Systematically Cross-Linked Human Hemoglobin: Functional Effects of 10 Å Spans between Beta Subunits at Lysine-82

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Abstract: The structure and properties of hemoglobin are altered by the introduction of cross-links of defined structure between specific residues. The bis methyl phosphates and bis 3,5-dibromosalicylates of 4-carboxy-*trans*-cinnamic acid as well as the bis methyl phosphate of 2,6-naphthalenedicarboxylic acid produce a 10 Å cross-link between the ϵ-amino groups of each β-lys-82 of human hemoglobin. The oxygen affinity of the modified proteins fits the correlation interpolated from those for shorter and longer cross-links. The oxygen binding curve shows a high degree of cooperativity. These results support the idea that the length of the semirigid cross-link in a structurally homogeneous series constrains the relaxation of the protein upon oxygen binding by a mechanism that is specifically reflected in the oxygen affinity, while interactions between hemes that affect cooperativity are not diminished.

Proteins assume defined shapes that are perturbed in the course of their function. Site-directed chemical modification of a protein presents the possibility of controlling the structure in order to alter function.[1−3] Such specifically altered proteins provide a basis for detailed studies of protein structure and function. Bifunctional and trifunctional semirigid anionic acylating agents react regioselectively to cross-link proteins, introducing the molecular equivalent of a clamp. This is exemplified by the reactions of bis(acyl methyl phosphates) derived from a series of unsaturated dicarboxylic acids that selectively cross-link human deoxy hemoglobin A between its two β subunits: at the ϵ-amino groups of lysine-82 of each subunit or at one of the lysine-82 amino groups and the N-terminal valine α amino group.[1,4] Some of these products have been observed in reactions of 3,5-dibromosalicylate esters of the same dicarboxylic acids.[5−7] Our analysis of the oxygen affinities of these cross-linked proteins reveals a correlation between the free energy of oxygen binding to hemoglobin and the molecular length of the cross-link.[2] We proposed that this is the result of differential translational restriction in the modified proteins. Subsequent X-ray crystallographic analysis of several of the structures substantiated this proposal.[8]

The selection of spans in the set of cross-linkers used in those studies was based on a limited set of aromatic and unsaturated dicarboxylic acids: fumaric acid, isophthalic acid, terephthalic acid, and (3,3′)-stilbene dicarboxylic acid. Molecular modeling of the cross-links between nitrogen atoms of the resulting modified protein gives distances of 6.1, 7.3, 7.5 and 13.2 Å respectively.[2]

The two types of cross-linked hemoglobin ($\alpha\alpha\beta$-lysine-82-X-β'lys-82) and ($\alpha\alpha\beta$-lysine 82-X-β'-valine-1) yield separate oxygen-affinity correlation. The slope are of opposite sign, and they intersect at a point corresponding to a span of about 10 Å. The 10 Å distance is not close to the data points for the cross-linked proteins but is in the middle of both correlations. Thus, in order to test one of the correlations as well as to provide materials for other studies we used molecular modeling to consider materials that would give a 10 Å cross-link. Our selection was based on a consistency with the structures in the correlation. In this paper we report the preparation of cross-linkers that produce materials in this ∼10 Å range and the properties of some of the materials resulting from their reaction with hemoglobin.

Experimental Section

Human hemoglobin A was obtained as the highly purified material in the deoxy state from Hemosol, Inc. The material was stored as the tetracarbonomonoxy form at 4 °C and converted to the deoxy form prior to reaction by photodissocation under wet nitrogen.

We calculated the approximate distance between amino groups of amides resulting from cross-linking between ϵ-amino groups of lysine residues of a protein using the bis methyl amide derivative of the core dicarboxyl compound as a model. The calculations were done under MS-DOS with the program Alchemy 3 (TRIPOS Associates) which uses the TRIPOS 5.2 force field[2] and the Alchemy minimizer (as described in the program manual).

Syntheses. General Methods. The reagents developed for these studies are methyl acyl phosphates and dibromosalicylates of known dicarboxylic acids. These derivatives are generally reactive and hygroscopic. They are principally characterized by their nmr spectra and by their hydrolysis products. The resulting cross-linked hemoglobins define the structure of interest.

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(*E*)-4-(Carboxylethenyl)benzoic Acid.[9] **[56148-65-3] (4-Carboxy-*trans*-cinnamic Acid).** We used the method of Rappoport[10] with minor changes. The stereochemical assignment is based on more recent work by Cervinka.[11] A mixture of 4-carboxybenzaldehyde (5.6 g, 37 mmol), malonic acid (5.7 g, 55 mmol), and piperidine (0.6 mL) in dry pyridine (30 mL) was stirred and heated for 90 min at 80 °C, and 2 hours at 100 °C, and then refluxed for 90 min. The solution was then poured into 3 N HCl (250 mL). The resulting solid was collected by filtration and washed with 250 mL of water and then with 250 mL of 95% ethanol. The product was dried under vacuum (6.1 g, 86%): mp 362−363 °C (decomposed), lit. 358 °C; IR (KBr) C═O 1688 cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.97 (ArH, 2 H, d, J = 8.1 Hz), 7.80 (ArH, 2 H, d, J = 8.4 Hz), 7.65 (HC═C, 1 H, d, J = 16.1 Hz), 6.65 (HC═C, 1 H, d, J = 16.1 Hz); ^{13}C NMR (DMSO-d_6) δ 167.47, 167.00, 142.79, 138.49, 131.99, 129.90, 128.39, 121.72.

(*E*)-4-(Carbonylethenyl)benzoyl Dichloride (4-Carboxy-*trans*-cinnamic Acid Dichloride). To a stirred suspension of 4-carboxy-*trans*-cinnamic acid (1.0 g) and a catalytic amount of dry dimethylformamide (2 drops) in dry dichloromethane (15 mL), oxalyl chloride (1.0 mL, 11.4 mmol) was added dropwise at room temperature under nitrogen. After addition, the reaction mixture was refluxed under nitrogen for 2 h. Solvent was removed under reduced pressure. The resulting yellow needles were recrystallized from dry ether (10 mL) giving 1.1 g (92%) of pale yellow crystals: mp 125−126 °C; IR (KBr) C═O 1740, 1770 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.19 (ArH, 2 H, d, J = 8.4 Hz), 7.87 (HC═C, 1 H, d, J = 15.6 Hz), 7.74 (ArH, 2 H, d, J = 8.5 Hz), 6.80 (HC═C, 1 H, d, J = 15.6 Hz); ^{13}C NMR ($CDCl_3$) δ 167.50, 165.63, 147.53, 139.16, 135.21, 131.86, 129.12, 126.03.

2,6-Naphthalenedicarbonyl Dichloride. To a stirred suspension of 2,6-naphthalenedicarboxylic acid (0.5 g) containing one drop of dimethylformamide in dry dichloromethane (10 mL) was added oxalyl chloride (0.44 mL) slowly under nitrogen at room temperature. After addition, the reaction mixture was refluxed under nitrogen for 4 h. Solvent was removed under reduced pressure. The resulting solid (yellow needles) was recrystallized from dry ether (8 mL (0.55 g, 94%): mp 189−190 °C; IR (KBr) C═O 1750 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.79 (2 H, s), 8.20 (2 H, d, J = 8.7 Hz), 8.12 (2 H, d, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$) δ 168.02, 135.19, 133.70, 133.60, 130.82, 126.69.

4-Carbonyl-*trans*-cinnamoyl Bis(dimethyl phosphate). This follows the general procedure for other acyl dimethyl phosphates.[4] A suspension of sodium dimethyl phosphate (1.58 g, 10.7 mmol) and 4-carbonyl-*trans*-cinnamoyl dichloride (1.2 g, 5.2 mmol) was stirred in dry tetrahydrofuran (15 mL) under nitrogen at room temperature for 2 h. The solution was filtered, and solvent was removed, leaving the product as a solid. Recrystallization from dry benzene and ether gives the product as white flakes (1.9 g, 90%): mp 78−79 °C; IR (KBr) C═O 1700, 1730 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.11 (ArH, 2 H, d, J = 8.1 Hz), 7.83 (HC═C, 1 H, d, J = 16.1 Hz), 7.67 (ArH, 2 H, d, J = 8.4 Hz), 6.55 (HC═C, 1 H, dd, J = 15.8 Hz, J_{P-H} = 1.8 Hz), 4.01 (OCH_3, 6 H, d, J_{P-H} = 11.7 Hz), 3.97 (OCH_3, 6 H, d, J_{P-H} = 11.6 Hz). ^{13}C NMR ($CDCl_3$) δ 160.19 (d, J_{P-C} = 7.9 Hz), 160.00 (d, J_{P-C} = 8.0 Hz), 146.98, 139.06, 131.25, 129.80 (d, J_{P-C} = 8.6 Hz), 128.63, 119.20 (d, J_{P-C} = 9.3 Hz), 55.62 (d, J_{P-C} = 3.0 Hz), 55.57 (d, J_{P-C} = 2.8 Hz); ^{31}P NMR ($CHCl_3$) δ −4.6 (s, decoupled), −4.9 (s, decoupled).

2,6-Naphthalenedicarbonyl Bis(dimethyl phosphate). A suspension of sodium dimethyl phosphate (0.7 g, 4.7 mmol) and 2,6-naphthalenedicarbonyl dichloride (0.55 g, 2.2 mmol) was stirred in dry tetrahydrofuran (10 mL) under nitrogen at room temperature for 3 h. Solvent was removed under reduced pressure. The resulting white powder was dissolved in dry benzene (10 mL). The mixture was filtered through a sintered-glass funnel and washed with dry benzene (10 mL). The solvent was removed under reduced pressure, leaving the product as a solid. Recrystallization from benzene and ether yielded the product as white flakes (0.9 g, 95%): mp 97−99 °C; IR (KBr) C═O 1740 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.68 (2 H, s), 8.15 (2 H, dd, J = 8.5 Hz, J_{P-H} = 1.4 Hz), 8.09 (2 H, d, J = 8.6 Hz), 4.06 (OCH_3, 12 H, d, J_{P-H} = 11.7 Hz); ^{13}C NMR ($CDCl_3$) δ 160.52 (d, J_{P-C} = 8.3 Hz), 134.90, 132.27, 130.29, 127.97 (d, J_{P-C} = 8.5 Hz), 126.41, 55.56 (d, J_{P-C} = 5.3 Hz); ^{31}P NMR ($CHCl_3$) δ −4.6 (s, decoupled).

4-Carbonyl-*trans*-cinnamoyl Bis(sodium methyl phosphate) (CCMP). A solution of sodium iodide (1.65 g, 11 mmol) in dry acetonitrile (8 mL) was added to an acetonitrile (8 mL) solution of 4-carbonyl-*trans*-cinnamoyl bis(dimethyl phosphate) (1.9 g, 4.7 mmol) in a 25-mL round-bottom flask at room temperature under nitrogen. The solution was shaken, and the flask was left for 12 h, during which time the product precipitated as a pale yellow powder. Filtration followed by washes with dry acetonitrile (2 × 8 mL) and methylene chloride (2 × 8 mL) resulted in an off-white powder that was dried under reduced pressure. The powder was recrystallized by dissolving in 40 mL of methanol with gentle heating. Then 60 mL of 1:1 (v/v) ethanol:propan-2-ol was added, and the solution was allowed to stand for 30 min. The resulting white crystals were collected by vacuum filtration and dried *in vacuo* (1.8 g, 90%); mp 278−280 °C; IR (KBr) C═O 1700, 1730 cm^{-1}; ^1H NMR (D_2O) δ 7.98 (ArH, 2 H, d, J = 8.4 Hz), 7.71 (HC═C, 1 H, d, J = 15.9 Hz), 7.68 (ArH, 2 H, d, J = 8.1 Hz), 6.55 (HC═C, 1 H, dd, J = 16.0 Hz, J_{P-H} = 1.7 Hz), 3.60 (OCH_3, 3 H, d, J_{P-H} = 11.5 Hz), 3.57 (OCH_3, 3 H, d, J_{P-H} = 11.5 Hz). ^{13}C NMR (D_2O) δ 165.22 (d, J_{P-C} = 7.7 Hz), 164.71(d, J_{P-C} = 7.1 Hz), 147.30, 140.58, 132.22, 131.58, 130.25, 121.96 (d, J_{P-C} = 6.0 Hz), 55.11 (d, J_{P-C} = 2.6 Hz), 54.95; ^{31}P NMR (D_2O) δ −5.0 (s, decoupled), −5.2 (s, decoupled). Anal calc for $C_{14}H_{12}O_{10}P_2Na_2$: C, 33.98; H, 2.85. Found: C, 34.07; H, 2.85.

2,6-Naphthalenedicarbonyl bis(sodium methyl phosphate) (NMP) was prepared from 2,6-naphthalenedicarbonyl bis(dimethyl phosphate) (0.9 g, 2.1 mmol) and sodium iodide (0.7 g, 4.7 mmol) in acetonitrile (10 mL) as above. The crude product was recrystallized by dissolving in 20 mL of methanol with gently heating. Then 40 mL of 1:1 (v/v) ethanol:propan-2-ol was added, and the solution was allowed to stand for 30 min. The resulting off-white crystals were collected by vacuum filtration and dried *in vacuo* (0.9 g, 96%): mp 290−292 °C; IR (KBr) C═O 1710 cm^{-1}. ^1H NMR (D_2O) δ 8.46 (2 H, s), 7.91 (4 H, s), 3.67 (OCH_3, 6 H, d, J_{P-H} = 11.4 Hz).^{13}C NMR (D_2O) δ 164.95 (d, J_{P-C} = 7.7 Hz), 135.93, 133.02, 131.65, 130.39 (d, J_{P-C} = 7.0 Hz), 127.56, 55.29 (d, J_{P-C} = 4.7 Hz). ^{31}P NMR (D_2O) δ −4.6 (s, decoupled).

***tert*-Butyl-3,5-dibromosalicylate** was prepared as described previously.[6,12−14]

4-Carbonyl-*trans*-cinnamoyl Bis(*tert*-butyl-3,5-dibromosalicylate). To a solution of *tert*-butyl-3,5-dibromosaliylate (1.34 g, 3.8 mmol) in dry tetrahydrofuran (5 mL) was added 3.8 mL of 1.0 M potassium *tert*-butoxide in tetrahydrofuran. The mixture was stirred for 15 min at room temperature. 4-Carbonyl-*trans*-cinnamoyl dichloride (0.41 g, 1.8 mmol) in tetrahydrofuran (5 mL) was added dropwise over 15 min. Stirring was continued for 2 h at room temperature. The solution was filtered and concentrated. The residue was recrystallized from ethanol (15 mL) to give the product (1.32 g, 85%): mp 208−210 °C; IR (KBr) C═O 1710, 1720, 1740 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.30 (ArH, 2 H, d, J = 8.3 Hz), 7.92−8.04 (ArH, 4 H, m), 7.97 (HC═C, 1 H, d, J = 15.8 Hz), 7.77 (ArH, 2 H, d, J = 8.4 Hz), 6.83 (HC═C, 1 H, d, J = 16.0 Hz), 1.52 ($C(CH_3)_3$, 9 H, s), 1.39 ($C(CH_3)_3$, 9 H, s). ^{13}C NMR ($CDCl_3$) δ 163.15, 162.85, 162.01, 161.87, 146.58, 146.53, 145.86, 139.10, 138.73, 133.59, 133.53, 131.06, 130.50, 129.11, 128.84, 128.55, 119.44, 119.34, 119.24, 119.18, 83.10, 28.02, 27.86.

4-Carbonyl-*trans*-cinnamoyl Bis(3,5-dibromosalicylate)(CCDS). 4-Carbonyl-*trans*-cinnamoyl bis(*tert*-butoxy-3,5-dibromosalicylate) (0.5 g, 0.6 mmol) was dissolved in anhydrous trifluoroacetic acid (10 mL) at room temperature. After 10 min, the mixture was diluted with ether (2 mL) and then placed in ice bath for 20 min. The crude product was collected by vacuum filtration and recrystallized from tetrahydrofuran/trifluoroacetic acid to give the product (0.36 g, 84%): mp 237−239 °C; IR (KBr) C═O 1700, 1740 cm^{-1}. ^1H NMR (DMSO-d_6) δ 8.03−8.33 (ArH, 8 H, m), 8.02 (HC═C, 1 H, d, J = 16.0 Hz), 7.16 (HC═C, 1 H, d, J = 16.1 Hz). ^{13}C NMR (DMSO-d_6) δ 163.62, 163.35, 163.26, 162.97, 146.81, 146.72, 145.90, 139.17, 138.82, 133.44, 133.34, 130.60, 130.43, 129.81, 129.75, 129.38, 128.08, 127.93, 119.56, 119.48, 119.30,

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119.13. Anal calc for $C_{24}H_{12}O_8Br_4$: C, 38.54; H, 1.62. Found: C, 38.33; H, 1.77.

Biochemical Methods. Biochemical preparations and materials follow earlier descriptions.[2,15,16]

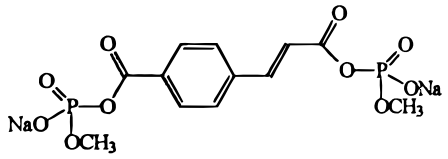
Cross-Linking Reactions. A solution of 1.4 g of carbonmonoxy hemoglobin in 20 mL of water was added to 20 mL of 0.1 M borate, pH 9.0. Oxygen was passed over the rotating solution to remove carbon monoxide. Nitrogen was flushed over the solution in three evacuation-fill cycles to produce deoxy hemoglobin. The solution was kept under nitrogen. The reagent (2 or 5 equiv) was placed into a total volume of 7 mL of 0.05 M pH 9.0 borate. Dioxane was substituted for 2 mL of buffer if needed to dissolve the reagent. The solution was flushed with nitrogen and kept under nitrogen. The preparation of cross-linker solution was done within 10 min preceding reaction with hemoglobin. The reagent solution was injected slowly into the deoxyhemoglobin solution at 35 °C under humidified nitrogen. Two equivalents of reagent was used in order to assure completion of the reaction and to compensate for competing hydrolysis. The rotating flask was left for 2 h at this temperature. At the end of the reaction period, the flask was flushed with carbon monoxide to stabilize the product and placed on ice. Reagents and low molecular weight byproducts were then removed by gel filtration.

Separation and Characterization of Modified Hemoglobins. The procedures described by Jones[15] were used. Modified hemoglobins were separated by ion exchange chromatography. The various native and modified globin chains were separated by HPLC on reversed-phase C-4 columns. The modifications of the major product were identified by digestion of the globin chains with trypsin and endoproteinase Glu-C, peptide mapping, and amino acid analysis. Ion spray mass spectrometry of the HPLC-separated globin chains in the major product was used to identify the nature of chemical modifications by changes in molecular mass. Each peak gave a characteristic mass.

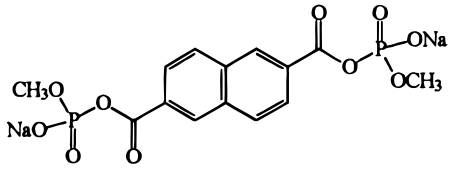
Measurement of Functional Properties of Isolated Hemoglobins. The hemoglobin−oxygen equilibrium properties of modified hemoglobin were measured as previously reported.[3] The separated major component was isolated by ion-exchange chromatography, and its oxygen binding properties was measured. The oxygen pressure at which the modified hemoglobin has half its total oxygen capacity utilized, P_{50}, and the Hill coefficient at oxygen pressure = P_{50} were determined in 50 mM Bis-Tris (pH 7.4) at 25 °C and 55 μM heme, 0.1 M chloride. The effect of pH on oxygen affinity (Bohr effect) was measured between pH 6.0 and pH 9.0 and then calculated for the change associated with a change from pH 7 to pH 8. The effect of chloride on oxygen affinity was measured over a concentration range of sodium chloride from 0.007 to 0.5 M.

Results

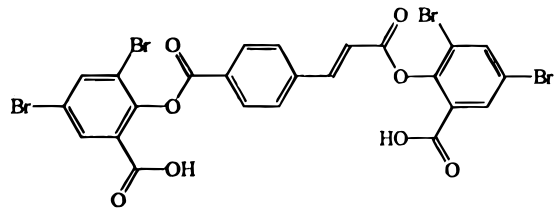
In our earlier studies we used conjugated and aromatic bifunctional anionic acylating agents to introduce length-defined cross-links into the BPG-binding site of hemoglobin between amino groups of different subunits.[2−4,7,13] The reagents were bis methyl acyl phosphates[4] and bis(3,5-dibromosalicylates).[7,12,13] The examples we prepared spanned the following distances (Å): 5.3 (fumaryl), 6.6 (isophthalyl), 7.6 (terephthalyl), and 11.6 (3,3′-stilbene dicarboxylic) between reacting amino groups. The correlation of oxygen affinity and cross-link spans was linear for two series of cross-links but with opposite slopes. The data plots cross at 10 Å, where the properties coincide with those of native hemoglobin. Since that point was not experimentally accessible, we sought a route to cross-linkers for the 10 Å span. To be consistent with the types of compounds we used for other cross-linkers we chose 2,6-naphthyl dicarboxylic acid and 4-carboxy-*trans*-cinnamic acid as our basis. Calculations with molecular mechanics minimization give maximum spans of 9.9 Å between the amino groups of *N*,*N*′-dimethyl-2,6-naphthyl bis dicarboxamide and 9.8 Å for the amino groups in *N*,*N*′-dimethyl-4-carboxy-*trans*-cinnamic bis carboxamide. These were converted to the corresponding bis(methyl acyl phosphates) (**1** and **2**). We also prepared 4-carboxy-*trans*-cinnamic acid bis(3,5-dibromosalicylate) (**3**).



1 - CCMP



2 - NMP



3 - CCDS

Reactions of Deoxyhemoglobin with Reagents. Deoxyhemoglobin was combined with the cross-linking reagents, and the products were analyzed by ion-exchange separation of modified proteins as well as by reversed-phase HPLC under conditions that separate hemes and globin chains.[2,15] Reaction with 2 equiv of each cross-linker (35 °C, 2 h, pH 9 borate) leaves <10% unmodified hemoglobin. Increasing the concentration of cross-linker to 5 eq gives little more conversion and a more complex collection of products. The reactions produce a diverse collection of modified hemoglobins. Analysis by ion exchange and reversed-phase HPLC reveals two major products resulting from reaction with **CCDS**, seven with **CCMP**, and five with **NMP**. The structures of the major products were determined by tryptic-Glu-C digestion of the globin chains along with amino acid analysis.[15]

Peptide Patterns of Modified Globin Chains. Figure 1 shows the results of reversed-phase HPLC analysis of the major β chain product obtained from the reaction of hemoglobin with **CCDS**. For comparison, Figure 2 is the digest pattern of unmodified hemoglobin. The peptides in Figure 2 designated as βT-9 contains the β-lysine 82 residue. Peptide βT-9 and the next peptide in the β chain sequence, βT-10a′, are not present in the modified peptide pattern in Figure 1. The new peptide in Figure 1 corresponds to the cross-linked residues as determined by amino acid analysis. When the ϵ-amino group of lysine residue is acylated by the 4-carbonyl-*trans*-cinnamoyl group, the residue will no longer serve as a substrate for trypsin catalyzed hydrolysis. The β82 lysyl residue is normally hydrolyzed by trypsin to form the βT-9 and βT-10a peptides or βT-10a′ peptide in the case of additional treatment with Glu-C endoproteinase. Thus, the absence of the peaks arising from βT-9 and βT-10a′ in Figure 1 indicates that the ϵ-amino groups of the β82 lysyl residues have been blocked. This is consistent with a bis-amide cross-link between the ϵ-amino group of the lysyl residue 82 of one β chain and the ϵ-amino group of the

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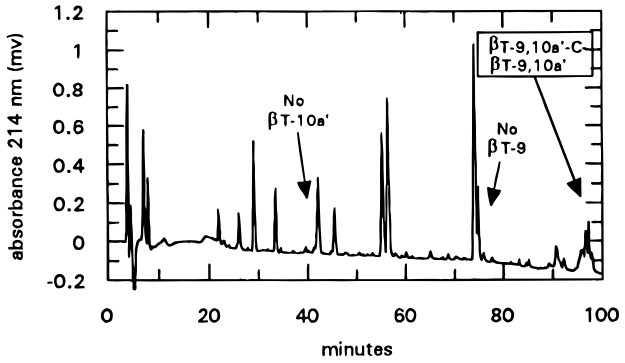

Figure 1. Tryptic-Glu-C peptides of β chains obtained from the major modified hemoglobin formed from the reaction of deoxy hemoglobin with **CCDS**.

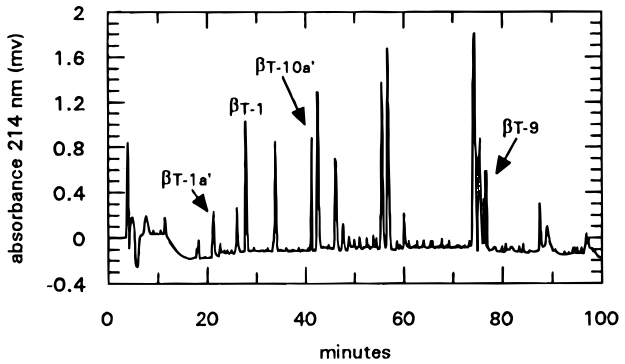

Figure 2. Tryptic-Glu-C peptides of unmodified β chains of hemoglobin.

Table 1. Oxygen Binding Properties of Cross-Linked Hemoglobins

Hb structures	cross-link	P_{50}, Torr	n_{50}	% Bohr eff.	% Cl^- eff.
native Hb	none	5.0	3.0	100 (−0.54)	100 (−0.44)
ααβ-82-N-82β	naphthyldicarbonyl	6.7	3.0	83	43
ααβ-82-C-82β	carboxycinnamyl	7.2	3.3	85	43

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

lysyl residue 82 of the other β chain. The new peptide confirms the assignment. The chemical formula of the hemoglobin from which this modified globin was obtained is designated αα(β82-C-β82) where "C" represents the 4-carbonyl-*trans*-cinnamoyl group. The product containing the naphthalene dicarboxylic cross-link between the same residues is designated αα(β82-N-β82).

The modified proteins that were isolated and analyzed contain cross-links between the lysyl 82 residues of each of the β subunits. Both products have the same span in the cross-link, and both have the same oxygen affinity ($P_{50} \sim 7$ Torr, Table 1). These values fit the correlation we observed for longer and shorter cross-links (Figure 3).

Both species show a high degree of cooperativity in binding oxygen. The Hill coefficients (n_{50}) are about the same as for the unmodified protein. The effect of protons (Bohr effect) and the effect of chloride ion on oxygen affinity are reduced in the modified hemoglobins. However, the Bohr effect is reduced to a lesser extent than is the chloride effect.

Discussion

The two modified hemoglobins contain cross-links that are chemically different between β subunits at lysine-82. Yet, the oxygen affinities are very close to each other and significantly different from those containing homologous cross-links. The hydrophobicity of the cross-linker in both cases is similar to those in the other cases in Figure 3, as is the degree of unsaturation but the oxygen affinities are very different. We had observed that the oxygen affinity is related to the length of the cross link span. The properties of the two hemoglobins with 10 Å spans fits into the correlation at the predicted location.

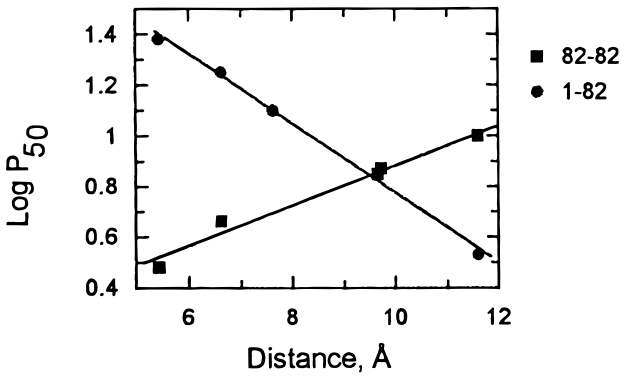

Figure 3. Oxygen affinity of cross-linked hemoglobins plotted as a function of the span of the cross-link between β subunits.[2] The data points for the two (β-82-Lys-X-Lys-β-82 cross-linked) species in the present study are at 9.8 and 9.9 Å (Table 1).

Most of the structure within hemoglobin associated with the Bohr effect is linked to that portion of the structure to which chloride ion binds to affect oxygen affinity.[17,18] This corresponds to the cationic core of the protein which includes the protonated amino group of β lysine 82.[19,20] Acylation of this residue on both subunits by cross-linking reduces the availability of this residue to interact with chloride.

The Hill coefficients for both species are close to that of unmodified hemoglobin (Table 1). Although the Hill coefficient is a macroscopic property, it originates from the interactive effects of oxygen binding to each heme.[21] If the communication between sites is blocked or reduced by the cross-link, then cooperativity should be affected. We see in the present case that cooperativity as measured by the Hill coefficient is maintained or possibly enhanced by the cross-link. The cross-link necessarily restricts movement of the globin chains. Clearly, the interactions responsible for changes in oxygen affinity are not simply results of free motions of the protein chains but are distinctly channeled. The cross-links permit the channels of communication to be maintained while affecting the overall position of the total equilibrium. Since oxygen affinity is reduced in the cross-linked protein, the full cooperative effect occurs over a smaller range of energy differential.

The structural basis of the physical effects of cross-links in hemoglobin can be understood in terms of insights from by X-ray crystallography.[8] The general restriction of motion permits higher energy states to be maintained upon binding of oxygen. The two species produced in the present study have been crystallized, and their detailed structures have been determined.[22] The location of the cross-linkers is consistent with the results we have presented. Detailed analysis of the complete structures places these hemoglobin derivatives in

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context with respect to conformational pathways through which hemoglobin undergoes structural changes upon oxygenation.[8,22]

Conclusion

Derivatives of naphthalenedicarboxylic acid and 4-carboxycinnamic acid produce cross-links within hemoglobin that span approximately 10 Å. The resulting modified proteins have oxygen-binding properties that fit a linear free energy relationship to the length of the cross-link. This suggests that a single coordinate along the cross-link can be analyzed for its relationship to the overall free energy change in the protein associated with oxygen binding. It also shows that a simple systematic change to a protein's structure can be used to introduce systematic changes in a measurable property.

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