH 8.0, and 57.7 μL of TPCK-trypsin (13 mg mL^{-1}) was added. Digestion was carried out for 24 h at 37 °C with gentle agitation. Digestion was terminated by freezing the samples and then lyophilizing them to a powder. The digested globin chains were analyzed by reverse phase HPLC using a C18 Phenomenex column (4.5 × 250 mm, 5 μm, 300 A) and 5% developer A (CH_3CN, 0.1% TFA) and 95% developer B (H_2O, 0.1%TFA), and a linear gradient was employed to give a final composition of 50% developer A and 50% developer B over a period of 100 minutes at 1 mL per minute while monitoring at 214 nm. The fractionated tryptic peptides were then analyzed by matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF).

Oxygen-Binding (P_{50}) Studies. A rapid method for measurement of hemoglobin oxygen equilibrium curves using enzymatic oxygen depletion[43] was adapted for use in the Hemox-Analyzer (TCS Medical Products Co., Huntington Valley, PA). Oxygen equilibrium curves were recorded by deoxygenation of air-equilibrated hemoglobin in 0.1 M bis-Tris buffer + 0.1 M NaCl (pH 7.4, 37 °C) in the spectral cuvette of the instrument. The conversion of protocatechuic acid to 3-carboxy-*cis*,*cis*-muconate by protocatechuate 3,4-dioxygenase is an effective oxygen scavenging reaction with several buffer formulations that can achieve oxygen partial pressures below 4 Torr. This system of oxygen removal eliminates potentially destructive bubbling of protein solutions with nitrogen and oxygen and provides complete curve generation in less than 4 min. Curves generated with fresh whole blood using the enzyme system in TCS's proprietary medium for red blood cells are typical of those reported in the literature for this instrument using standard gas bubbling techniques.

During each run, data was downloaded to a computer for subsequent analysis. Postprocessing was carried out in MATLAB (version 6.5.1 for Windows; The Math Works, Inc., Natick, MA). Oxygen dissociation curves were fitted to a modified Adair equation and the Hill equation[44] using a multidimensional unconstrained nonlinear minimization subroutine (Nelder-Mead),[45,46] which is a component of MATLAB's Optimization Toolbox. The partial pressure of oxygen (Torr) where hemoglobin is half-saturated (P_{50}) was calculated from the fitted dissociation curves by nonlinear minimization, using the Gauss−Newton method[47] in MATLAB.

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Supporting Information Available: Elemental microanalytical data for compounds **1**, **3**, **4**, **5**, **7**, and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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