A Site-Specific Tetrafunctional Reagent for Protein Modification: Cross-Linked Hemoglobin with Two Sites for Further Reaction

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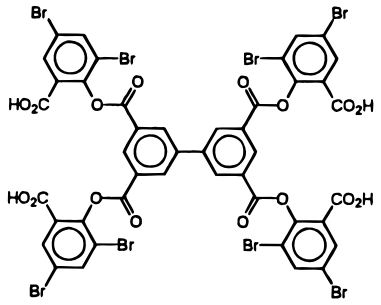
Abstract: A tetrafunctional site-directed reagent for protein modification has the potential for introducing specific cross-links by reaction at two of its four reactive sites. The remaining reactive groups on the link within the protein are available for further reaction with added reagents. The first example of such a designed multifunctional cross-linker, 3,5,3′,5′-biphenyltetracarbonyl tetrakis(3,5-dibromosalicylate), BTDS (**1**), was prepared by treatment of the tetra *tert*-butyl ester with trifluoroacetic acid. The ester was from reaction of the acid chloride of biphenyl 3,5,3′,5′-tetracarboxylic acid with *tert*-butyl-3,5-dibromosalicylate. BTDS contains four anionic sites each adjacent to four electrophilic sites. The reaction of BTDS with deoxy human hemoglobin A generates in high yield the biphenyl bis carboxamide at the ϵ-amino of lysine-82 of each of the β subunits (BBS-Hb). The remaining ester groups from the biphenyl cross-link are available to react with other nucleophiles. This is demonstrated by efficient reaction with ethylenediamine. The use of multifunctional cross-linkers presents opportunities for introduction of probes and bioactive materials.

Site-specific chemical modification of proteins[1] can be achieved with reagents that combine regioselectivity and chemoselectivity.[2] For example, anionic electrophiles modify hemoglobin at the cationic site that normally binds 2,3-diphosphoglycerate.[3] The use of bifunctional versions of such reagents makes it possible to accomplish site-directed cross-linking of a protein.[4−10] While bifunctional anionic electrophilic reagents are very selective in their reactions with hemoglobin, trifunctional reagents are even more selective and efficient, probably due to their greater charge density and directed alignment from the third anionic electrophilic site.[11,12] In addition, the trifunctional reagents can combine cross-linking of a protein with introduction of a bioconjugation site, permitting the addition of other species such as biophysical probes and drugs.[13] Where the introduction of bioconjugation sites is desired, the trifunctional reagents lose that possibility if the third group reacts with a nucleophile on the protein.[11] If the third group does not react, there still is only one site available for reaction at the introduced site on the modified protein. Specific applications may require multiple additions of the same exogenous molecule or addition of several entities.

Based on these considerations, we sought a tetrafunctional reagent that would improve possibilities for introducing reactive sites into a specifically cross-linked protein. We know from reactions of bi- and trifunctional reagents that meta-substituted benzene derivatives give maximal yields of cross-linked protein with desirable properties. Therefore, we designed the tetrafunctional reagent to have meta substitution, the tetra-3,5-dibromosalicyl ester derived from biphenyl 3,5,3′,5′-tetracarboxylic acid (**1**, BTDS or 3,5,3′,5′-biphenyltetracarbonyl tetrakis(3,5-dibromosalicylate)).



1, BTDS

Experimental Section

Commercial reagents and chemicals were used without further purification. Solvents were dried prior to use. Reagents for preparation of buffers were of analytical grade or better. Purified human hemoglobin A was from Hemosol, Inc. Proton NMR spectra were obtained at 400 MHz and ^{13}C NMR spectra were obtained at 100 MHz.

3,5,3′,5′-Biphenyltetracarbonyl tetrakis(3,5-dibromosalicylate) (1, BTDS). The preparation involves generation of 3,5,3′,5′-biphenyltetracarboxylic acid by oxidative coupling of 5-bromo-*m*-xylene.[14] This is converted to the acid chloride and then to the tetrakis *tert*-butyl-

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3,5-dibromosalicylate. A solution of *tert*-butyl-3,5-dibromosalicylate[12,15] (1.4 g, 0.0040 mol) and potassium *tert*-butoxide (0.45 g, 0.0040 mol) in 30 mL of dry tetrahydrofuran was stirred at room temp for 20 min. To this was added a solution of 0.40 g (0.0010 mol) of 3,5,3′,5′-biphenyl tetrakis(carbonyl chloride) in 15 mL of anhydrous tetrahydrofuran. Stirring was continued overnight. Water was added, producing a white precipitate. The product was isolated by extraction with ether, drying over magnesium sulfate, filtration, and evaporation. The resulting white solid (3,5,3′,5′-biphenyltetracarbonyl tetrakis(*tert*-butyl-3,5-dibromosalicylate)) was dissolved in 20 mL of anhydrous trifluoroacetic acid and left at room temp for 1 h. Then 20 mL of ether was added to the solution, producing a white precipitate. This was filtered and dried in vacuum (1.1 g, 76%): IR (KBr) 3079 (br s, ν_{OH}), 1736 (s, $\nu_{C=O}$), 1202 (s, ν_{C-O}) cm^{-1}; ^1H NMR (dioxane-d_8) δ 9.01 (2 H, t, $J = 1.6$ Hz, ArH), 8.80 (4 H, d, $J = 1.6$ Hz, ArH), 8.21 (8 H, dd, $J = 4.0$ Hz, ArH), 2.57 (4 H, br s, CO_2H); ^{13}C NMR (dioxane-d_8) δ 163.56, 162.96, 148.26, 141.65, 140.29, 134.85, 134.72, 131.85, 131.48, 127.55, 120.01; UV (dioxane) $\lambda_{max,1} = 230$ nm, $\epsilon_{max,1} = 133\,000$, $\lambda_{max,2} = 296$ nm, $\epsilon_{max,2} = 14\,000$; MS (negative FAB): 1441 (found), 1441 (M − 1 calcd for $C_{44}H_{18}O_{16}Br_8$), parent peak, 100%; isotopic distribution for observed consistent with eight bromines and two isotopes (1435, 11%; 1436, 5.6%; 1437, 39%; 1438, 19%, 1439, 78%; 1440, 38%, 1441, 100%; 1442, 47%; 1443.83%; 1444, 38%, 1445, 45%; 1446, 19%, 1447.15%); other major peaks in the molecular envelope: 1163 (and isotopic peaks), $C_{37}H_{16}O_{14}Br_6$ (calcd for M − 1 less one ester group), 80%; 885 (and isotopic peaks), $C_{30}H_{14}O_{12}Br_4$ (calcd for M − 1 less two ester groups), 80%. The purity of the material was assessed by HPLC on a C-18 Vydac reversed-phase column (9.25 × 0.46 cm). The acetonitrile developer contained 0.1% trifluoroacetic acid. The effluent was monitored at 220 nm. The chromatogram indicated that BTDS is at least 98% pure since only a single peak was observed.

Reaction of BTDS with Hemoglobin. A solution of human hemoglobin A (CO form, 2.0 mL, 1.40 mM) in 50 mM pH 6.5 Bis-Tris buffer was passed through a Sephadex G-25 column (2.5 × 15 cm) equilibrated with 50 mM pH 8.0 sodium borate buffer. The CO hemoglobin was converted into oxyhemoglobin by irradiation under flowing oxygen at 0 °C for 2 h. The oxyhemoglobin was deoxygenated under flowing nitrogen at 38 °C for 2 h. BTDS (0.0081 g, 5.6×10^{-6} mol) was dissolved in 1.0 mL of dioxane, and 2.8 mL of 50 mM pH 8.0 sodium borate buffer was added. Oxygen was removed from the solution by evacuation followed by addition of nitrogen, three times. The reagent solution was then added under nitrogen to the deoxyhemoglobin solution. The reaction mixture was kept at 38 °C for 2 h, and humidified nitrogen flowed through the rotating flask continuously. At the end of the reaction period, the flask was flushed with carbon monoxide. The solution of modified CO hemoglobin was passed through a Sephadex G-25 column (2.5 × 15 cm) equilibrated with 0.1 M pH 7.2 MOPS buffer to remove any unreacted reagent. C-4 reversed-phase HPLC columns were used to separate heme and the globin chains. The cross-linked β subunits were identified by ion spray mass spectrometry of the peak from the HPLC. M^+: 32 585 (found), 32 586 (calcd for β_2-bis(biphenyl dicarboxamide)bis(3,5-dibromosalicylate)). The reaction of carbonmonoxy hemoglobin with BTDS was carried out in a sealed vial saturated with carbon monoxide.

Determination of the Structure of Modified Hemoglobin. Heme and globin chains were separated by reversed-phase HPLC on 330-Å pore size C-4 Vydac columns (250 × 4.6 mm for analysis and 250 × 12 mm for preparation), using developers containing 0.1% trifluoroacetic acid and various gradients of acetonitrile (starting at 20% and ending at 60%) in water.[16] The effluent was monitored at 220 nm. Globin chains were recovered from the effluent by lyophilization.

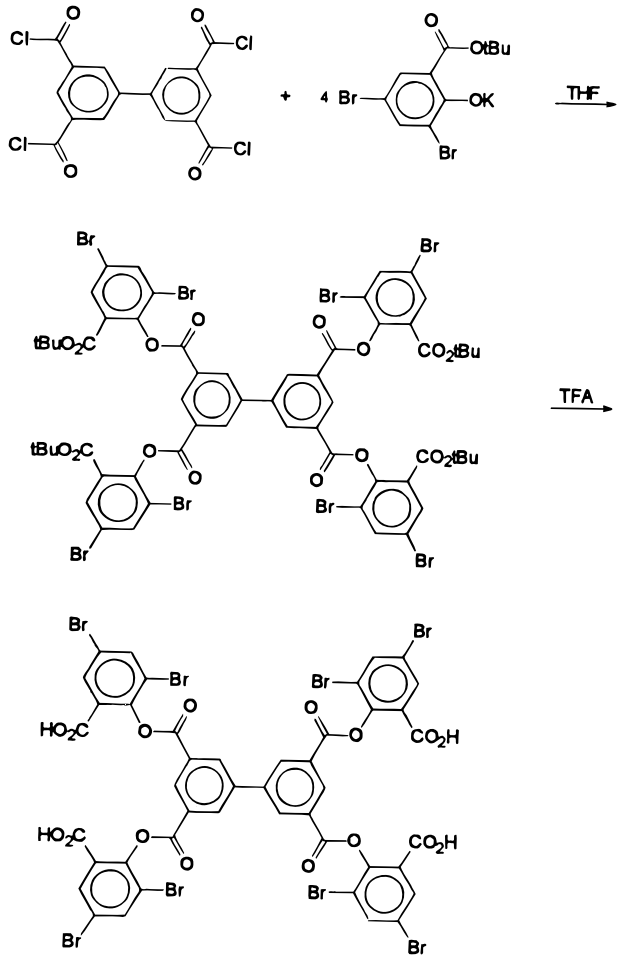
Globin chains were dissolved in 8 M urea and kept at room temperature for 2 h.[16] The solution was then diluted with 80 mM pH 8.5 ammonium bicarbonate buffer to a final concentration of 2 M urea. Trypsin (2% of total protein) was added, and the solution was allowed to stand at room temp for 24 h. Test tubes containing the hydrolysate were placed in boiling water for 2 min. The solution was then diluted with 80 mM pH 8.5 ammonium bicarbonate buffer to a final concentration of 1 M urea, and endoproteinase Glu-C (1% of total protein) was added. The solution was allowed to stand at room temperature for 72 h. Before injection onto the HPLC column, the hydrolysates were passed through a 0.45-μm filter.

Peptide fragments were separated on a C-18 Vydac reversed-phase HPLC column (9.25 × 0.46 cm).[16] Developers contained 0.1% trifluoroacetic acid and gradients of acetonitrile (starting at 0% and ending at 100%) in water. The effluent was monitored at both 220 and 280 nm.

Reaction of Cross-Linked Hemoglobin with Ethylenediamine. The solution of modified hemoglobin was passed through a column of Sephadex G-25 (2.5 × 15 cm) that had been equilibrated with 50 mM pH 8.0 sodium borate to remove unreacted reagent. A solution of 0.23 mL of 2.0 M ethylenediamine (pH 8, adjusted with HCl) was added to the sample. The reaction mixture was saturated with carbon monoxide, sealed, and kept at 37 °C for 2 h. The solution was then passed through the re-equilibrated Sephadex column to remove the amine. The product was isolated and analyzed as described for the initial modification. The modified globin chain isolated from the C4 column was analyzed by ion spray mass spectrometry.

Functional Properties of Modified Hemoglobin. Cross-linking affects the structure and oxygen-binding properties of hemoglobin.[4,10,11,17] In order to determine the changes due to the cross-link, the residual ester groups on the cross-link were removed by spontaneous hydrolysis in buffer, a reaction that has been studied in detail for related modified hemoglobins.[17] Functional properties were measured as described previously.[17]

Scheme 1



Results

The synthesis of BTDS is readily accomplished from the acid chloride. The route is summarized in Scheme 1.

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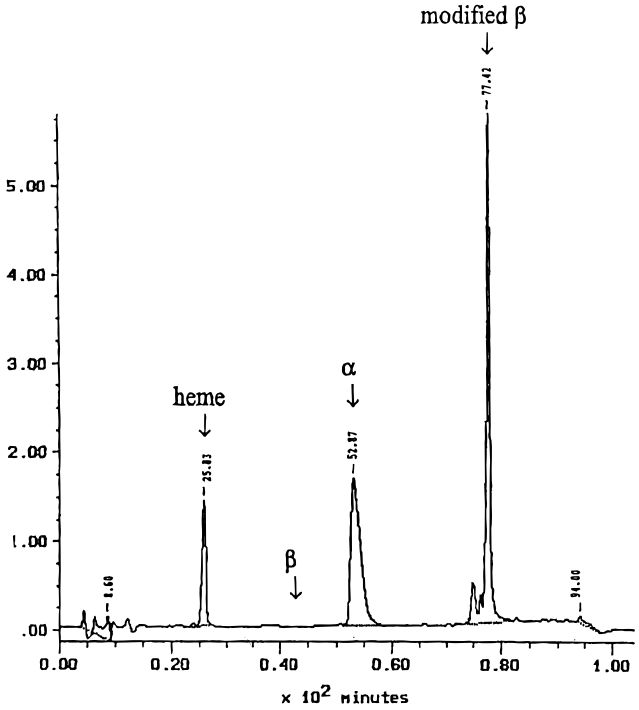

Figure 1. Globin chain separation after reaction of deoxy hemoglobin with BTDS.

Based on earlier work with other dibromosalicylates, we expected the reagent to react with the ϵ-amino groups of lysine-82 of each of the β subunits of human hemoglobin in the site that binds 2,3-diphosphoglycerate.[3,12,17] Combination of BTDS and carbonmomoxy hemoglobin at pH 8 gave no reaction according to HPLC analysis, consistent with the general inaccessibility of the 2,3-diphosphoglycerate binding site when hemoglobin is in the R state (which is favored by binding of CO or oxygen).[18] When hemoglobin is in the deoxy state, the 2,3-diphosphoglycerate binding site is accessible, and the reagent can react with the most accessible amino groups. Thus, the combining of 1 equiv of hemoglobin and 2 equiv of BTDS (pH 8.0) gives complete conversion of all the hemoglobin to a derivative. Analysis of the product was achieved by separation of globin chains and hemes by HPLC on a C-4 reversed-phase column (Figure 1). This indicates the formation of a single modified protein.

The site of the modification of the product was determined by a combination of mass analysis and digestion patterns. The hemes and the globin chains were separated by HPLC. The ion spray mass spectrum of the material eluting in the zone marked "modified β" in the C-4 chromatogram (Figure 1) indicated that two β chains are cross-linked as biphenyltetracarbonyl bis amides with two 3,5-dibromosalicylate ester groups remaining unreacted. In order to determine the site of modification of the protein chain, enzymic digestion patterns were analyzed.[11,16] Comparison of results from modified and unmodified proteins reveals the site of modification. The β chains of the product and native β chains were treated with trypsin followed by endoproteinase Glu-C. Trypsin hydrolyzes peptide bonds at the C-terminal side of amino acids with positively charged residues (lysine and arginine). The resulting peptides are further hydrolyzed by endoproteinase Glu-C at the C-terminal side of glutamate residues.[11,16]

In the C-18 reversed-phase HPLC, the peptide pattern of the chromatogram of the cross-linked β subunits contains a sharp peak at 95 min that is not present in that of the native protein. The peptide fragments βT-9 and βT-10a′ are found in the chromatogram from the unmodified β chain but are absent in that from the modified protein. This peptide results from cleavage adjacent to lysine-β-82. Therefore, we can conclude that in the modified chains, the ϵ-amino group of both lysine-β-82 residues have been acylated. This, combined with the mass spectral data, requires that a bis amide cross-link between the amino groups has formed specifically. The product is thus a bis(biphenylamide salicylate) derivative of hemoglobin (abbreviated BBS-Hb).

We reacted BBS-Hb with ethylenediamine to test the availability of the ester sites for reaction. A 220-fold excess of the amine was used to favor competition against ester hydrolysis (which is catalyzed by hydroxide). The reaction was followed by HPLC, and the chromatogram shows a single product results with very little unreacted ester remaining. The ion spray mass spectrum of the peak corresponds to the bis(ethylenediamine) derivative of the cross-linked β subunits, 32 109 (calcd 32 114). Scheme 2 illustrates the reaction of hemoglobin with BTDS followed by the reaction with excess ethylenediamine.

The functional properties of the cross-linked protein were determined after the ester groups from the reagent were hydrolyzed. This is necessary since the functional measurements are slow. Competing hydrolysis converts the tetraester into a mixed collection of partial hydrolysis products. (We designate the protein in which the α subunits are unmodified and the β subunits are cross-linked with the unreacted biphenyl carboxyl groups unesterified ($\alpha\alpha\beta\beta'$(82-lysyl,82′-lysyl)-bis-(biphenyl tetracarboxyl)bisamide or simply BB-Hb; see Scheme 2). Table 1 shows that oxygen binds cooperatively to the cross-linked hemoglobin, as has been observed for other cross-linked hemoglobins. The oxygen affinity is significantly reduced compared to that of native hemoglobin. The alkaline Bohr effect

Scheme 2

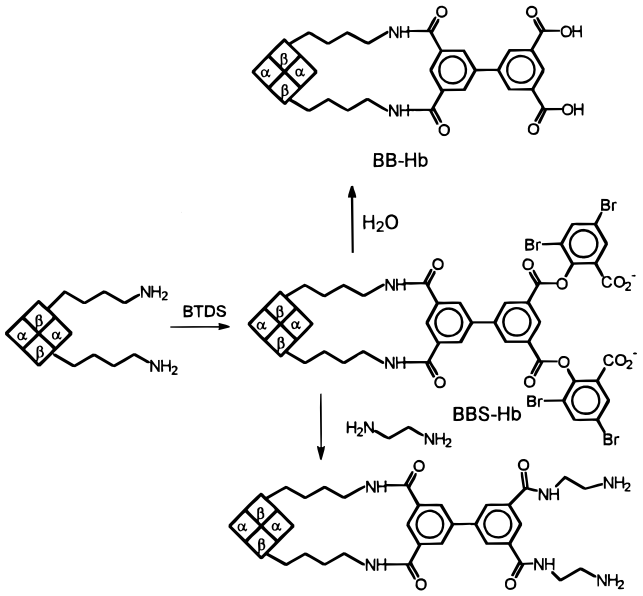

Table 1. Oxygen Binding Properties of Native and Cross-Linked Hemoglobin[a]

Hb structure	cross-link	P_{50} (torr)	n_{50}	% Bohr effect
$\alpha\alpha\beta\beta$	none	5.0	3.0	100 (−0.54)
$\alpha\alpha\beta$−82-B−82β	dicarboxybiphenyldicarbonyl	13.9	2.5	74

[a] Conditions for P_{50} and n_{50} are pH 7.4, 50 mM Bis-Tris, 0.1 M Cl^-, 55 μM heme at 25 °C.

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is also reduced to the same extent as has been observed for other lysine-82 acylated hemoglobins.[10]

Discussion

BTDS reacts with the ϵ-amino groups of the lysine-82 residues of both β chains of deoxy hemoglobin. This results in a cross-link between the two β subunits (Scheme 2), giving BBS-Hb with no significant byproducts. The two ester groups of BTDS that are not involved in the cross-linking reaction remain intact after the reaction with hemoglobin. These are available for reaction with added nucleophiles as demonstrated by aminolysis with ethylenediamine.

The degree of specificity of the modification is remarkable. Conversion of the native chains is complete, and analysis reveals only a single product although there are 24 amino groups per dimer available for reaction. Since other dibromosalicylates do not provide as extraordinarily high a degree of specificity,[19] the structure of the reagent must enhance its specificity. The size alone cannot be responsible since we have used other large molecules and these do not give similar results.[10] Therefore, selection is not simply the result of a steric effect. The mass-to-charge ratio is similar to that of bifunctional reagents derived from the dibromosalicylates of isophthalic acid. Isophthalyl bis-(3,5-dibromosalicylate) shows a much lower degree of specificity in its reaction with hemoglobin although it has the same leaving groups as BTDS.

Relatively high specificity has been achieved with trifunctional anionic acylating reagents.[12] Therefore, the total charge on the reagent is likely to be a significant factor. The four free carboxyl groups in BTDS should be ionized under the reaction conditions given that p*K*'s of the carboxyl groups should be <5. Thus, the reagent is a tetra anion, with the charge dispersed on its surface. Since the 2,3-diphosphoglycerate binding site in deoxyhemoglobin has widely dispersed positively charged groups,[20] there is the opportunity for a well-defined and oriented interaction. This results in high specificity and little or no reaction elsewhere. The fact that the reagent does not react with carbonmonoxy hemoglobin may indicate the degree to which electrostatic interactions are necessary for reaction. Alternatively, conformational differences between the deoxy and the carbonmonoxy forms may lead to different degrees of exposure of the reacting groups. The size of the central cavity is significantly smaller when oxygen or carbon monoxide is bound to the hemes, restricting the especially bulky reagent from reaching the amino residues in the 2,3-diphosphoglycerate binding site.[18]

There are two ways for BTDS to form the link between the lysine-82 residues of the two β subunits. The cross-link can be between groups on the same ring of the biphenyl or between acyl groups on each of the benzene rings (shown as A and B in Figure 2). These cannot be distinguished on the basis of the information we have available. The two will have the same molecular mass and the same peptide digestion pattern. X-ray crystal structure analysis of BB-Hb, currently in progress, will provide an answer along with much more structural detail.

The oxygen affinity of BB-Hb is lower than that of native hemoglobin while it retains a high degree of cooperativity (Table 1). We have shown that there is a correlation between the length of a cross-link and the oxygen affinity of the hemoglobin as measured by P_{50}.[10] The oxygen affinity of BB-Hb is much lower than would be predicted by the correlation if the cross-link is within the same benzene ring of the biphenyl. However, even if the link were between groups on different rings of the biphenyl, it still would not fit the correlation. This suggests that the additional anionic groups have a significant effect on reducing the oxygen affinity, beyond that of the cross-link. It is notable that the affinity is close to that considered ideal for a red cell substitute to be used for transfusions.[21]

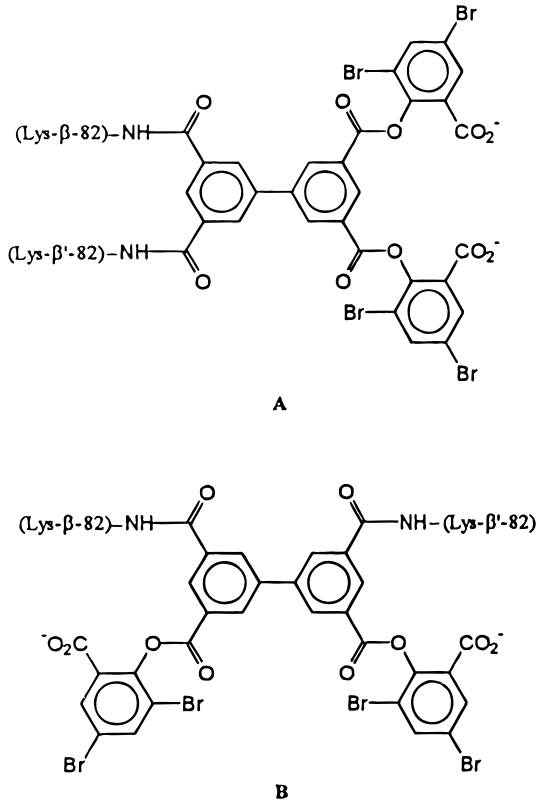

Figure 2. Schematic representation of possible structures of the cross-linked β subunits formed in the reaction of deoxyhemoglobin with BTDS.

Conclusions

Site-directed cross-linking of a protein with a tetrafunctional reagent extends the utility of chemical modification in creating specifically altered proteins. The reagent is highly selective and efficient. The product contains two unreacted ester groups that provide sites for further reaction. The two sites make possible the addition of species with useful physical properties as biophysical probes in combination with other species, such as for drug delivery. The resulting modified hemoglobin also provides efficient oxygen delivery that can be used in combination with the attachment process.

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