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Bis[2-(4-carboxyphenoxy)carbonylethyl]phosphinic Acid (BCCEP): A Novel Affinity Reagent for the β-Cleft Modification of Human Hemoglobin

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Abstract—The design, synthesis, and hemoglobin cross-linking studies of a novel organic reagent, bis[2-(4 carboxyphenoxy)carbonylethyl]phosphinic acid (BCCEP, **1**) have been reported. The reagent was designed with the aid of molecular modeling, employing crystal coordinates of human hemoglobin A_0. It was synthesized in three steps commencing from 4-*t*-butoxycarbonylphenol. The tri-sodium salt of **1** was employed to cross-link human oxyHb. While SDS–PAGE analyses of the modified hemoglobin product pointed to the molecular mass range of 32 kDa, the HPLC analyse suggested that the cross-link had formed between the β_1-β_2 subunits. The oxygen equilibrium measurements of the modified hemoglobin at 37 °C showed significantly reduced oxygen affinity ($P_{50} = 31.3$ Torr) as compared with that of cell-free hemoglobin ($P_{50} = 6.6$ Torr). The sigmoidal shape of O_2 curves of the modified Hb pointed to reasonable retainment of oxygen-binding cooperativity after the cross-link formation. Molecular dynamics simulation studies on the reagent-HbA_0 complex suggested that the most likely amino acid residues involved in the cross-linking are N-terminus Val-1 or Lys-82 on one of the-chains, and Lys-144 on the other. These predictions were consistent with the results of MALDI-MS analyses of the peptide fragments obtained from tryptic digestion of the cross-linked product.

Introduction

The current health scare of blood-borne diseases such as AIDS and hepatitis has heightened the interest in developing an alternative to blood for emergency transfusion. The shortage, brief shelf-life, and storage difficulties of whole blood, coupled with the necessity for typing, cross-matching, and thorough screening for bacteria and viruses, have prompted continuous efforts to develop an acellular oxygen carrier for well over half a century.[1] In this regard, the two intensely studied blood substitutes include fluorocarbon emulsions[1–3] and cell-free hemoglobin solutions.[1–4] However, owing to inadequate oxygen affinity and the absence of a metabolic mechanism other than the expired air for excretion, coupled with the associated immunotoxicity, the use of fluorocarbons as resuscitation fluids may be impractical.[2] This leaves hemoglobin as the best candidate for a blood substitute. Being a natural oxygen carrier, hemoglobin offers a number of obvious advantages including, but not limited to, its abundance, facile metabolism, and high tolerance by the body.[4]

There are, however, two major problems inherent in the use of cell-free hemoglobin that must be overcome before it can be employed in place of whole blood. First, separated from red blood cells, hemoglobin, which normally exists as a tetramer, easily dissociates into two α,β-dimers, and thus does not stay in circulation very long. It undergoes renal excretion within 1–4 h after

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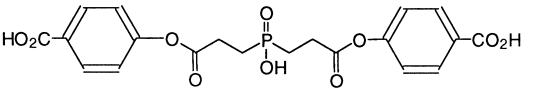
transfusion, often causing massive hemoglobinurea.[4] Second, cell-free Hb has too high an oxygen affinity, which prevents it from optimal delivery of oxygen, acquired from lungs, to tissues. A significant part of these problems has now been traced to the hemoglobin's natural allosteric regulator 2,3-diphosphoglycerate (DPG), a highly anionic small molecule that is present in intact erythrocytes, but deficient in Hb isolated from red blood cells. DPG aligns itself between the two β subunits (molecular mass = 16 kDa each) of the hemoglobin tetramer, and is surrounded by several positively charged amino acid residues residing on the β-subunits, thus forming an anionic sink that is commonly referred to as the β-cleft or the DPG pocket. Inside the red blood cells, the predominant function of DPG is to lower, and thus tune, the oxygen affinity of Hb by shifting the deoxy–oxy equilibrium toward the deoxy form. DPG favors the deoxy form over the oxy because of the relatively larger pocket size of the former. The covalent cross-linking of hemoglobin subunits with a DPG mimic, preferably in the β-cleft site, is anticipated to considerably alleviate these intrinsic problems of cell-free hemoglobin.[1]

Efforts have since been focused on intramolecular (interdimer)/intermolecular (inter-tetramer) cross-linking with multifunctional organic reagents such as dialdehydes,[5] bis-imidates,[6] diaspirins,[7,12c] triaspirins,[8a,8b] tetraaspirins,[8c] bis-isothiocyanates,[9] bis-enol-ethers,[10] bis-pyridoxal phosphates,[11] and bis/tris(methyl phosphates).[12] With only a few notable exceptions that are currently undergoing clinical trials,[1] the majority of modified hemoglobins still suffer from too high oxygen affinity, too low intravascular retention time or too facile autooxidation to MetHb that lacks the capacity to carry oxygen.[1] Although some reagents are known to be specific for the β-cleft, most of them are non-specific or random cross-linkers that yield heterogeneous mixtures of species, ranging in molecular mass from 64 kDa to 600 kDa.[1–4] The most desirable reagent would be one that not only possesses adequate physicochemical and stereoelectronic characteristics so as to be specifically directed to the DPG pocket (β-cleft) of hemoglobin like an affinity label, but also invokes optimally stabilizing Hb-reagent interactions even after the cross-link formation with little, if any perturbation of the native structure of hemoglobin. In this regard, even those Hb cross-linking reagents that are known to be specific for the β-cleft might be less than ideal. The reagent should, in addition, enable cross-linking under convenient, ambient, oxygenated reaction conditions noting that a number of earlier Hb cross-linkings have been performed on deoxyHb under stringent deoxygenated media. Finally, the reagent should be easy and inexpensive to synthesize on a large scale, considering the fact that several otherwise promising reagents were not pursued by industry because of perceived high costs of production.[1]

In an attempt to address these issues, we report herein our work on the design and synthesis of a novel bifunctional organic reagent, bis[2-(4-carboxyphenoxy)carbonylethyl]phosphinic acid (BCCEP, **1**), employing a combination of modern molecular modeling and synthetic organic techniques. Also reported are the hemoglobin cross-linking studies using BCCEP, along with physicochemical and functional data on the modified hemoglobin, as well as the elucidation of sites of cross-link employing MALDI MS analyses of tryptic digests of the cross-linked protein.



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REAGENT BCCEP

Materials and Methods

Molecular modeling studies

Molecular modeling was performed on a Silicon Graphics workstation, using the software Insight/Discover (Molecular Simulations, Inc., San Diego, CA, USA). The X-ray coordinates of human HbA_0,[13] imported from the Brookhaven National Laboratory, Upton, NY, USA, were employed for molecular modeling studies. Reagent **1** was designed as described under Results and Discussion. It was energy-minimized and docked into the β-cleft (DPG pocket) of hemoglobin, followed by energy minimization of the reagent-Hb complex. All atoms that were 12 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 complex was minimized to convergence using consecutive Steepest Descent and Conjugate Gradient (VAO9A and Newton) minimization protocols (see Figure 1). In order to simulate the natural environment even further, a femtosecond molecular dynamics simulation (5000 iterations) at 300 K was performed on the above energy-minimized protein-ligand complex by soaking the latter in an aqueous layer of 5 Å thickness all around the complex. There were no morse or cross terms. The distance ranges of the ester carbonyl carbon atoms of the reagent from the appropriate ε-amino nitrogen atoms of lysine residues or the α-amino nitrogen atom of the N-terminus valine were computed from graphs derived from the dynamics trajectories.

Synthesis of reagent BCCEP (1). Commercial reagents were employed without further purification. Organic reagents and solvents were purchased from Fischer

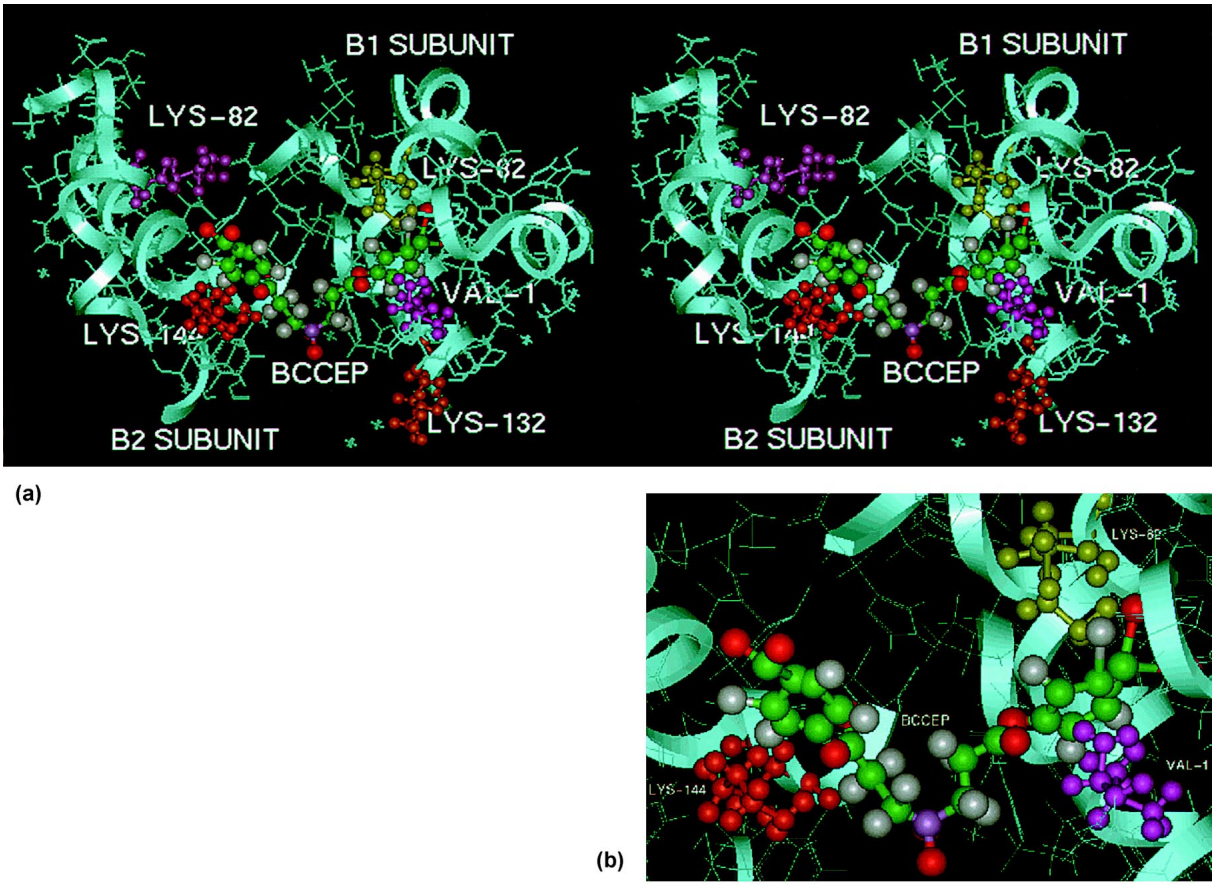
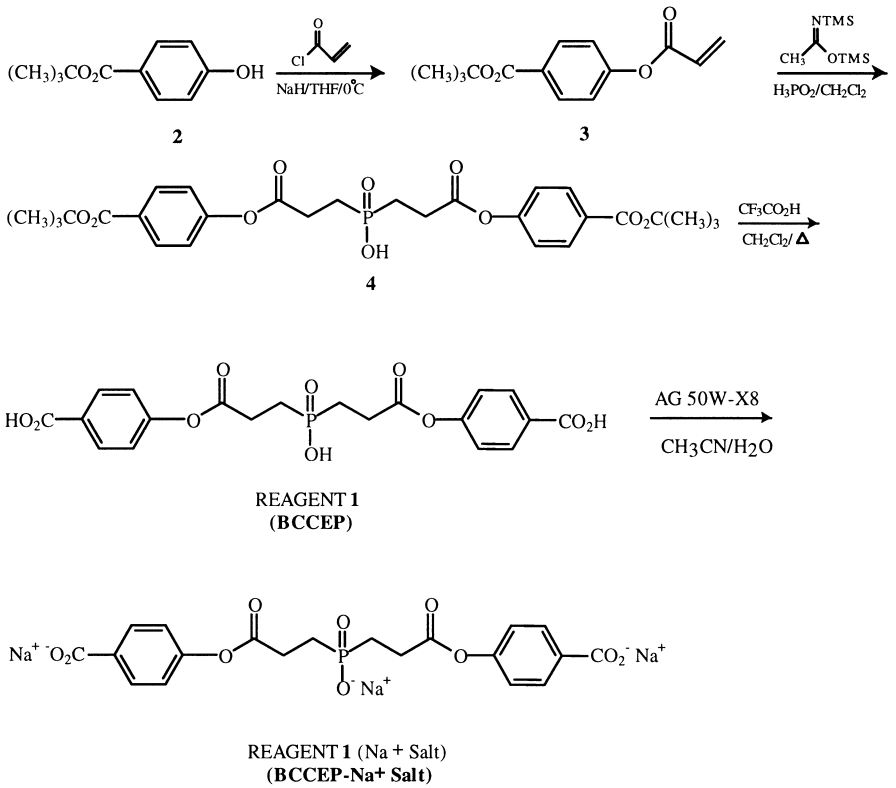


Figure 1. (a) A stereoview of the energy-minimized BCCEP-HbA_0 complex. Only those amino acid residues of SFHb that lie within a 12 Å radius from the reagent in the β-cleft are shown. (b) A closeup view of the above in monoview with the reagent and the three concerned amino acid residues shown in solid render.

Scientific Co., Aldrich Chemical Co., or Lancaster Synthesis, Inc., and the solvents were dried before use. The purity of samples of newly synthesized materials was assessed by a combination of NMR spectroscopy, mass spectrometry, analytical thin layer chromatography, and elemental analysis. The latter was performed by Atlantic Microlab, Inc., Norcross, Georgia. The synthesis of Reagent **1** is outlined in Scheme 1.

4-*t*-Butoxycarbonylphenyl acrylate (3). 4-Hydroxy-*t*-butyl benzoate (**2**)[14] (4 g, 20.6 mmol) was dissolved in anhydrous THF (250 mL) under nitrogen, and the solution was cooled in an ice bath. To this was added NaH (0.5 g, 21 mmol, 96% dispersion in mineral oil). The suspension was allowed to stir under nitrogen for 15 min, after which acryloyl chloride (1.9 g, 21 mmol) was added slowly under nitrogen. The reaction mixture was allowed to stir for an additional 4 h and allowed to come to room temperature. It was then diluted with 200 mL of CH_2Cl_2 and washed with water (3×200 mL). The organic layer was dried over $MgSO_4$, filtered, and the filtrate evaporated in a rotavapor to give the crude product which was purified over silica gel to give a colorless oil (4.1 g, 80%); ^{1}H NMR ($CDCl_3$) (8.03 (d, 2 H, Ar-H), 7.18 (d, 2 H, Ar-H), 6.63 (d, 1 H, CH = CH_2), 6.32 (dd, 1 H, CH = CH_2), 6.03 (d, 1 H, CH = CH_2), 1.59 (s, 9H, CH_3); ^{13}C NMR ($CDCl_3$) δ 164.53, 163.51, 153.67, 132.60, 130.65, 129.38, 127.41, 121.06, 80.74, 27.88.

Bis[2-(4-*t*-butoxycarbonyl)phenoxycarbonylethyl]phosphinic acid (4). To an ice cold stirred solution of hypophosphorus acid (0.17 g, 2.6 mmol) in dry CH_2Cl_2 was added *N,O*-bistrimethylsilylacetamide (1.85 g, 9.1 mmol) and the above acryloyl ester (1.3 g, 5.2 mmol), and the reaction mixture was allowed to stir for 1 h. It was partitioned between CH_2Cl_2 and H_2O, and the organic layer was washed with aq. HCl (0.1N, 3×100 mL). The organic layer was then dried with $MgSO_4$, filtered, and the filtrate evaporated to give an oil. Upon trituration



Scheme 1.

with 2-propanol, a white solid precipitated out (1.4 g, 96%) which was directly used for the next reaction without further purification, mp 147–149 °C; ^{1}H NMR ($CDCl_3$) δ 7.99 (d, 2 H, Ar-H), 7.18 (d, 2 H, Ar-H), 4.90 (br s, 1 H, P-OH), 2.88 (m, 4 H, CH_2), 2.18 (m, 4 H, CH_2), 1.50 (s, 18 H, two C_4H_9). Anal. calcd for $C_{28}H_{35}PO_{10}$: C, 59.78; H, 6.27. Found: C, 59.98; H, 6.30.

Bis[2-(4-carboxyphenoxy)carbonylethyl]phosphinic acid (1). A solution of **4** (0.1 g, 0.17 mmol) was refluxed with 1 mL of CF_3CO_2H in anhy. CH_2Cl_2 (25 mL) for 3 h. After evaporation of the solvent on a rotavapor, a white solid (**1**) was obtained which was recrystallized from 2-propanol, yield (0.7 g, 92%), mp 230–232 °C; ^{1}H NMR (DMSO-d_6) δ 8.00 (d, 4 H, Ar-H) 7.26 (d, 4 H, Ar-H), 4.90 (br s, 1 H, P-OH) 2.85 (m, 4 H, CH_2), 2.05 (m, 4 H, CH_2); ^{13}C NMR: δ 170.83 (d), 166.58 (s), 154.00 (s), 130.82 (s), 128.35 (s), 121.98 (s), 26.99 (s), 23.97 (d); MS (FAB) m/z 451 (MH^+). Anal. calcd. for $C_{20}H_{19}PO_{10}\cdot H_2O$: C, 51.29; H, 4.52. Found: C, 51.26; H, 4.48.

Trisodium salt of bis[2-(4-carboxyphenoxy)carbonylethyl] phosphinic acid. An ion exchange column (2.5×25 cm), packed with the resin AG 50W-X8 (H^+ form), was converted into the Na^+ form by equilibrating with a solution of 1 N NaOH (200 mL). Reagent **1** (0.07 g, 0.15 mmol), dissolved in 50 mL of EtOH, was loaded and the column was then eluted with an additional 200 mL of water. Evaporation of solvent gave an oil which, upon addition of CH_3CN, precipitated out as a powder (0.07 g, 90%). The solid was dried under vacuum and used for Hb modification without further purification; ^{1}H NMR (D_2O) δ 7.92–7.84 (m, 4 H, Ar-H), 7.160 (t, 2 H, Ar-H), 6.89 (br, t, 2 H, Ar-H), 2.84 (br m, 2 H, CH_2), 2.49–2.47 (br m, 2 H, CH_2), 1.95–1.77 (m, 4 H, CH_2).

Hemoglobin cross-linking studies. Hemoglobin was isolated from red blood cells according to literature procedure.[15] The stroma-free hemoglobin was a gift from the Walter Reed Army Institute of Research, Washington, D. C. The Hb cross-linking was carried out at ambient temperature (~25 °C) under oxygenated conditions in a PBS buffer (phosphate-buffered saline solution made by dissolving 8 g of NaCl, 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, 0.1 g of $MgCl_2$, 0.1 g of $CaCl_2$, and 0.1 g of KCl in a liter of H_2O) (pH 7.4) using a fourfold molar excess of reagent **1** (Na^+ salt) as compared to Hb (0.5 mM). After the reaction time of 1 h, the crude mixture was dialyzed against 0.2 M glycine buffer (pH 8.0). The sample was then applied to a DEAE-cellulose column equilibrated with the same buffer. The unreacted

Hb and the cross-linked products were eluted using a 30–150 mM NaCl gradient. The spectrophotometric readings of the eluted fractions at λ_{max} 540 nm gave two distinct peaks, corresponding to the unmodified Hb and the cross-linked product (see Figure 2). The fractions were concentrated individually in an Ultrafiltration Cell^R (Amicon). Both peaks were analyzed by HPLC (Figure 3), SDS–PAGE (Figure 4), and IEF (Figure 5). SDS–PAGE and IEF were run on Bio-Rad^R Mini Protean II and Mini IEF Cell, respectively. HPLC analyses were performed on a Perkin-Elmer Series 400 Liquid Chromatograph system (Vydac C_4 protein column, pore size 300 μ, particle size 10 μ, employing a gradient of 35–50% CH_3CN-H_2O, containing 0.1% TFA, as an eluting solvent with a flow rate of 1 mL/min, over a period of 75 min. The MALDI MS analysis of the cross-linked product (Figure 6) was performed as described under Mass Specral Analyses below.

Oxygen equilibrium (P_{50}) measurements. The oxygen equilibrium curves (Figure 7) were recorded by reoxygenation of nitrogen-equilibrated hemoglobin in 0.1 M Bis-Tris buffer (pH 7.4, 37 °C) in the spectral cuvette of a Hemox Analyzer (TCS Medical Products Co., Huntington Valley, PA, USA). During each run, data was downloaded to a computer for subsequent analysis. Oxygen dissociation curves were fitted to the Adair equation[16] by unconstrained non-linear optimization, using the Nelder-Mead simplex search algorithm[17,18] implemented in MATLAB (version 4.0 for Windows; The Math Works, Inc., Natick, MA, USA). The partial pressure of oxygen where hemoglobin was half-saturated (P_{50}) was calculated from the fitted dissociation curves by non-linear minimization, using the Gauss–Newton method[19] in MATLAB.

Tryptic digestion analyses. Tryptic digestion was performed[20] on an isolated cross-linked β-chain (0.2 mg) of Figure 2, at a trypsin:protein concentration of 1:100 (w/w), for 4 h in 0.1 M NH_4HCO_3 buffer, pH 8.5. For comparison, the tryptic digestion of the uncross-linked, pure β-chain of Figure 3a was also carried out under the same reaction conditions. The peptides were separated by reverse phase HPLC on a Vydac C_4 large-pore column, using a linear gradient (130 mins) between 5% CH_3CN in H_2O containing 0.1% TFA (Solution A) and 50% CH_3CN in H_2O, containing 0.1% TFA (Solution B), which ran at a flow rate of 1 mL/min. The fragments obtained were collected manually from the HPLC column as they eluted out and lyophilized individually. The HPLC elution profiles of the uncross-linked and cross-linked β-chains are shown in Figure 8a and b, respectively.

Mass spectral analyses. Mass spectral analyses of tryptic fragments were carried out[21] on a Kratos/Shimadzu Kompact MALDI III Instrument (Manchester, UK), equipped with a Nitrogen Laser (337 nm) and a desktop

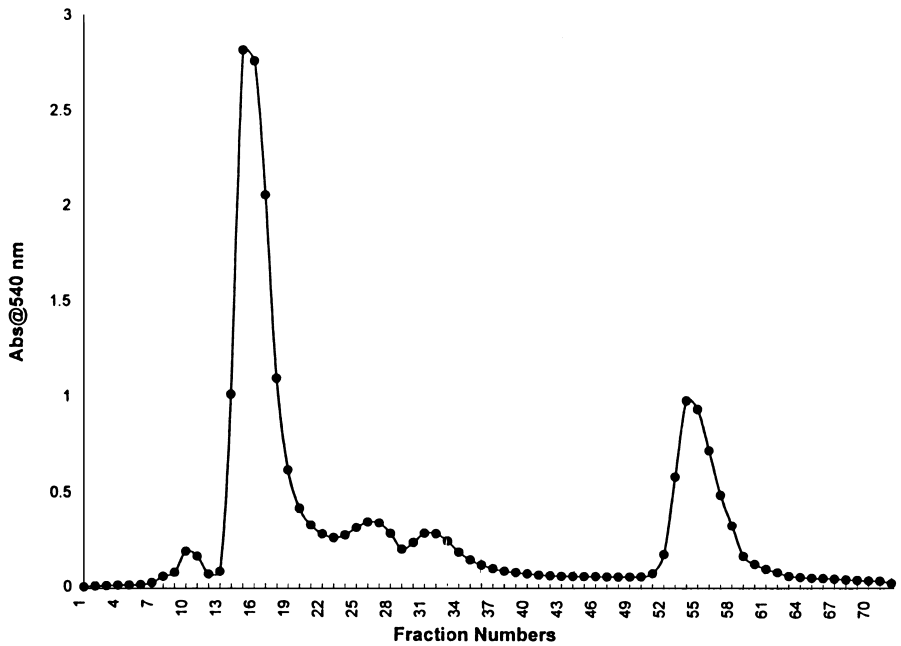


Figure 2. The elution profile, monitored at λ_{max} 540 nm, of the product of reaction of oxyhemoglobin with reagent BCCEP, separated by chromatography on a DEAE-cellulose column. The first peak at fraction numbers 14–20 is due to native Hb. The peak at fractions 52–60 is due to modified Hb, cross-linked between β_1–β_2 subunits.

computer (Sun Computer Inc.). All experiments were performed using α-cyano-4-hydroxycinnamic acid (Aldrich) as the matrix. The matrix solution consisted of a mixture of 7:3 aqueous 0.1% trifluoroacetic acid:-acetonitrile. Each lyophilized tryptic fragment was dissolved in H_2O (10 μL), and 0.5 μL of the solution was applied to a sample well, followed by an equal volume of the matrix solution. The samples were allowed to dry at room temperature before placing into the spectrometer.

Results and Discussion

The reagent BCCEP (**1**) was designed through the use of molecular modeling (Insight/Discover from MSI),

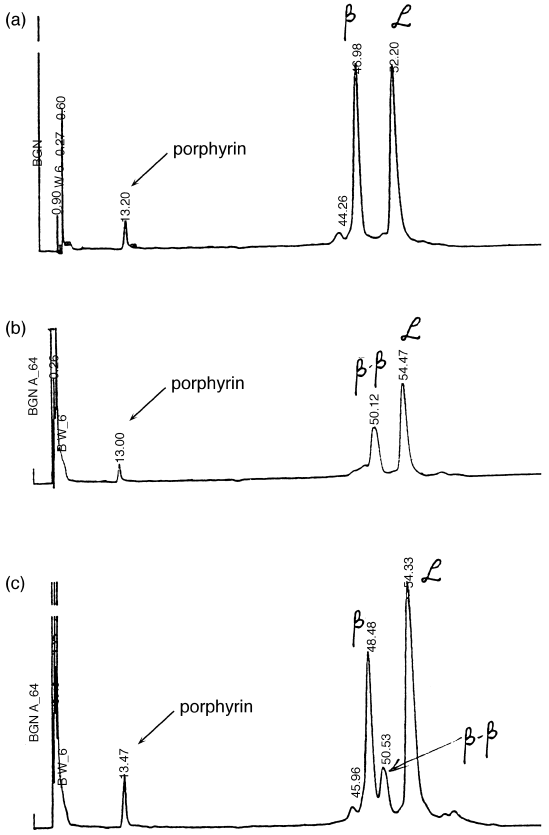


Figure 3. HPLC chromatogram of (a) stroma free Hb (SFHb), (b) cross-linked product, and (c) a mixture of SFHb and cross-linked product. Peaks in (c) corresponding to retention times 48.48 min (β-chain), 50.53 min (β-β) and 54.33 (α-chain) were characterized by SDS–PAGE, which showed molecular mass ranges of 16 kDa, 32 kDa, and 16 kDa, respectively. The HPLC was performed on a Vydac C_4 protein column, pore size 300 μ, particle size 10 μ, employing a 75 min linear gradient of 35–50% CH_3CN/H_2O containing 0.1% trifluoroacetic acid, as an eluting solvent, with a flow rate of 1 mL/min.

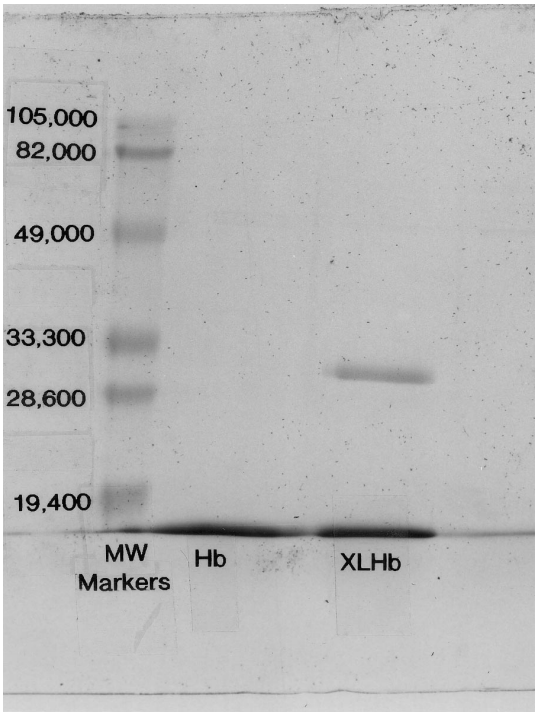


Figure 4. SDS–PAGE analyses of the modified product obtained by DEAE-cellulose chromatography. Lane 1: Molecular weight markers, Lane 2: SFHb, Lane 3: cross-linked Hb (retention time 50.53 min on HPLC).

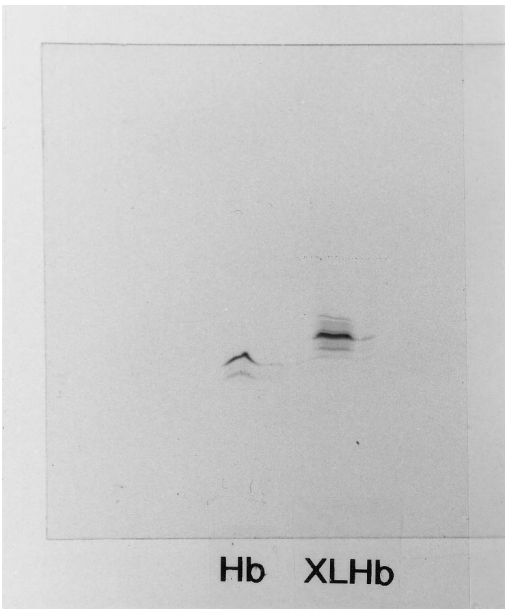


Figure 5. Isoelectric focussing analysis (IEF) of stroma-free hemoglobin (SFHb) and BCCEP-modified hemoglobin.

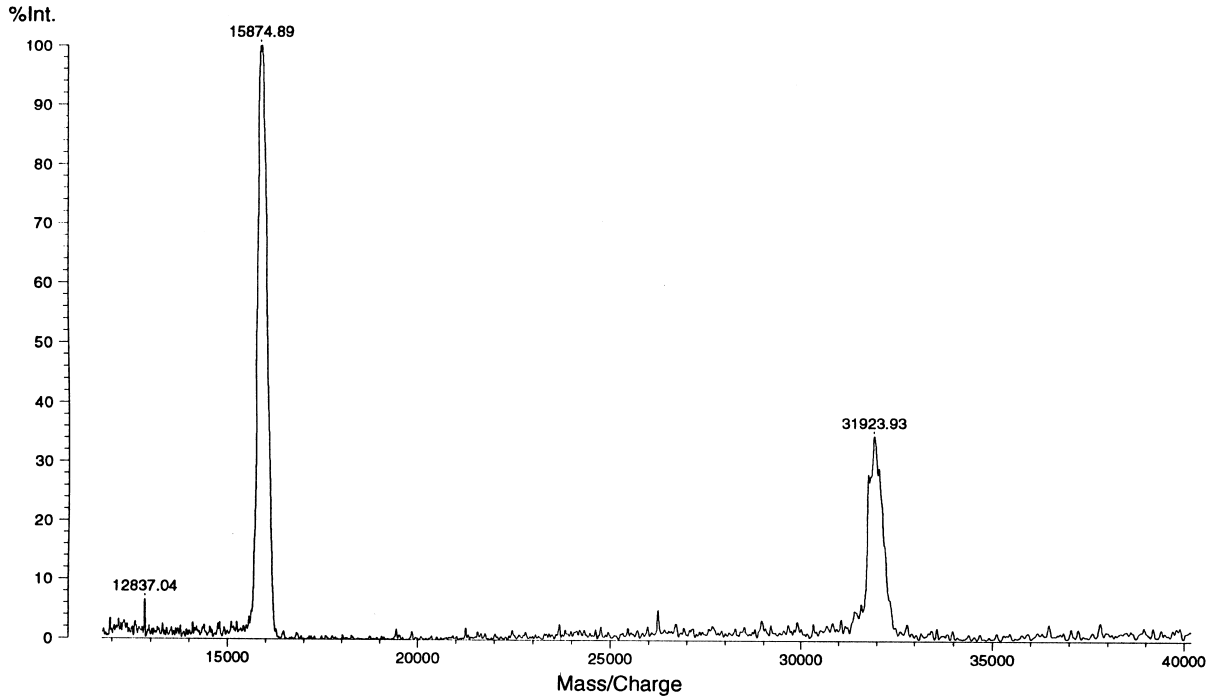


Figure 6. The MALDI MS analyses of the β chains of Figure 3c.

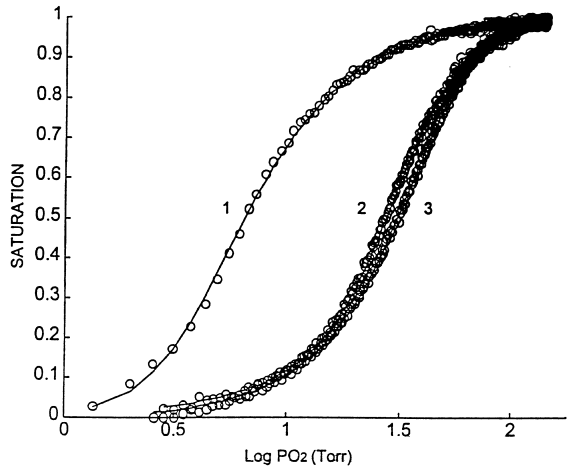


Figure 7. The oxygen equilibrium curves of unmodified stroma-free Hb (SFHb) (1) as contrasted with those of α-α cross-linked Hb (2), and BCCEP-cross-linked Hb (3) in 0.1 M Bis-Tris, pH 7.4, at 37 °C.

employing crystal coordinates of human HbA_0,[13] imported from the Brookhaven National Laboratory. While molecular modeling techniques employing crystal coordinates of Hb have been used for extensive investigations of allosteric modifications of hemoglobin,[22,23] drug delivery of hemoglobin bioconjugates,[24] and biophysical characterizations of genetic variants,[25] mutants,[26] self-association,[27] and packing[28] of hemoglobins, the documented use of molecular modeling for the de novo design of hemoglobin cross-linking reagents is somewhat rare.

The initial structural framework for the cross-linking reagent was laid down by assessment of the nature and dimension of the DPG pocket, the identification of the target amino acids on the diagonally opposed β_1 and β_2 subunits for cross-linking, as well as the tether length, geometry, and the electrophilic functional groups required for efficient cross-linking. To this end, the modeling studies were first carried out using DPG itself as the ligand. The extent and scope of potentially stabilizing hydrophobic and hydrophilic Hb–reagent interactions elicited by a variety of functional groups when present on the reagent were then explored by performing energy minimizations and dynamics simulations on each new version of the reagent–Hb complex so as to arrive at the most suitable functional groups for introduction into the reagent's skeletal framework. In this context, it must be pointed out that several other potentially promising reagents besides BCCEP were also suggested by molecular modeling. The choice of **1** over others for the present study, principally aimed at exploring the feasibility of using molecular modeling techniques for the design of hemoglobin cross-linking reagents, was largely based on the anticipated facile synthesis of the reagent.

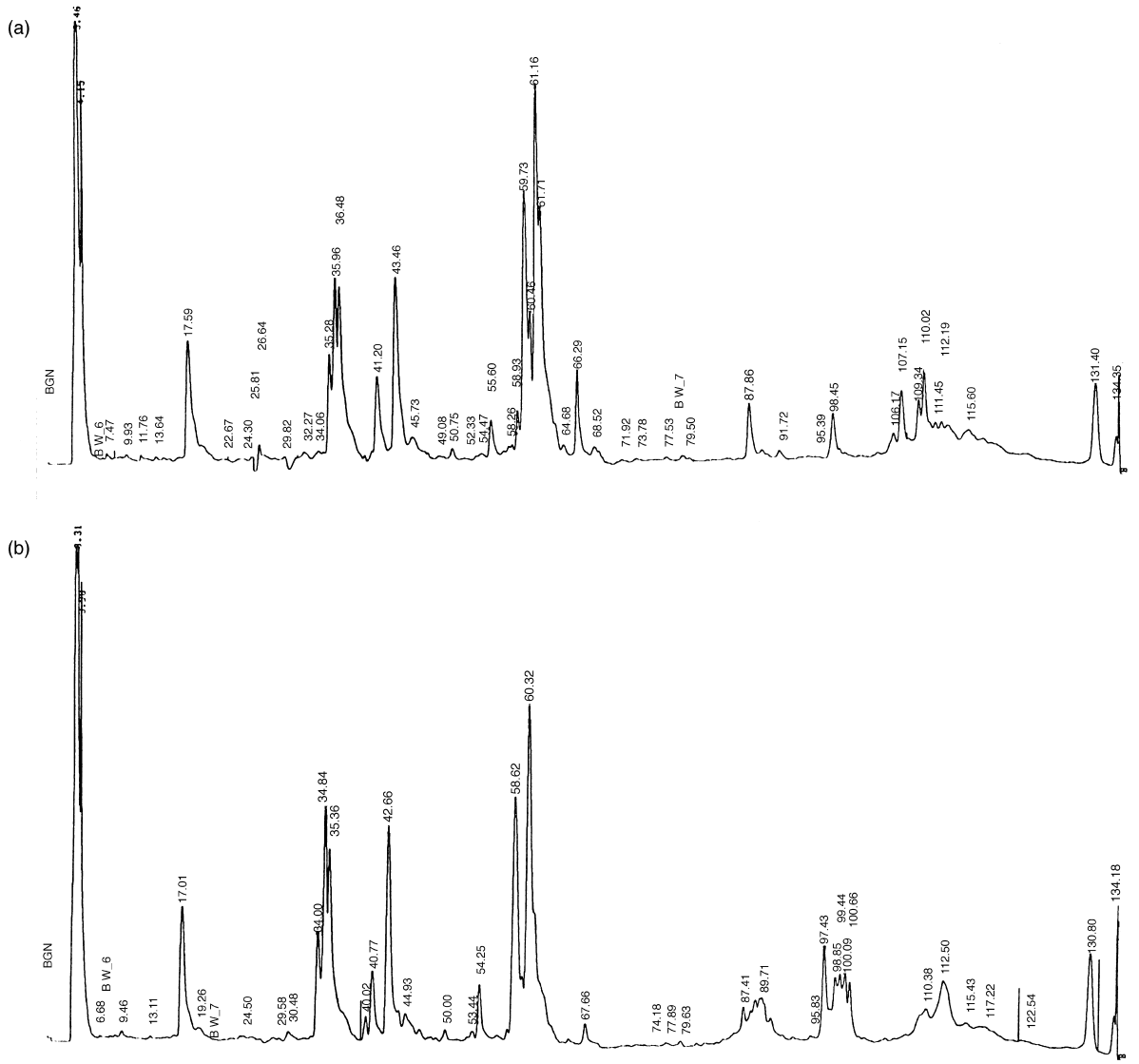


Figure 8. The HPLC profiles of the tryptic digests of (a) pure β-chain, and (b) the cross-linked β_1-XL-β2 chain. The peptides were separated on a reverse phase Vydac C_4 column, using a linear gradient of CH_3CN-H_2O, containing 0.1% CF_3CO_2H.

Reagent BCCEP (**1**) is equipped with two activated ester functionalities to serve as the two cross-linking sites. The two end carboxylate groups and the central phosphinic hydroxyl are anticipated to provide the necessary anionic charges at biological pH for the molecule to be specifically drawn to the β-cleft of hemoglobin. Initial docking and energy-minimization studies revealed that four different possibilities existed for the reagent to align itself within the β-cleft: (a) between β_1-Val-1 and β_2-Lys-144, (b) between β_1-Lys-82 and β_2-Lys-144, (c) between β_1-Lys-132 and β_2-Lys-144, and (d) between β_1-Lys-82 and β_2-Lys-82. The energy-minimization and molecular dynamics simulation studies performed individually on these four different arrangements of the BCCEP-Hb complex, suggested that the best fit with least energy (total Insight energy = 737.097 kcals, average absolute derivative = 0.00012, standard deviation of absolute derivative = 0.00012, and average RMS derivative = 0.00017) and maximum reagent–Hb interactions (see below) occurs when BCCEP is aligned between Lys-144 on the β_2 subunit and midway to N-terminus Val-1 and Lys-82 on the β_1 subunit (intermediate between arrangements a and b described above), as shown in Figure 1. The energy-minimized BCCEP–Hb complex with this arrangement revealed the existence of several hydrogen-bonding interactions between the protein and

the ligand: (a) Reagent-*p*-Ph-CO_2^-...HO-Thr-87-β_2 (1.45 Å), (b) Reagent-P(O_2)CH_2CH_2C(O)O...HN-His-143-β_2 (2.41 Å), (c) Reagent-PO_2^-...$H_2N(\varepsilon)$-Lys 144-β_2 (1.92 Å), (d) Reagent-*p*-Ph-CO_2^-...HN-(CO)-Asp-79-β_1, (2.22 Å), (e) Reagent-*p*-Ph-CO_2^-(1)...HN-(CO)-Leu-81-β_1 (1.93 Å), (f) Reagent-*p*-Ph-CO_2^-(2)...HN-(CO)-Leu-81-β1 (1.97 Å), (g) Reagent-*p*-Ph-CO_2^-(2)...$H_2N(\varepsilon)$-Lys-82-β_1 (1.86 Å), and (h) Reagent-*p*-Ph-CO_2^-(2)...HN-(α)Lys-82-β1 (2.20 Å). The two end phenyl groups of the reagent fit snugly into the two hydrophobic pockets created by the following amino acids, 14 on each side of the two β-subunits: Val-1, Leu-78, Asp-79, Asn-80, Leu-81, Lys-82, Thr-84, Phe-85, Ser-89, Lys-132, Ala-135, Val-133, Leu-141, and His-143 on the β_1-subunit, whereas Leu-78, Leu-81, Lys-82, Thr-84, Phe-85, Ala-86, Thr-87, Ser-89, Ala-135, Gly-136, Val-137, Ala-140, His-143, and Lys-144 on the β_2-subunit.

A femtosecond molecular dynamics simulation (300 K, 5000 iterations) of the above energy-minimized BCCEP–Hb complex in the preferred arrangement, soaked with an aqueous layer of 5 Å thickness all around, showed the concerned lysine residues on each β-subunit, lying in close proximity to the cross-linking sites of BCCEP. The range of distance between the appropriate ester carbonyl of BCCEP and the ε-amino group of lysine residues or the α-amino group of N-terminus valine, as computed from the graphs derived from dynamics trajectories, indicated that the closest contacts of β_1-Val-1 (5.61 Å), β_1-Lys-82 (5.83 Å), and β_2-Lys-144 (3.62 Å) are all well within the combined Van der Waals' radii of the two bonding atoms of the concerned amino and carbonyl functions of Hb and BCCEP, respectively. Thus, the most preferred amino acids to participate in cross-linking by BCCEP appeared to be Val-1 and Lys-82 on the β_1-subunit and Lys-144 on the β_2-subunit, which correspond to arrangements (a) and (b) described above. While the latter two are the suggested preferred arrangements, our simulation studies did not totally rule out the arrangements (c) and (d) described earlier, which would require the involvement of an additional amino acid on each of the β chains: Lys-132 on β_1 and Lys-82 on β_2. However, we did not find any experimental evidence, based on MALDI MS analyses of tryptic digests of the cross-linked product (see below), supporting these latter two arrangements.

The reagent BCCEP (**1**) was synthesized (Scheme 1) by condensation of *p-t*-butoxycarbonylphenyl acrylate (**3**), prepared from *p-t*-butoxycarbonylphenol (**2**) by reaction with acryloyl chloride, with hypophosphorous acid in the presence of a silylating reagent, bis(trimethylsilyl) acetamide. The product, upon removal of the *t*-butyl protecting groups with trifluoroacetic acid in refluxing methylene chloride, gave reagent BCCEP as a crystalline solid in 72% overall yield.

The reagent was converted to its tri-sodium salt by ion-exchange chromatography, and was used for cross-linking human HbA_0. Cross-linking was carried out under oxygenated conditions at ambient temperature (~25 °C) in a PBS buffer (pH 7.4), using a fourfold molar excess of reagent **1** (Na^+ salt) over Hb. The same reaction, however, failed to proceed in deoxygenated media inspite of otherwise identical experimental conditions. After extensive dialysis of the reaction mixture, the product was separated from the unmodified Hb by DEAE–cellulose chromatography (see Figure 2), concentrated, and analyzed by high performance liquid chromatography (HPLC) (Figure 3), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 4), and isoelectric focusing (IEF) (Figure 5). The HPLC profile of the cross-linked product (Figure 3b), as contrasted with that of the unmodified Hb (Figure 3a), suggested that the modification was on the β-chain of Hb. The SDS–PAGE analyses of the modified product (see Figure 4) clearly pointed to the presence of cross-linked product with a molecular mass of 32 kDa, corresponding to the dimers of Hb subunits. The SDS–PAGE analyses of the individual α and β chains of the modified product of Figure 3b revealed molecular masses of ≈16 and 32 kDa, respectively, confirming that the modification was on the β chain. The most convincing evidence for the cross-link came from the MALDI MS analysis of the β chains of Figure 3c which revealed (see Figure 6) molecular masses corresponding to the unmodified chain (m/z 15874.89 Da) and the BCCEP-modified β chain [obsd. m/z 31923.93; calcd. m/z 31923.78 = 2×15874.89 (two β chains) + 176 (the cross-linking fragment -C(O)$(CH_2)_2$-P(O)(OH)-$(CH_2)_2$-C(O)- of BCCEP)-2 (two ε- or α-amino H's removed upon cross-linking)].

The modified hemoglobin was further analyzed for oxygen affinity (P_{50})[29] characteristics at 37 °C. For the sake of comparison, P_{50} values were also measured under the same conditions for the uncross-linked, stroma-free Hb (SFHb) as well as for the α-α cross-linked Hb.[7] The graphs of log PO_2 vs % Hb saturation for (1) SFHb, (2) α-α cross-linked Hb[7], and (3) the BCCEP-modified Hb are shown in Figure 7. The computed P_{50} value for the modified Hb was 31.3 Torr, as contrasted with that of cell-free Hb (6.6 Torr) and the α-α cross-linked Hb (27.9 Torr). Thus, the BCCEP-modified as well as the α-α cross-linked Hb's showed significantly lowered oxygen affinity as compared with cell-free hemoglobin. This was further attested by the observed right-shifting of O_2 equilibrium curves of both cross-linked products relative to that of cell-free Hb. Furthermore, the sigmoidal shapes of O_2 curves of the BCCEP-modified Hb, comparable to those of the α-α cross-linked Hb,[7] pointed to reasonable retainment of oxygen binding cooperativity after the cross-link formation. Whereas the Hill Coefficient n,[30] generated

from the Hill plots of P_{50} values, would be a quantitative indicator of oxygen-binding cooperativity, the computed value of $n = 2.0$ from such plots for the BCCEP-modified Hb is, nevertheless, less meaningful in view of the anticipated and observed (see below) multiple cross-linking sites, and the consequent heterogeneity of the BCCEP-modified Hb.

In order to determine the location of the cross-links, the tryptic digestion analyses of both the pure uncross-linked β-chain and the cross-linked β_1-XL-β_2 chain were carried out, employing standard procedures and reaction conditions.[20] The peptide fragments were separated by reverse-phase HPLC on a Vydac C_4 protein column. A comparison of the HPLC profile of the tryptic fragments of the pure β-chain (Figure 8a) with that of the cross-linked product (Figure 8b) revealed that the new peptide fragments were mainly clustered into two broad humps, each consisting of 5–6 individual peaks, approximately centered at retention times of 89.71 and 99.44 minutes. In addition, the broad peak at the highest region of the HPLC profiles of Figure 8a and b, starting at approximate retention time of 106 minutes, showed some conspicuous differences.

The matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS)[21] of each of the tryptic fragments of Figure 8a and b was performed to determine their compositions. Where it was not possible to isolate a totally pure fragment peak (e.g., the clusters of retention times 89.71 and 99.44 mentioned above) the MALDI MS were run on the peak of highest possible purity, which normally consisted of two or three components. However, the presence of multiple components in a peak did not hinder determination of composition of individual peptide fragments or cross-linking sites using MALDI MS analyses. In this regard, a recent report of successful MALDI MS analyses, carried out directly on the crude tryptic digests of hemoglobin, is noteworthy.[21] Listed in Tables 1 and 2 are the retention times and observed average masses (MH^+ ions) by MALDI MS analyses of predominant peptide fragments of Figure 8a and b, respectively, along with the calculated average masses and tentative structural analyses of each of those fragments.

The MALDI MS analyses of the tryptic digests of native β-chain (see Figure 8a and Table 1) showed the expected (MH^+) ions for those tryptic peptides whose masses exceeded 900 Da. The MALDI mass assignments are normally difficult for peptides of masses considerably lower than 1 kDa because of interfering low mass matrix ions.[31] Thus, tryptic fragments T_6 (mass = 245), T_7 (411), T_8 (146), and T_{15} (318) were not analyzed. While ions corresponding to T_1 (952), T_2 (932), T_3 (1314), T_4 (1274), T_5 (2059), T_9 (1670), T_{13} (1378), and T_{14} (1149) were directly observed, the fragments T_{10} (1421) and T_{11} (1126) could only be seen as a single peptide ($T_{10} + T_{11} - H_2O$) at m/z 2529. Likewise, fragment T_{12} (1720) was observed either as a cysteine dimer (cystine) with a disulfide bridge at m/z 3438 or as part of a three-peptide fragment ($T_{12} + T_{13} + T_{14} - 2H_2O$), resulting from incomplete tryptic digestion, at m/z 4234. In view of the absence of disulfide bridges in normal human hemoglobin in the native state, the formation of cystine from Cys-112 of T_{12} fragment may be due to air oxidation during digestion, sample preparation, or analyses. Another important observation in the MALDI mass spectra was the presence of Na and K ions in a number of observed fragment peaks. Since neither of these ions was present in the matrix of mass spectral analysis or during tryptic digestion, they must have come from the phosphate buffer solutions employed during the isolation and purification of hemoglobin from red cells. One other important point to be noted is that the typical mass accuracy available in conventional MALDI MS analyses is one mass per thousand.[32]

The MALDI MS analyses of tryptic digest fragments of the cross-linked β_1-XL-β_2 chain (see Figure 8b and Table 2) provided evidence for the sites of cross-links in the modified hemoglobin. The most important clues came from the major peaks of retention times 98.85 and 99.44 minutes. The observed MH^+ ions from the two peaks at m/z 6255 and m/z 6207, respectively (see Figure 9a,b and Table 2), corresponded to fragments (T_1–T_2)-XL-(T_{12}–T_{14}) and (T_8–T_{10})-XL-(T_{13}–T_{15}). These fragments, along with other shorter, tell-tale fragments (see below), undeniably suggested that the cross-links involved an amino acid residue taken from each of the three different groups of tryptic peptides containing residues 1–17 (T_1–T_2) (group I), 60–95 (T_6–T_{10}) (group II), and 105–146 (T_{12}–T_{15}) (group III). Since the first two groups are each connected to the common third group, it appeared that the cross-links involved one of the amino acids from residues 1–17 or 60–95 on one of the β chains, and an amino acid from residues 105–146 on the other β chain. This is indeed consistent with the results of molecular modeling described above, which suggested the possibility of cross-link formation between Val-1 or Lys-82 on one of the β-chains, and Lys-144 on the other (refer to arrangements a and b above). While there are several other amino acid residues that are capable of cross-linking, besides Val-1 (group I), Lys-82 (group II), and Lys-144 (group III), especially the lysines that are present in each of the three groups, for example, Lys-8 and Lys-17 (group I), Lys-61, Lys-65, Lys-66, and Lys-95 (group II), and Lys-120, Lys-132 (group III), their considerably distant locations (≥ 15 Å) from the cross-linking sites of BCCEP in the β-cleft render them the unlikely candidates for cross-linking. Although cross-links are theoretically possible

Table 1. MALDI MS analyses of the peptide fragments obtained from the tryptic digestion of the uncross-linked, pure β-chain of human hemoglobin

Retention time (min)	Calcd av. mass[a]	Obsd av. mass[b]	Fragment residues	Tentative structural analysis of tryptic fragment
17.59	952.1	951.9	1–8	T_1
	974.1	974.3	1–8	$(T_1\text{-}1)+Na$
	990.2	990.2	1–8	$(T_1\text{-}1)+K$
35.28	1149.4	1149.8	133–144	T_{14}
	1171.4	1171.5	133–144	$(T_{14}\text{-}1)+Na$
	1187.5	1187.1	133–144	$(T_{14}\text{-}1)+K$
35.96	1378.6	1378.4	121–132	T_{13}
	1400.5	1400.4	121–132	$(T_{13}\text{-}1)+Na$
	1416.7	1416.4	121–132	$(T_{13}\text{-}1)+K$
36.48	1126.2	1126.7	96–104	T_{11}
	1314.4	1314.3	18–30	T_3
	1400.5	1400.4	121–132	$(T_{13}\text{-}1)+Na$
41.20	1449.7	1450.0	133–146	$(T_{14}+T_{15})-H_2O$
	1471.7	1471.7	133–146	$(T_{14}+T_{15}\text{-}H_2O)-1+Na$
	1487.8	1488.1	133–146	$(T_{14}+T_{15}\text{-}H_2O)-1+K$
43.46	932.1	932.6	9–17	T_2
	954.1	954.5	9–17	$(T_2\text{-}1)+Na$
	970.2	970.0	9–17	$(T_2\text{-}1)+K$
58.93	1798.1	1799.1	66–82	$(T_8+T_9)-H_2O$
59.73	1274.5	1274.4	31–40	T_4
61.16	2059.3	2059.7	41–59	T_5
	2081.3	2081.1	41–59	$(T_5\text{-}1)+Na$
	2097.4	2097.1	41–59	$(T_5\text{-}1)+K$
61.71	1669.9	1669.8	67–82	T_9
66.29	2529.8	2529.2	83–104	$(T_{10}+T_{11})-H_2O$
87.86	4234.0	4234.9	105–144	$(T_{12}+T_{13}+T_{14})-2H_2O+Na$
98.45	3438.2	3439.6	2×(105–120)	$(T_{12})_2$–2 (dimer of T_2 with a disulfide bridge)

[a]The average mass of peptide fragment was calculated using the computer program, GPMAW (General Protein Mass Analyzer for Windows), Ver. 2.0, available from Lighthouse 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 observed mass was calibrated against protein nerve growth factor (PNGF), $MH^+ = 2004.3$.

between β_1-Lys-132 and β_2-Lys-144 (arrangement c) and between β_1-Lys-82 and β_2-Lys-82 (arrangement d), we found no evidence for such cross-links in the MALDI MS analyses of any of the tryptic digest fragments of modified hemoglobin (see Table 2). Therefore, we believe that the cross-links lie between β_1-Val-1 and β_2-Lys-144 (arrangement a) and between β_1-Lys-82 and β_2-Lys-144 (arrangement b). Furthermore, in view of the relatively large number of tryptic fragments that corresponded to arrangement b (see Table 2), the cross-link appears to be predominantly between β_1-Lys-82 and β_2-Lys-144 (arrangement b).

The other peptide fragments of Figure 9a corroborated the above notion. The observed MH^+ ions at *m*/*z* 3426 [(66–82)-XL-(133–146)], 3856 [(62–82)-XL-(133–146)], 4068 [(60–82)-XL-(133–146)], 4505 [(66–82)-XL-(121–144)], 5103 [(60–82)-XL-(121–144)], and 5888 [(66–95)-XL-(121–144)] are consistent with the notion that the cross-link is between one of the amino acids from residues 60–95 and one from residues 121–146. Also, the fragment ions at *m*/*z* 5583 and *m*/*z* 3198 of respective retention times 100.09 and 115.43 min corresponded to the cross-linked fragments (67–104)-XL-(133–144) and (66–82)-XL-(133–144), respectively. Lys-82 and Lys-144 were also part of the cross-linked tryptic fragments, (66–82)-XL-(105–144) and (66–95)-XL-(121–146), which matched the observed MH^+ ions at *m*/*z* 6267 and *m*/*z* 6286, respectively, both of which came from the tryptic digest peak of retention time 97.43 min.

Table 2. MALDI MS Analyses of the peptide fragments obtained from the tryptic digestion of the cross-linked, β_1-BCCEP-β_2 chain of human hemoglobin

Retention time (min)	Calcd av. mass[a]	Obsd av. mass[b]	Fragment residues	Tentative structural analysis of tryptic fragment
17.01	952.1	951.6	1–8	T_1
	974.1	972.8	1–8	$(T_1\text{-}1) + Na$
	990.2	988.4	1–8	$(T_1\text{-}1) + K$
34.00	1149.4	1148.3	133–144	T_{14}
34.84	1378.6	1377.8	121–132	T_{13}
35.36	1126.2	1127.3	96–104	T_{11}
	1314.4	1315.4	18–30	T_3
	1400.5	1401.6	121–132	$(T_{13}\text{-}1) + Na$
40.02	1144.2	1145.7	(1–8) + Reagent	$(T_1\text{-}1) + C(O)\text{-}(CH_2)_2\text{-}P(O)(OH)\text{-}(CH_2)_2\text{-}CO_2H$
42.66	970.2	970.6	9–17	$(T_2\text{-}1) + K$
	1449.7	1451.7	133–146	$(T_{14} + T_{15})\text{-}H_2O$
	1701.9	1701.9	133–146 + Reagent	$(T_{14} + T_{15}\text{-}H_2O\text{-}1) + C(O)\text{-}(CH_2)_2\text{-}P(O)(ONa)\text{-}(CH_2)_2\text{-}CO_2K$
	1906.5	1907.1	67–82 + Reagent	$(T_9\text{-}1) + C(O)\text{-}(CH_2)_2\text{-}P(O)(ONa)\text{-}(CH_2)_2\text{-}CO_2Na$
44.93	1641.8	1642.7	133–146 + Reagent	$(T_{14} + T_{15}\text{-}H_2O\text{-}1) + C(O)\text{-}(CH_2)_2\text{-}P(O)(OH)\text{-}(CH_2)_2\text{-}CO_2H$
	1663.8	1664.3	133–146 + Reagent	$(T_{14} + T_{15}\text{-}H_2O\text{-}1) + C(O)\text{-}(CH_2)_2\text{-}P(O)(ONa)\text{-}(CH_2)_2\text{-}CO_2H$
	1679.9	1680.4	133–146 + Reagent	$(T_{14} + T_{15}\text{-}H_2O\text{-}1) + C(O)\text{-}(CH_2)_2\text{-}P(O)(OK)\text{-}(CH_2)_2\text{-}CO_2H$
	1721.9	1721.7	133–146 + Reagent	$(T_{14} + T_{15}\text{-}H_2O\text{-}1) + C(O)\text{-}(CH_2)_2\text{-}P(O)(OK)\text{-}(CH_2)_2\text{-}CO_2K$
54.25	2026.1	2026.6	67–82 + Reagent	$(T_9\text{-}1) + C(O)\text{-}(CH_2)_2\text{-}P(O)(ONa)\text{-}(CH_2)_2\text{-}C(O)\text{-}(OC_6H_4)\text{-}CO_2Na$
58.62	1274.5	1276.3	31–40	T_4
60.32	1286.3	1287.0	1–8 + Reagent	$(T_1\text{-}1) + C(O)\text{-}(CH_2)_2\text{-}P(O)(ONa)\text{-}(CH_2)_2\text{-}C(O)\text{-}(OC_6H_4)\text{-}CO_2H$
	1669.9	1671.3	67–82	T_9
	2004.1	2002.2	67–82 + Reagent	$(T_9\text{-}1) + C(O)\text{-}(CH_2)_2\text{-}P(O)(ONa)\text{-}(CH_2)_2\text{-}C(O)\text{-}(OC_6H_4)\text{-}CO_2H$
	2079.2	2076.4	1–17 + Reagent	$(T_1 + T_2\text{-}H_2O\text{-}1) + C(O)\text{-}(CH_2)_2\text{-}P(O)(ONa)\text{-}(CH_2)_2)\text{-}CO_2H$
	2081.3	2081.6	41–59	$(T_5\text{-}1) + Na$

(continued)

Table 2—*contd*

87.41	4235.01	4235.9	105–144	$(T_{12}+T_{13}+T_{14}\text{-}2H_2O\text{-}1)+Na$
	4241.8	4240.8	67–104	$(T_9+T_{10}+T_{11}\text{-}2H_2O\text{-}2)+Na+K$
89.71	2273.6	2273.3	62–82	$(T_7+T_8+T_9\text{-}2H_2O\text{-}3)+2Na+K$
	5295.1	5297.5	62–95 XL 133–146	$(T_7+T_8+T_9+T_{10}\text{-}3H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(OK)\text{-}(CH_2)_2\text{-}C(O)+(T_{14}+T_{15}\text{-}H_2O\text{-}1)+K$
97.43	3431.9	3432.5	66–95 + Reagent	$(T_8+T_9+T_{10}\text{-}2H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(OH)\text{-}(CH_2)_2\text{-}CO_2K$
	6268.2	6267.7	66–82 XL 105–144	$(T_8+T_9\text{-}H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(ONa)\text{-}(CH_2)_2\text{-}C(O)+(T_{12}+T_{13}+T_{14}\text{-}2H_2O\text{-}1)+K+Na$
	6286.1	6286.5	66–95 XL 121–146	$(T_8+T_9+T_{10}\text{-}2H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(ONa)\text{-}(CH_2)_2\text{-}C(O)+(T_{13}+T_{14}+T_{15}\text{-}2H_2O\text{-}1)+2K$
98.85	3421.7	3426.9	66–82 XL 133–146	$(T_8+T_9\text{-}H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(OH)\text{-}(CH_2)_2\text{-}C(O)+(T_{14}+T_{15}\text{-}H_2O\text{-}1)$
	3853.4	3856.05	62–82 XL 133–146	$(T_7+T_8+T_9\text{-}2H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(OK)\text{-}(CH_2)_2\text{-}C(O)+(T_{14}+T_{15}\text{-}H_2O\text{-}1)$
	4064.7	4068.5	60–82 XL 133–146	$(T_6+T_7+T_8+T_9\text{-}3H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(OH)\text{-}(CH_2)_2\text{-}C(O)+(T_{14}+T_{15}\text{-}H_2O\text{-}1)$
	4504.1	4505.6	66–82 XL 121–144	$(T_8+T_9\text{-}H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(ONa)\text{-}(CH_2)_2\text{-}C(O)+(T_{13}+T_{14}\text{-}H_2O\text{-}1)$
	5102.9	5103.29	60–82 XL 121–144	$(T_6+T_7+T_8+T_9\text{-}3H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(OH)\text{-}(CH_2)_2\text{-}C(O)+(T_{13}+T_{14}\text{-}H_2O\text{-}1)$
	5885.7	5888.6	66–95 XL 121–144	$(T_8+T_9+T_{10}\text{-}2H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(OH)\text{-}(CH_2)_2\text{-}C(O)+(T_{13}+T_{14}\text{-}H_2O\text{-}1)$
	6252.2	6255.2	1–17 XL 105–144	$(T_1+T_2\text{-}H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(OH)\text{-}(CH_2)_2\text{-}C(O)+(T_{12}+T_{13}+T_{14}\text{-}2H_2O\text{-}1)$
99.44	6207.9	6207.03	66–95 XL 121–146	$(T_8+T_9+T_{10}\text{-}2H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(ONa)\text{-}(CH_2)_2\text{-}C(O)+(T_{13}+T_{14}+T_{15}\text{-}2H_2O\text{-}1)$
100.09	3073.5	3073.9	67–95	$T_9+T_{10}\text{-}H_2O$
	5583.4	5583.8	67–104 XL 133–144	$(T_9+T_{10}+T_{11}\text{-}2H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(OH)\text{-}(CH_2)_2\text{-}C(O)+(T_{14}\text{-}1)+2K$
115.43	3198.6	3198.9	66–82 XL 133–144	$(T_8+T_9\text{-}H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(OK)\text{-}(CH_2)_2\text{-}C(O)+(T_{14}\text{-}1)+K$
	3431.9	3432.5	66–95 + Reagent	$(T_8+T_9+T_{10}\text{-}2H_2O\text{-}1)+C(O)\text{-}(CH_2)_2\text{-}P(O)(OK)\text{-}(CH_2)_2\text{-}CO_2H$

[a]The average mass of peptide fragment was calculated using the computer program, GPMAW (General Protein Mass Analyzer for Windows), Ver. 2.0, available from Lighthouse 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 observed mass was calibrated against protein nerve growth factor (PNGF), $MH^+ = 2004.3$.

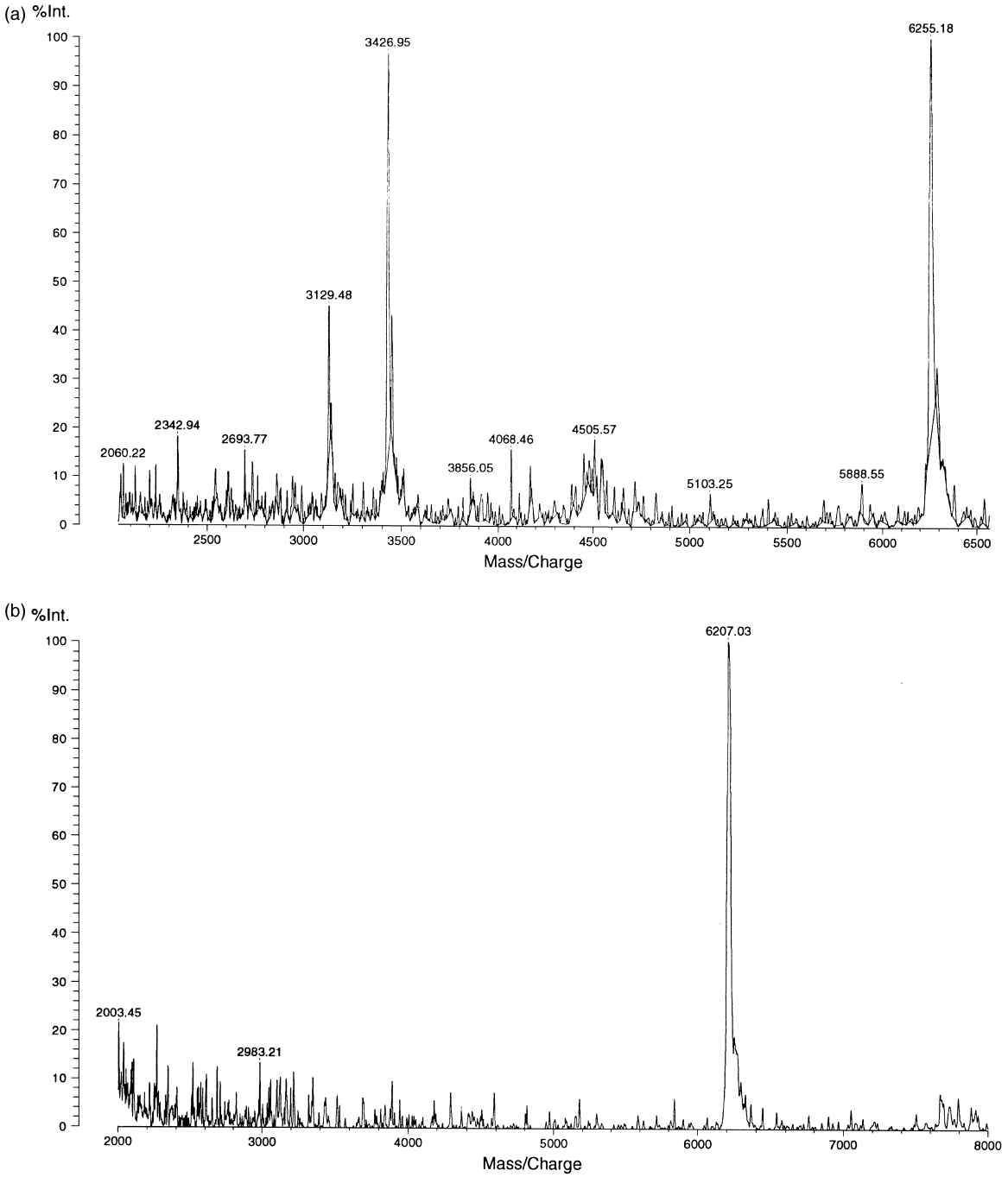


Figure 9. The MALDI MS analyses of tryptic fragments of retention times (a) 98.85 min, and (b) 99.44 min.

Several other fragment ions listed in Table 2 gave further insight into not only the process of cross-linking but also the subsequent hydrolysis that seemed to occur in a number of instances. Many of the above-mentioned tryptic peptides involved in cross-linking also exhibited ions that indicated that the peptides contained the reagent BCCEP either as a covalent mono adduct or as the product of hydrolysis of the latter. For example, the observed fragment ion at m/z 1287 from the HPLC peak of retention time 60.32 min corresponded to the covalent mono adduct of BCCEP with T_1. Similarly, the fragment ions at m/z 2002 and m/z 2026 of respective

retention times 60.32 and 54.25 min corresponded to the covalent monoadduct of mono- and disodium salt of BCCEP with T_9, respectively. The partially hydrolyzed T_1-BCCEP and T_2-BCCEP adducts were seen at m/z 1145 and m/z 1907 at retention time 40.02 and 42.66 minutes, respectively. Likewise, the HPLC peaks of retention time 44.93 min revealed several ions that matched the tryptic fragment of residues 133–146 (T_{14}–T_{15}) with a partially hydrolyzed BCCEP as a covalent mono adduct. The latter was seen in several different forms (see Table 2): as the parent ion at m/z 1641, a mono sodium salt at 1663, a mono potassium salt at 1679, and a dipotassium salt at 1721. The same adduct was also seen as a monosodium–monopotassium salt at m/z 1701 at retention time 42.66 min.

The above tryptic fragments that correspond to covalent mono adducts of BCCEP with T_1 (m/z 1287) and T_9 (m/z 2002 and 2026) lend further support to the notion based on molecular modeling described earlier (see Figure 1) that one end of the reagent BCCEP aligns itself midway between the N-terminus Val-1 (T_1) and Lys-82 (T_9) on one of the β chains, while the other end lies close to Lys-144 (T_{14}) on the other β chain. In this orientation, there is an equal probability that at any one moment, both Val-1 and Lys-82 belonging to the same subunit will be modified by BCCEP forming a mono adduct each. However, only one of the mono adducts can form a cross-link with Lys-144 of the other β-subunit at any instant, leaving the other monodduct intact. This is further corroborated by the fact that no such *intact* covalent mono adducts of BCCEP were observed with Lys-144 (T_{14}).

While there were only three intact covalent mono adducts of BCCEP with T_1 and T_9, as described above, there was an abundance of partially hydrolyzed BCCEP adducts with T_1 and T_9, as well as with T_{14}. These species could arise from hydrolysis of the above intact mono adducts as well as from that of the cross-linked products, presumably during tryptic digestion, HPLC separation or sample preparation for MALDI MS analysis. While the latter two processes employed acidic conditions (0.1% TFA), the first one was performed under a basic medium (pH 8.5). Although it is not clear, and was not explored further, as to at what stage it might have taken place, the hydrolysis could, nevertheless, satisfactorily explain why the cross-linker-free tryptic fragments of T_1, T_9, and T_{14} were detected in spite of their implicated involvement in cross-linking. Apparently, upon hydrolysis, the cross-linked product would initially produce a partially hydrolyzed mono adduct, containing a tryptic fragment from either the β_1 or β_2 side plus the one-end-hydrolyzed reagent part. A second hydrolysis of such a mono adduct would produce the reagent-free peptide, as observed. Similar hydrolysis of the intact mono adducts would produce the partially hydrolyzed mono adducts of T_1 and T_9, along with the respective cross-linker-free fragments.

Finally, in view of formation of a number of partially digested peptide fragments from apparently incomplete tryptic digestion of the cross-linked product, it was decided to repeat the experiment by extending the time of digestion to 24 h from the 4 h duration that was employed earlier, although incomplete tryptic digestion of modified hemoglobin has been documented.[21] Since the digestion conditions employed for both native and cross-linked β chains have been nearly identical, it seems that the modification of an amino acid residue by BCCEP causes difficulty for trypsin to access the vicinity of the modified residue, resulting in partially digested fragments. If this is the case, the products obtained from the prolonged digestion should not be much different from the ones obtained from the 4 h digestion. If, on the other hand, the complete hydrolysis of the reagent occurred during the prolonged period of digestion, resulting in liberation of cross-linker-free peptide fragments as discussed above, then trypsin should be able to further degrade these peptides into smaller fragments in the usual manner. Indeed, the MALDI MS of the tryptic fragments obtained from the 24 h tryptic digestion turned out to be a lot less informative than the ones from the 4 h digestion. Most of the cross-linked tryptic fragments obtained earlier from the shorter digestion were now absent, and many of the observed ions were found to be due to self-digestion of trypsin. It appears, therefore, that the cross-link formed by BCCEP is prone to hydrolysis under conditions employed (pH 8.5) for tryptic digestion. Nevertheless, the cross-linked product was found to be stable for indefinite periods of time when suspended in a PBS (pH 7.4), as evidenced by HPLC profiles taken at intervals of several days. As there are no prior reports of hydrolysis, to the best our knowledge, of such cross-linkers that form bis-amide linkages with hemoglobin subunits, we speculate that the central phosphinic acid might be playing a crucial role in facilitating the process of hydrolysis by providing an anchimeric assistance via formation of a five-membered ring intermediate. Nonetheless, this can only be a tentative explanation in the absence of careful and thorough scrutiny of the mechanism of hydrolysis.

Conclusion

We have designed and synthesized a novel bifunctional organic reagent aided by modern molecular modeling and synthetic organic methods. The reagent BCCEP has a number of desired characteristics for a hemoglobin cross-linker. It is, like DPG, specific for the β-cleft

modulation of hemoglobin. It can be conveniently synthesized in three easy steps from readily available, inexpensive starting materials, has an indefinite shelf life as a crystalline solid, can be easily converted into its trisodium salt for usage in an aqueous medium, and employed to cross-link Hb under ambient oxygenated media, requiring no special precautions or reaction conditions. The oxygen affinity of the cross-linked product is considerably lower ($P_{50} = 31.3$ Torr) as compared with that of cell-free Hb ($P_{50} = 6.6$ Torr), and is somewhat comparable to the oxygen affinity of whole blood ($P_{50} = 27$ Torr). The cross-linked product also retains some of the oxygen-binding cooperativity characteristics of the native protein, as revealed by the sigmoidal shape of O_2 curve that is comparable to that of the α-α cross-linked hemoglobin.[7] We have also successfully elucidated the sites of cross-links, employing the MALDI MS analyses of tryptic digests of the cross-linked product.

Further structural modification of BCCEP must take into consideration that while the reagent is very specific for the β-cleft, and is also specific for Lys-144 of one of the β subunits, it has somewhat less specificity for the residues lying on the other β subunit, interacting with both Val-1 and Lys-82. Consequently, achievement of absolute specificity, if necessary, may involve interaction of the reagent with only one of the latter two amino acid residues from the same Hb subunit, although it is not obvious at this point if there is any distinct advantage of achieving such a goal beyond that of the cleft-specificity, provided that the cross-linking reaction is uniformly reproducible and that the oxygen affinity as well as the oxygen-binding cooperativity of the modified Hb are adequate. In that regard, while the oxygen affinity of BCCEP is in the desired range for a blood substitute, its cooperativity characteristics could further benefit from structural improvements. In any event, our preliminary molecular modeling exercises call for interchanging and/or adding carboxy groups to the *ortho* and/or *meta* positions of the terminal phenyl groups of BCCEP so as to potentially realize the described total specificity.

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