Modification of Human Hemoglobin with Methyl Acyl Phosphates Derived from Dicarboxylic Acids. Systematic Relationships between Cross-Linked Structure and Oxygen-Binding Properties†

Richard T. Jones,*,‡ Charlotte G. Head,‡ Thomas S. Fujita,‡ Daniel T.-B. Shih,*,‡ Jolanta Wodzinska,§ and Ronald Kluger*,§

Department of Biochemistry and Molecular Biology, School of Medicine, Oregon Health Sciences University, Portland, Oregon 97201, and Lash Miller Chemical Laboratories, Department of Chemistry, University of Toronto, Toronto, Canada M5S 1A1

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ABSTRACT: Human hemoglobin was reacted with five dicarboxylic acid bis(methyl phosphate) reagents under different ligand conditions. The bis(methyl phosphate) reagents tested were derived from fumaric, isophthalic, terephthalic, *trans*-stilbene-3,3′-dicarboxylic, and *trans*-stilbene-4,4′-dicarboxylic acids. These acyl phosphate mixed anhydrides are anionic electrophiles and will react with N-terminal amino and lysyl ε-amino groups to form amides. The major and many of the minor reaction products that result have been isolated and structurally characterized by globin chain and peptide analysis. Products which are not cross-linked, intrachain linked, and interchain singly and doubly cross-linked occur in proportions which depend upon the reaction conditions and reagent. Modifications of the β chains were limited to the amino groups of β1Val, β82Lys, and, to a minor extent, β144Lys. In the case of the smaller reagents, the amino groups of α1Val, α99Lys, and, to a minor extent, α139Lys were modified. The oxygen binding affinities of most of the major modified hemoglobins have been measured and are characterized by P_{50} values from about $^1/_2$ to over 5 times that of unmodified human hemoglobin. Most show strong cooperativity with Hill coefficients (n) of 2.0 or greater. Several of the products that are cross-linked between the β1Val of one chain and the β82Lys of the other chain have oxygen affinities in a physiologically useful range for oxygen transport and delivery. An inverse linear correlation has been found between the log of P_{50} and bridging distances for the hemoglobins cross-linked between β1Val of one chain and the β82Lys of the other chain. There is a positive correlation (with a smaller slope) for the bridge length of the β82Lys cross-linked hemoglobins with the log of their P_{50}'s.

The introduction of specific chemical modifications into human hemoglobin has been the goal of a large number of studies for several purposes (Manning, 1991; Vandergriff & Winslow, 1991). Originally, modifications were made to identify structural elements and molecular mechanisms important in the reversible binding of oxygen, CO_2, and allosteric effectors (Kilmartin & Rossi-Bernardi, 1969; Kilmartin & Fogg, 1973). Covalent modification of hemoglobin continues to be an important approach to the medical treatment of sickle cell disease (DeFuria et al., 1972; Klotz & Tam, 1973; Manning, 1991). Chemically modified hemoglobins have also been considered for use in solutions as oxygen transporters for transfusion and perfusion purposes (Vandegriff & Winslow, 1991). Cross-linking of hemoglobin is necessary to prevent dissociation into αβ dimers (leading to vascular escape) (Bunn et al., 1969). Specific modification is needed to reduce the high intrinsic oxygen affinity of hemoglobin outside the erythrocyte in the absence of its principal allosteric effector, 2,3-DPG[1] (Benesch & Benesch, 1967; Chanutin & Curnish, 1967). Approaches to these problems have involved chemically cross-linking hemoglobin in ways that prevent its dissociation into αβ dimers and, with the same or other modifications, alter its oxygen binding to obtain products with physiologically useful oxygen transport properties. (Manning, 1991; Vandegriff & Winslow, 1991). A number of chemicals have been used for cross-linking and modification of hemoglobin with some success (Benesch et al., 1975; Arnone et al., 1977; Zaugg et al., 1977; Walder et al., 1977, 1979; Chatterjee et al.; 1986, Kavanaugh et al., 1988).

A new approach to cross-linking of hemoglobin that utilizes dicarboxylic acid bis(methyl phosphates) as an anionic (and thus cation-directed) acylating reagent was proposed by Kluger et al. (1990). From the apparently site-specific modifications of hemoglobin at β1Val, β82Lys, and β144Lys that were obtained with methyl acetyl phosphate (Ueno et al., 1986,

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* Authors to whom correspondence may be addressed.

‡ Oregon Health Sciences University.

§ University of Toronto.

[1] Abbreviations: Bis-tris, bis(2-hydroxyethyl)aminotris(hydroxymethyl)methane; deoxyHb, deoxyhemoglobin; DIDS, *trans*-4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DPG, 2,3-diphosphoglycerate; FMP, fumaroyl bis(methylphosphate); Hb, hemoglobin; HbCO, carbon monoxide hemoglobin; HPLC, high-performance liquid chromatography; IPMP, isophthaloyl bis(methyl phosphate); MSMP, *trans-m-* or 3,3′-stilbenedicarbonoyl bis(methyl phosphate); P_{50}, partial pressure (torr) at which the fractional saturation of hemoglobin equals 50%; PSMP, *trans-p-* or 4,4′-stilbenedicarbonoyl bis(methyl phosphate); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TMMP, trimesoyl tris(methyl phosphate); TPMP, terephthaloyl bis(methyl phosphate).

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1989), the possibility of cross-linking hemoglobin between different combinations of these residues with a bifunctional bis(methyl phosphate) was recognized (Kluger et al., 1990). A series of these reagents in which the bridging units are analogues of other cross-linkers and some are novel have been synthesized.

This paper presents the structural and preliminary functional characterization of the major and some minor products that are obtained from reacting hemoglobin under several different conditions with five different dicarboxylic bis(methyl phosphate) reagents. These studies reveal the versatility of the method, the value of systematic characterization, and the possibilities for selective control of production of desired modifications.

MATERIALS AND METHODS

Hemoglobin Preparation and Modification. Hemoglobin solutions were prepared by the method of Drabkin (1949) as modified by Kavanaugh et al. (1988), treated with carbon monoxide (CO), equilibrated with deionized water, stripped of all the remaining ions with a deionization column (Nazaki & Tanford, 1967), and stored at 0 °C on ice.

The diacyl bis(methyl phosphates) of the following dicarboxylic acids were prepared and analyzed as previously reported (Kluger et al., 1990): fumaric (FMP), isophthalic (IPMP), terephthalic (TPMP), *trans-m-* or 3,3′-stilbenedicarboxylic (MSMP), and *trans-p-* or 4,4′-stilbenedicarboxylic (PSMP).

Chemical modifications of hemoglobin were done using hemolysate diluted with 0.1 M Bis-tris-HCl buffer at pH 7.2 to 1 mM Hb (tetramer) and cross-linking reagent at between 2 mM and 5 mM. The temperature of the reaction was either 35 °C or 60 °C, and the duration of reaction was 2–3 h. At the higher temperature, the cross-linking reagent was added slowly by infusion over $^1/_2$ to 2 h. The reactions were run with hemoglobin in the carbon monoxide form (HbCO) or deoxygenated form (deoxyHb) without 2,3-diphosphoglycerate (DPG) or with it at a concentration of 5 mM. The cross-linking reagents were then removed by gel filtration through Sephadex G-25. Further details are presented in Kluger et al. (1992).

Analytical and Preparative Separations of Hemoglobins. Both cation- and anion-exchange HPLC procedures were used to separate the modified hemoglobins for analytical and some preparative purposes. The cation system utilized SynChropak CM300 columns (250 × 4.1 mm for analytical and × 10 mm for preparative from SynChrom, Inc., Linden, IN), developers containing 30 mM Bis-tris, pH 6.4, and various gradients of sodium acetate starting at 30 mM and ending at 300 mM (Huisman, 1987). Anion-exchange HPLC separations were done with SynChropak AX300 columns (250 × 4.1 mm for analytical and × 10 mm for preparative) and developers containing 15 mM Tris–acetate at pH 8.0 and various gradients of sodium acetate starting at 10 mM and ending at 150 mM (Huisman, 1987). Most preparative isolation and purification of separate hemoglobin components was done by standard ion-exchange chromatography using DEAE-Sephacel (Huisman & Dozy, 1965) and CM-Sephadex (Schroeder & Huisman, 1980). Hemoglobin was detected in the effluent by its absorbance at either 420 nm or 540 nm. Dilute hemoglobin solutions were concentrated by ultrafiltration using either a pressure ultrafiltration chamber or Amicon CF25 25 000-MW centriflo membrane cones (Amicon Corp., Danvers, MA).

Analysis of Modified Proteins. Globin chain separation by C4 reversed-phase HPLC, enzyme hydrolysis of globins by trypsin and endoproteinase GluC from *Staphylococcus aureus* V8, peptide separation by C18 reversed-phase HPLC, amino acid analysis, and SDS–PAGE estimation of molecular weights were all done as described previously (Kluger et al., 1992). For the separation of peptides, the HPLC effluent was monitored at 214 nm to detect most peptides and in addition at 280 nm to detect tyrosyl- and tryptophanyl-containing peptides, at 306 nm to detect peptides containing stilbene groups, or at 258 nm to detect phthalylated peptides. During the course of these studies the Vydac C4 columns obtained from the Separations Group changed due to alteration in the manufacturer's preparation of the silica. Newer columns bind globin chains more strongly than earlier columns; therefore, the starting concentration of acetonitrile was increased to obtain comparable elution profiles.

Measurement of Functional Properties of Isolated Hemoglobins. The hemoglobin–oxygen equilibrium properties of modified hemoglobins were measured in terms of P_{50} and Hill's coefficient, n, by the automatic recording method of Imai et al. (1970) as described in Shih and Jones (1986).

RESULTS

Influence of Reaction Conditions and Ligand State of Hemoglobin on the Products Formed. The number of modified hemoglobins, chromatographic elution positions, and relative amounts of hemoglobin products obtained for hemoglobin reacted with the diacyl bis(methyl phosphate) reagents vary with the ligand state of the hemoglobin and with the presence or absence of 2,3-DPG. Examples of this are shown in Figure 1 for the reaction of isophthaloyl bis(methyl phosphate) (IPMP) at 35 °C with deoxyHb in the absence of 2,3-DPG (Figure 1A), HbCO (Figure 1B), and deoxyHb with 5 mM 2,3-DPG (Figure 1C). The first peak in each case is unmodified Hb A. (The structural identities of all of the major and many minor products have been determined and are described later.) Comparable differences between the reaction products for HbCO and deoxyHb conditions have been found for the other dicarboxylic bis(methyl phosphate) reagents. The effect of the presence of DPG on the reaction of deoxyHb with the fumaroyl bis(methyl phosphate) (FMP) and terephthaloyl bis(methyl phosphate) (TPMP) reagents was found to be similar to that for IPMP.

Effect of Structure of the Cross-Linking Reagent on Product Formation. Comparisons of the chromatographic behavior of the modified hemoglobins produced with different diacyl bis(methyl phosphate) reagents are shown in Figure 2. The FMP, IPMP, and *trans-m-* or 3,3′-stilbenedicarbonoyl (MSMP) reagents were reacted at a concentration of 2 mM to 1 mM deoxyHb. The chromatogram shown for the TPMP reagent was reacted at 6.7 mM TPMP because the lower concentration resulted in relatively little product formation. The results using the 4,4′-stilbenedicarbonoyl (PSMP) are not presented because of the very low yield of products. The separations were made using an analytical SynChropak AX300 anion-exchange HPLC column. The elution patterns of the different reaction mixtures are complex but similar to one another. The structural identity of the major and most minor modified hemoglobin components was determined as described below.

The number, kinds, and relative amounts of modified globin chains obtained by reacting hemoglobin with the bis(methyl phosphate) reagents was also found to vary with the ligand state of the hemoglobin, the presence or absence of 2,3-DPG,

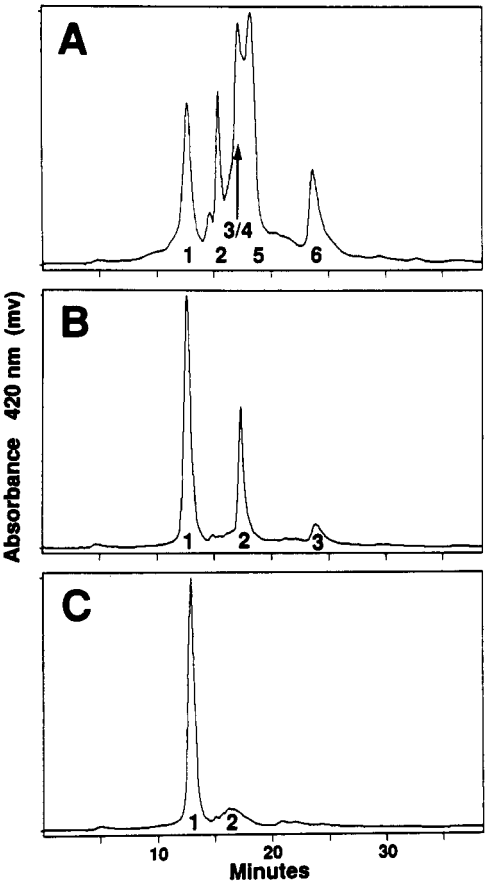

FIGURE 1: AX300 anion-exchange HPLC separations of isophthalyl modified hemoglobins detected at 420 nm. Reactions were carried out at 35 °C for 2 h with Hb at 1 mM and IPMP at 2 mM in 0.1 M Bis-tris-HCl buffer at pH 7.2. The ligand state of hemoglobin was (A) deoxyHb, (B) HbCO, and (C) deoxyHb in the presence of 5 mM DPG.

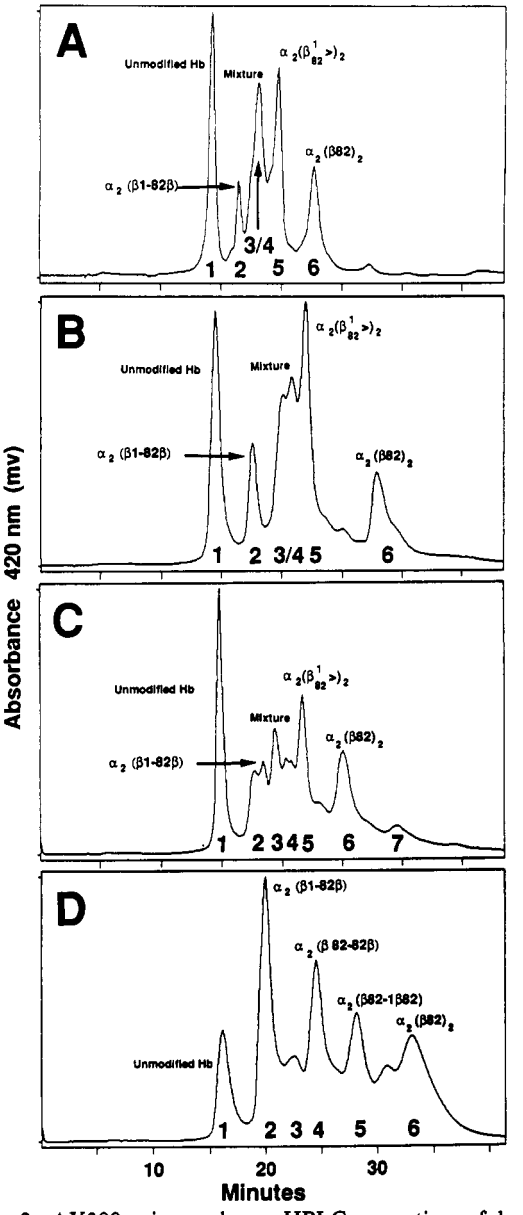

FIGURE 2: AX300 anion-exchange HPLC separations of deoxyHb reaction mixtures detected at 420 nm. Reactions were carried out at 35 °C for 2 h with Hb at 1 mM and diacyl bis(methyl phosphate) reagent at 2 mM (except TPMP was at 6.7 mM) in 0.1 M Bis-tris-HCl buffer at pH 7.2. The modifying reagents were (A) FMP, (B) IPMP, (C) TPMP, and (D) MSMP.

and the structure of the cross-linking reagent. Figure 3 shows a comparison of the chromatographic behavior of the globin chains of the mixtures of modified hemoglobins resulting from reacting deoxyHb at 35 °C with four different dicarboxylic bis(methyl phosphate) reagents. The reaction conditions were the same as or similar to those used for the study of modified hemoglobins shown in Figure 2. Variations in the elution times of globin chains as reflected in the positions of unmodified α and unmodified β chains are due to slight differences in the developer conditions which sometimes occurs when the chromatograms are run at different times. The sequences of elution of the different modified β chains were similar for the four cross-linking reagents. All modified β chains eluted slower than unmodified β chains. Cross-linked β chains move slower than singly modified but un-cross-linked β chains. The position of elution of modified chains appears to be influenced by the size of the dicarboxylic acid group with the 3,3′-stilbenedicarbonyl being the slowest and fumaryl the fastest. Although modification of the α chains was not evident with the stilbene reagents, the other three reagents did produce one or more different modifications of the α chains.

General Approach to Structural Characterization of Modified Hemoglobins by Peptide Analysis. Single hemoglobin components were isolated from the complex reaction mixtures by preparative chromatography and rechromatography. The extent of purification of fractions at each step was assessed by analytical anion-exchange and globin chain HPLC separations. These analytical procedures were also used to identify the elution positions of the hemoglobins and their modified globin chains shown in Figures 2 and 3. The globin chains of the modified hemoglobins isolated by preparative, reverse-phase HPLC (Vydac C4 columns) procedures were used for SDS–PAGE studies to estimate the molecular size of the globin fraction and for further structural analyses.

The presence and exact nature of the structural modifications of each globin chain were determined by hydrolyzing the isolated chain with trypsin generally followed by further hydrolysis with GluC endoproteinase. The resultant peptides were separation by reversed-phase HPLC (Vydac C18 column), and their amino acid compositions were determined. The peptide patterns of each modified globin chain were examined for the decrease or loss of normal, unmodified peptides and the appearance of new, modified peptides. Where no alterations in peptide patterns or elution of the globin chains were noted, the chain was concluded to be unmodified. From the amino acid composition of the modified peptides plus the

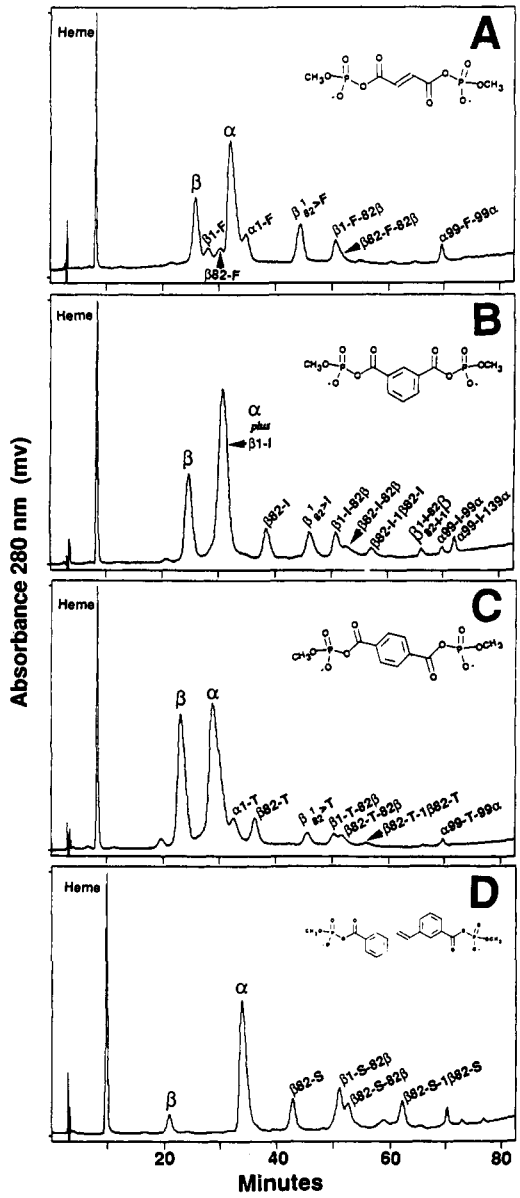

FIGURE 3: Globin chains of unmodified hemoglobin and deoxyHb reaction mixtures separated by reversed-phase HPLC on a large pore C4 column with detected at 220 nm. Reactions were done as described in the legend for Figure 2. The modifying reagents were (A) FMP, (B) IPMP, (C) TPMP, and (D) MSMP.

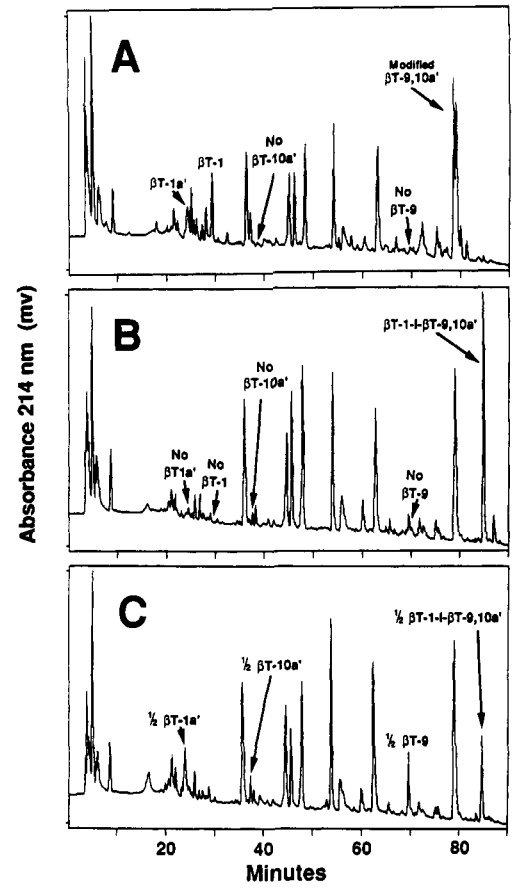

FIGURE 4: HPLC peptide patterns of enzyme hydrolysates of β chains from IPMP modified Hb components isolated by ion-exchange and reversed-phase HPLC. The hydrolysis was done with trypsin followed by Glu-C endoproteinase. The separation was with a C18 reversed-phase column and an ammonium acetate acetonitrile gradient. Peptides were detected by absorption at 214 nm. Those containing isophthalyl groups were identified by their absorption at 258 nm (data not shown). The modified β chains are from the following modified hemoglobins shown in Figure 2B: (A) zone 6, (B) zone 5, and (C) zone 2.

decrease or absence of one or more normal peptides, the structural modifications of the globin chains were deduced. In some cases, the results of the SDS–PAGE analysis of the purified hemoglobin component or isolated chain have been required to conclude that a cross-linking is between two globin chains rather than within one globin chain.

The first hemoglobin component to elute from the anion-exchange column chromatograms of each of the reaction mixtures (see Figure 2) has unmodified α and β chains and is unreacted hemoglobin. Most of the other isolated hemoglobins were found to have modified β chains with unmodified α chains (although some α modification does occur with fumaryl, isophthalyl), and terephthalyl reagents).

Figure 4 shows the peptide patterns of the β globin chains isolated as described above from three different hemoglobins components modified with IPMP (see Figure 2B for elution positions of hemoglobins and Figure 3B for elution positions of the modified chains). These chromatograms show changes in the amounts of the tryptic-GluC peptides βT-1, βT-9, and βT-10a′ (Gly-Thr-Phe-Ala-Thr-Leu-Ser-Glu) and the presence of one or more modifications of these peptides. The identities of the unmodified and modified peptides have been confirmed by amino acid composition analysis. In the case of the modified peptides, the presence of the isophthalyl group was detected by its absorption at 258 nm.

The peptide pattern of Figure 4A is of the modified globin chain from the major hemoglobin component in zone 6 of Figure 2B. This shows a β chain peptide pattern with a normal amount of βT-1 plus βT-1a (Val-His-Leu-Thr-Pro-Glu), no βT-9 or βT-10a′, and the presence of a modified peptide that has the amino acid composition of βT-9,10a′ as shown in Table I as AX6 CM1 II. The UV absorption of this peptide indicated the presence of one isophthalyl group per mole of βT-9,10a′. The participation of the ϵ-amino groups in an amide bond prevents normal enzymatic hydrolysis by trypsin. Because the SDS–PAGE studies of the isolated chain and the hemoglobin component from which it came indicates that the modified chain migrates as a monomer, it is concluded that this hemoglobin has two unmodified α chains and two β chains each modified as an isophthalamide of its β82Lys residue but not cross-linked. The formula of this modified hemoglobin can be written as $\alpha_2(\beta 82\text{-I})_2$, where "I" represents an isophthalyl group.

The peptide pattern of Figure 4B is of the modified globin chain from the main hemoglobin components in zone 5 of

Table I: Amino Acid Composition of Modified Peptides from Tryptic + Glu-C Hydrolysis in Urea of β Chains of Four Isophthalyl Modified Hemoglobins

	modified peptides from				normal peptides		
amino acids	AX4 CM1 I	AX6 CM1 II	AX5 CM1 II	AX3 CM3 II	βT-1	βT-9,10a′	βT-1,9,10a′
Asp		2.9	3.1	3.1		3	3
Glu	2.0	1.1	3.2	1.1	2	1	3
Ser		1.7	1.9	1.8		2	2
Gly		3.2	3.3	3.2		3	3
His	0.9	0.9	1.7	1.0	1	1	2
Thr	1.0	1.9	3.1	1.9	1	2	3
Ala		3.2	3.3	2.9		3	3
Pro	1.0		1.1		1		1
Val	1.0	1.1	1.9	1.1	1	1	2
Leu	1.0	4.9	5.8	4.9	1	5	6
Phe		2.1	2.0	2.0		2	2
Lys	1.0	1.1	2.0	1.0	1	1	2
modified tryptic peptide(s)	1	9, 10a′	1, 9, 10a′	9, 10a′			
elution times (min) in TFA	43	84	82	91			
chain structure	β1-I	β82-I	β1-I-82β	β82-I-82β			

Figure 2B. This also shows a β chain pattern but without any normal βT-1, βT-1a, βT-9, or βT-10a′. A single modified peptide was found with an amino acid composition of βT-1, βT-9, and βT-10a′ as shown in Table I as AX5 CM1 II. Its UV absorption indicated the presence of one isophthalyl group per mole of peptide. Because β1Val is in the βT-1 peptide and β82Lys is at the C-terminus of βT-9, this modified peptide results from a cross-linking between the α-amino group of β1Val of one βT-1 peptide and the ε-amino group of β82Lys of a βT-9,10a′ peptide. The SDS–PAGE studies of this modified β chain and its hemoglobin indicated the presence of only monomer globin chains (no interchain cross-linking); therefore, the structural change must be an intrachain linkage between the amino groups of β1Val and the β82Lys of the same chain. This can be represented as $\alpha_2(\beta^1{}_{82}{>}I)_2$.

In the case of the globin chain in Figure 4C (zone 2 of Figure 2B), only half of the normal amounts of unmodified βT-1, βT-9, and βT-10a′ were found along with half a mole of the same cross-linked βT-1 to βT-9,10a′ peptide found for the modified β chain of the $\alpha_2(\beta^1{}_{82}{>}I)_2$ hemoglobin described above. From this peptide pattern and the SDS–PAGE studies which show that interchain cross-linking has occurred, it can be concluded that this modified hemoglobin has two unmodified α chains and two β chains cross-linked by a single isophthalyl group between the β1Val of one chain and the β82Lys of the other chain. Its formula is α2β1-I-82β.

Peptide patterns of the modified β chains from two different hemoglobins present in zones 3 and 4 (Figure 2B) of reaction mixtures of deoxyHb treated with IPMP are shown in Figure 5 (see Figure 3B for the elution positions of the modified chains). These modified hemoglobins were isolated first by preparative anion-exchange HPLC (SynChropak AX300) followed by rechromatography by preparative cation HPLC (SynChropak CM300). TFA rather than ammonium acetate was used for the HPLC developer gradient for the peptide separations. Figure 5A results from β chains cross-linked by a single IPMP between the β82Lys of one chain and the β82Lys of the other chain. The UV absorption of the modified peptide indicated the presence of one isophthalyl group per 2 mol of βT-9,10a. Its composition is also listed in Table I as AX3 CM3 II. This modified βT-9,10a peptide is concluded to consist of two dipeptides, βT-9,10a cross-linked by a single isophthalyl group between the ε-amino groups of the lysyl residues corresponding to position 82 of each β chain. The formula of the hemoglobin from which this modified globin was obtained can be represented as α2(β82-I-82β). This

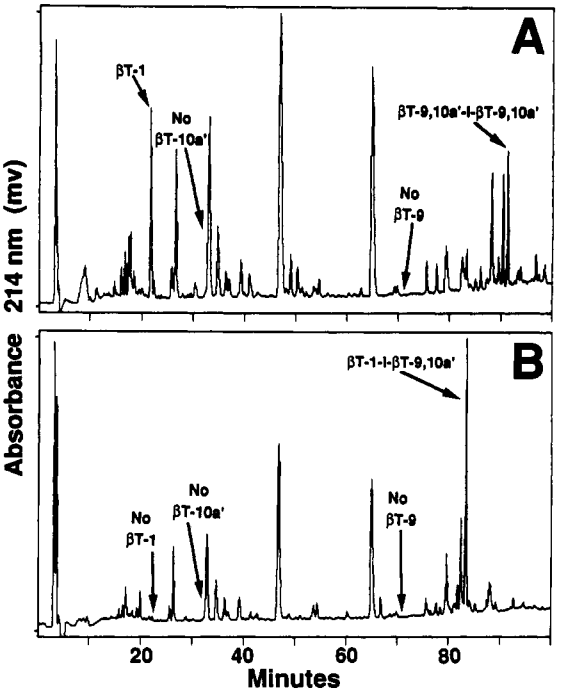

FIGURE 5: HPLC peptide patterns of enzyme hydrolysates of β chains from two IPMP-modified Hb components isolated by preparative HPLC chromatography. The procedures used for hydrolysis and separation of peptides are the same as for Figure 4 except for the use of 0.1% TFA in the place of 10 mM ammonium acetate in the HPLC developers. The modified β chain for 1A was from zone AX3 CM3 II which was the second globin zone from an HPLC chain separation of the third hemoglobin zone from a SynChropak CM300 rechromatography of the third zone from a SynChropak AX300 preparative HPLC chromatogram of IPMP-modified deoxyHb. The modified β chain for 1B was from zone AX4 CM2 VI and corresponds to the globin peak that elutes at 67 min in Figure 3B.

hemoglobin component was found to be one of the two major modified hemoglobins when HbCO was reacted with IPMP but only a minor component when deoxyHb is reacted with IPMP.

The peptide pattern in Figure 5B shows that its globin chain has two cross-linkages with one isophthalyl group between the β1Val of one chain and the β82Lys of the other and a second isophthalyl group between the β1Val of the second chain and the β82Lys of the first chain. This peptide pattern and the amino acid composition of its modified peptide are identical to those of the β chain that is internally linked between the β1Val and the β82Lys of the same chain. The peptide

Table II: Structures of Modified Globins[a]

globin structures	fumaryl	isophthalyl	terephthalyl	3,3′-stilbene-dicarboxyl
β1-X	d	d	d	
β82-X	dd, cc	dd, c	d, c	dd, c
α1-X	dd, p	p	d, p	
$\beta^{1}{}_{82}$>X	dddd	ddd	dd	
β1-X-82β	dd	ddd	dd	ddd, c
β82-X-82β	d, ccc	d, ccc	dd, cc	dd, cc
$\beta^{1}{}_{82}$>X-82β				
β1-X-82β82-X		d	d	dd, c
β1-X-82β1-X			d	
β82-X-144β				d, cc
$\beta^{1\text{-}X\text{-}82}_{82\text{-}X\text{-}1}\beta$	d	d	d	
α99-X-99α	dd, pp	d, pp	d	
α1-X-139α	d			
α99-X-139α	d	dd, pp		

[a] Notes: d = deoxyHb reacted with each reagent; c = HbCO reacted with each reagent; p = deoxyHb reacted in the presence of 2,3-DPG with FMP & IPMP only. One letter indicates a minor component, <5% of product was formed. Two letters indicate a component of >5% but <10% of product was formed. Three letters indicate 10–20% of product was formed. Four letters indicate >20% of product was formed.

pattern but not the modified peptide differs from that of the singly cross-linked globin with the formula β1-I-82β described above (Figure 4C). The singly cross-linked globin chains show the presence of half the normal amounts of βT-1, βT-9, and βT-10a′ rather than the complete absence of these normal tryptic peptides.

Structures of Globin Chains Found in Hemoglobins Treated with Four Different Diacyl Bis(methyl phosphate) Cross-Linking Reagents. The structures determined for the modified globin chains of the major and some minor hemoglobins that have been isolated from the reaction of hemoglobins in various ligand conditions with four diacyl bis(methyl phosphate) reagents are identified in Table II. In addition to unmodified globin chains (not shown in Table II), the major modified globin chains found for the reaction of FMP with deoxyHb (in the absence of 2,3-DPG) were β82-F, $\beta^{1}{}_{82}$>F, and β1-F-82β. Smaller amounts of β1-F, α1-F, β82-F-82β, double-cross-linked $\beta(1\text{-F-}82)_2\beta$, α99-F-99α, α1-F-139α, and α99-F-139α were also demonstrated. The reaction of FMP with deoxyHb in the presence of 2,3-DPG did not produce any modified β chains but did produce α99-F-99α and α1-F as the main modified chain products. The main modified products found from the reaction of FMP with HbCO were β82-F and β82-F-82β. All of these were also found as minor components in the reaction of deoxyHb with FMP.

The structures of the globin chains isolated from the reaction of hemoglobin in various ligand states with IPMP are also shown in Table II. The major modified globin chains from the reaction of IPMP with deoxyHb (in the absence of DPG) were β82-I, $\beta_{82}{}^{1}$>I, and β1-I-82β. Smaller amounts of the following modified globins were also found: β82-I, β82-I-82β, β82-I-1β82-I, double cross-linked $\beta(1\text{-I-}82)_2\beta$, α99-I-99α, and α99-I-139α. The major modified globin chains formed by treatment of HbCO with IPMP was found to be β82-I-82β. Some β82-I was also identified. The main modified globin chains formed from IPMP reacting with deoxyhemoglobin in the presence of 2,3-DPG were α99-I-99α and α99-I-139α.

The structures of globin chains of modified hemoglobins resulting from treating deoxyHb with TPMP are also shown in Table II. The globins which were modified by reaction of deoxyHb and HbCO with the TPMP reagent were the same as those modified by the IPMP reagent, indicating that the reagents have similar specificities. Although chromatograms of the modified globin chains produced by reacting deoxyHb with TPMP in the presence of 2,3-DPG were similar to those of the isophthalyl reagent, only the α1-T and α99-T-99α structures were confirmed by peptide pattern analysis.

The structures of the globins isolated from the major hemoglobin components that can be separated from the reaction mixture resulting from treating deoxyHb with MSMP are also shown in Table II. Fewer modified hemoglobins and globin chains have been found in the reaction of MSMP with deoxyHb than for the other three bis(methyl phosphate) reagents that have smaller diacyl groups. The major MSMP globin chains that have been characterized are β82-S, β1-S-82β, β82-S-82β, β82-S-1β82-S, and β82-S-144β. A β chain internally linked from β1Val to β82Lys of the same chain has not been observed for the MSMP reacted hemoglobin. Presumably, these residues are too close to one another on the same chain to be linked by a single reagent molecule of the size of stilbene.

Reaction of hemoglobin with *p*- or 4,4′-stilbene-*trans*-dicarboxyl bis(methyl phosphate) (PSMP) under conditions used with *m*- or 3,3′-stilbene-*trans*-dicarboxylic acid bis-(methyl phosphate) (MSMP) resulted in very low yields of modified hemoglobins. Therefore, further studies of the structure and function of PSMP modified hemoglobins were not made.

Comparison of Modified Globin Chains Obtained with Diacyl Bis(methyl phosphate) Reagents. It is apparent from Table II that α chains are not accessible to the MSMP reagent but are accessible to the FMP, IPMP, and TPMP reagent, especially when the central cavity between the β chains is occupied by DPG. The β82Lys residue appears to be available to react with each of the reagents when the hemoglobin is in either the low oxygen affinity state (deoxyHb) or the high affinity state (HbCO) in the absence of 2,3-DPG. On the other hand, the β1Val residue appears to react with these reagents only when the hemoglobin is in the low affinity state in the absence of DPG. This residue does not react with any of the reagents when the hemoglobin is in the high affinity state.

One of the major modified hemoglobins formed under deoxy conditions with the FMP, IPMP, and TPMP but not the MSMP has intrachain linkage between the β1Val and β82Lys residues of the same chain. All of the diacyl bis(methyl phosphate) reagents so far tested form modified hemoglobins with cross-linking between the β1Val residue of one chain and the β82Lys of the other chain when the reaction is carried out with deoxyHb. This product is not formed with HbCO.

Additional modifications of the β1-X-82β cross-linked β chains have been demonstrated, at least for the isophthaloyl, terephthaloyl, and stilbenedicarbonoyl reagents. These reagents form small amounts of this cross-linked product with an additional group on the second β82Lys residue. In the case of the FMP, IPMP, and TPMP reagents, doubly cross-linked modifications have been found between the β1Val's of each chain and the β82Lys's of the opposite chain. Small amounts of an un-cross-linked product have been found with fumaryl, isophthalyl, and terephthalyl groups on the β1Val residue. More of the β82Lys modified but un-cross-linked product is formed compared to the β1Val modified product, especially with the IPMP and MSMP reagents. These findings are consistent with the β1Val residue being less reactive than β82Lys.

The main modified hemoglobins found after the reaction of HbCO with all of these reagents are those with the β82 residue modified, either with a single reagent group on each

Table III: Functional Properties of Modified Hemoglobins[a]

hemoglobin structures	P_{50} fumaryl	isophthalyl	terephthalyl	3,3'-stilbene-dicarboxyl
$\alpha_2(\beta 1\text{-X})_2$		6.6 (2.0)		
$\alpha_2(\beta 82\text{-X})_2$	9.8 (2.3)	8.7 (2.8)		6.8 (2.4)
				3.4 (2.1)[b]
$\alpha_2\beta 82\text{-X-}82\beta$	3.0 (2.2)	4.6 (2.4)		10.0 (2.8)
$\alpha_2\beta 1\text{-X-}82\beta$	24 (2.2)	17.8 (2.7)	12.5 (2.5)	3.4 (2.6)
$\alpha_2(\beta^1{}_{82}\text{>X})_2$	8.3 (2.0)	9.4 (2.6)		
$\alpha_2\beta 82\text{-X-}1\beta 82\text{-X}$				4.7 (2.0)
$\alpha_2\beta^{1\text{-X-}82}_{82\text{-X-}1}\beta$		22.5 biphasic		

[a] Normal unmodified Hb A: P_{50} = 5.0, n_{50} = 3.0. Conditions: 50 mM Bis-tris, 0.1 M Cl^-, ph 7.4, 25 °C, 55 μM heme. [b] Bis-tris appears to be present as an ester with one of the carboxyl groups of each molecule of 3,3'-stilbenedicarboxyl in this modified hemoglobin.

β82Lys or one bridging molecule, cross-linking between the β82 residues of the two β chains.

Small amounts of α99Lys cross-linked hemoglobins are formed in the reaction of the FMP, IPMP, and TPMP reagents with deoxyHb. Some α1Val modified but un-cross-linked hemoglobin was also found for these three reagents. In the case of the IPMP, a significant amount of α99-I-139α has been found.

No cross-linking between the two N-terminal valyl residues, i.e., β1-X-1β, has been found for the reactions of hemoglobin in any ligand state with any of these reagents presumably because the distances are too great.

Hemoglobin–Oxygen Equilibrium Studies of Modified Hemoglobins. The P_{50} and the n_{50} values for selected hemoglobins modified by dicarboxylic bis(methyl phosphate) reagents were measured. The oxygen affinities of these hemoglobins range from somewhat greater than, to lower than, that of unmodified, normal adult hemoglobin. As shown in Table III, the P_{50} values range from 3.4 torr for $\alpha_2\beta 1\text{-S-}82\beta$ to 24.0 for $\alpha_2\beta 1\text{-F-}82\beta$. The most significant and potentially useful changes in oxygen affinities are found for the singly and the doubly cross-linked hemoglobins with isophthalyl or terephthalyl bridging between β1Val and β82Lys. It is estimated that the P_{50} values for these modified hemoglobins would be at or above 27 torr at pH 7.4 and 37 °C (Imai, 1982). The effect on oxygen affinity of modifying hemoglobin with these bifunctional reagents differs with the size of the modifying group, whether or not the β chains are cross-linked, and whether the cross-linking is between β82Lys of each chain or β1Val of one chain and β82Lys of the other chain. In the case of un-cross-linked hemoglobins that have a modifying group on each β82Lys residue, there is a small but significant decrease in the log of P_{50} (increase in oxygen affinity) with increase in the size of the modifying group. The correlation is the same but the effect is much greater in the case of the series of hemoglobins cross-linked between β1Val of one chain and the β82Lys of the other chain. On the other hand, a direct correlation is observed between the log of P_{50} and the bridging distances for the $\beta 82\text{-X-}82\beta$ cross-linked hemoglobins. These patterns of effects are shown when comparisons are made of the log of P_{50} values versus the sizes or bridging distances of the modifying group (Figure 6).

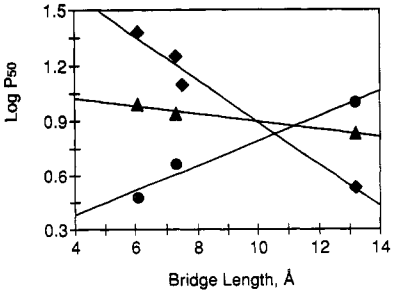

FIGURE 6: The correlation of oxygen affinity (log P_{50} in torr) on the ordinate with bridging distance in angstroms on the abscissa for three different types of hemoglobin modifications. (▲) represents the $\alpha_2(\beta 82\text{-X})_2$ un-cross-linked hemoglobins; (●) is the $\alpha_2\beta 82\text{-X-}82\beta$ cross-linked hemoglobins; and (♦) is the $\alpha_2\beta 1\text{-X-}82\beta$ cross-linked hemoglobins where X represents fumaryl (6.1 Å), isophthalyl (7.3 Å), terephthalyl (7.5 Å), and 3,3'-stilbenedicarboxylate (13.2 Å).

Table IV: Distances between Reactive Residues and Distances Spanned by Gross Linker Reagents

(A) Distances between β1Val-NH_2 and β82Lys-NH_2 in Oxy- and DeoxyHb

residues	distances (Å) deoxy-	oxy-
$\beta_1$1Val–$\beta_2$1Val	18.4	19.9
$\beta_1$1Val–$\beta_2$82Lys	11.5	15.5
$\beta_1$82Lys–$\beta_2$82Lys	9.3	10.7
$\beta_1$1Val–$\beta_1$82Lys	9.9	5.4

(B) Distances between Nitrogens for Selected Cross-Linkers in Diamide

cross-linker	distances (Å)
fumaryl	6.1
isophthalyl	7.3
terephthalyl	7.5
3,3'-stilbene	13.2
DIDS	16

DISCUSSION

Specificity and Sequence of Reaction of Dicarboxylic Bis(methyl phosphate) Reagents with Hemoglobin. These reagents are quite specific in their reaction with hemoglobin under the conditions used. Of the possible 24 amino groups per dimer that might react, only those of β1Val, β82Lys, α1Val, and α99Lys react to any significant extent. The degree of specificity of reaction of these bifunctional reagents is similar to that of the monofunctional reagent, methyl acetyl phosphate, reported by Ueno et al. (1989). Therefore, the specificity is associated primarily with the reagent's functional group rather than the acyl chain. As summarized in Table II, the reactivity of these residues is also influenced by the ligand state of the hemoglobin, the presence of 2,3-DPG, and the size of the bridging group of each reagent.

The distances between the various reactive residues have been examined and are listed in section A of Table IV [from X-ray crystallographic data of Fermi et al. (1984) and Shannan (1983) and molecular modeling]. Distances are shown for both the deoxy and oxy conformations of human hemoglobin. The distance spanned by each of several cross-linker reagents are given in section B of Table IV. The latter measurements are between nitrogen atoms assuming each reagent has reacted with two different amino groups. The bridging distances for the fumaryl, isophthalyl, and terephthalyl reagents are too short to form cross-links between β1Val of one chain and the β82Lys of the other or between the two β82Lys in either the oxy or deoxyHb conformation. However, because cross-linking between these amino groups is found with these reagents, movement of the side chains of these residues or the polypeptide backbone must occur, at least under the reaction conditions used. These results are consistent with the earlier observations of Arnone et al. (1977), where they found similar shortening of the distances between β1Val and β82Lys when cross-linked with nFPLP. Likewise, consistent with the earlier work of Kavanaugh (1987) and Kavanaugh et al. (1988), who

showed cross-linking between the two β1Val residues with DIDS, which has a span of about 16 Å, it can be concluded that the distances between the amino groups of some of these residues must be less than those shown in Table IV, at least for part of the time. Alternatively or in addition, these distances may change as the result of th electrostatic attraction of the cross-linking reagents as they bind in the central cavity between the two β chains.

From the relative amounts of the various hemoglobin products formed, it can be concluded that the ϵ-amino group of β82Lys reacts more readily with each of the bis(methyl phosphate) reagents than does the α amino group of the β1Val residue. Once a molecule of one of the dicarboxyl bis(methyl phosphate) reagents reacts with a β82Lys residue, the other acyl methyl phosphate group appears to react either with the other β82Lys in HbCO or, in the case of deoxyHb, with the β1Val of the other chain or, for the smaller bridging groups, with the β1Val of the same chain. The fact that appreciable amounts of β82-X are formed with each of the reagents reacting with either deoxyHb or HbCO is probably due to hydrolysis of the second methyl phosphate group from the reagent before cross-linking can occur. As noted before, reactions with the β1Val amino group have only been found when the hemoglobin is reacted in the deoxy conformation in the absence of 2,3-DPG. This sequence of reactions of amino groups with these dicarboxyl bis(methyl phosphate) reagents is the same as observed for trimesoyl tris(methyl phosphate) (Kluger et al., 1992). We propose that the mechanisms are the same for the first two steps.

In the case of reactions of hemoglobin with dicarboxyl bis-(methyl phosphate) reagents under conditions that do not result in the modification of all β chains, hybrid molecules like $\alpha_2\beta\beta$82-X may be formed. These have not been detected by either electrophoresis or chromatography, presumably because of dissociation into dimers (αβ and αβ82-X) and rearrangement into $\alpha_2\beta_2$ and $\alpha_2(\beta\beta2\text{-X})_2$ tetramers which are separated by virtue of charge differences. The existence and rearrangement of hybrid molecules has been reported for abnormal human hemoglobins (Bunn & McDonough, 1974), and we postulate that these occur with these chemically modified hemoglobins. However, under conditions where little or no unmodified β chains remain in the reaction mixture, it is clear from the structures identified that two molecules of the reagent can react with amino groups in the DPG site or central cavity of a single hemoglobin molecule. From a consideration of the relative amounts of the different modified hemoglobins obtained for the various bifunctional reagents, it is apparent that two of the smaller dicarboxyl bis(methyl phosphates) can be accommodated in the central cavity more readily than the larger reagents like those containing stilbene. However, even the latter can form a minor component with two stilbenes per tetramer, namely, $\alpha_2\beta$82-S-1β82-S.

Functional Properties of Dicarboxylic Bis(methyl phosphate) Modified Hemoglobins and Structure Function Relationships. All three of the $\alpha_2(\beta82\text{-X})_2$ modified hemoglobins that were studied exhibit low oxygen affinity (increased P_{50}). These modifications should be the same as or similar to those of the pseudo-cross-linked human hemoglobin reported by Bucci et al. (1989) using mono(3,5-dibromosalicyl) fumarate. They propose that the free carboxyl groups of the two fumaryl residues interact electrostatically either with the two β143 histidyl residues or with the two β1 valyl and two β2 histidyl residues of the opposite chains. Whichever interaction occurs, all would be expected to stabilize the tetramer structure.

The P_{50} values found for the trimesyl (or benzene-1,3,5-tricarbonyl [B]) cross-linked hemoglobins measured under the same conditions as used for the present study and reported by Kluger et al. (1992) are very similar to those found for the isophthalyl cross-linked hemoglobins, namely, 4.8 torr for $\alpha_2\beta$82-B-82β, 17.1 torr for $\alpha_2\beta$1-B-82β, and 18.1 torr for $\alpha_2\beta^1{}_{82}$>B-82β. In the case of the first two modifications ($\alpha_2\beta$82-B-82β and $\alpha_2\beta$1-B-82β), the third carboxyl group of the trimesyl cross-linker is free and negatively charged. This extra negative charge in the central cavity does not appear to alter the effect of the identical bridging distance of the isophthalyl and trimesyl cross-linkers on oxygen affinity (P_{50} of $\alpha_2\beta$82-I-82β is 4.6 compared to 4.8 for $\alpha_2\beta$82-B-82β and $\alpha_2\beta$1-I-82β is 17.8 compared to 17.1 for $\alpha_2\beta$1-B-82β).

The effect on oxygen affinity of modifying hemoglobin with the bifunctional reagents studied differs with the size of the modifying group, whether or not the β chains are cross-linked, and whether the cross-linking is between β82Lys of each chain or the β1Val of one chain and the β82Lys of the other chain. The mechanisms by which these chemical modifications affect the oxygen affinity of hemoglobin are not yet clear. In all cases, the net change in charge in the central cavity compared to unmodified hemoglobin should be the same for the different bridging groups. Therefore, alteration in charge cannot explain the differences in P_{50}'s that are observed with a change in bridging distances. In the case of the $\alpha_2\beta$1-X-82β cross-linked hemoglobins, the effect on oxygen binding of cross-linking between the β1Val of one chain and the β82Lys of the other chain is much larger than for the other types of modifications. The oxygen equilibrium is shifted considerably toward the low affinity state in all the cross-linked hemoglobins studied, except the stilbene-modified one. This may be explained by the steric confinement in this region of the molecule. In native Hb A, the distance between β1Val of one chain and β82Lys of the other chain is about 4 Å shorter in the unliganded conformation (11.5 Å) than the liganded conformation (15.5 Å). A cross-linker with the bridging distance close to or shorter than 11.5 Å should stabilize a structure that would favor the unliganded, low affinity state. Conversely, a cross-linked hemoglobin with the bridging distance close to or more than 15.5 Å should stabilize the strucrture of the liganded, high affinity state. In fact, a linear correlation between cross linker's bridging distance and the logarithm of the oxygen affinity, log P_{50} is observed (Figure 6). However, this steric confinement model does not explain the functional properties of the other types of cross-linked hemoglobins. For example, an opposite trend in the relationship of oxygen equilibrium to bridging distances is observed for the series of $\alpha_2\beta$82-X-82β cross-linked hemoglobins. Therefore, it appears that the mechanisms by which these different types of chemical modifications affect the oxygen affinity of hemoglobin must be different from one another.

The finding that the n values equal or exceed 2.0 indicates that considerable cooperativity is retained by all of these modified hemoglobins. This suggests that these chemical modifications do not freeze the molecule completely. Considerable flexibility must remain in these modified structures, and shifts in the conformational equilibrium must occur for each modified hemoglobin. Further functional studies are being pursued to gain better insights into oxygen transport efficiency and structure–function relationships of these tetramerically stabilized hemoglobins.

Conclusions. These systematic and detailed studies of the structural modifications and functional properties of hemoglobins obtained by reacting deoxyHb and HbCO with

different dicarboxylic bis(methyl phosphate) reagents and trimesoyl tris(methyl phosphate) (Kluger et al., 1992) provide new insights into structure–function relationships and a new rationale for designing cross-linked hemoglobins for potential use as substitutes for blood transfusion. Many of the cross-linked hemoglobins obtained show that distances between the amino groups of β1Val and β82Lys of the same and opposite chains of native hemoglobin must vary substantially from the most probable ones observed by X-ray crystallography for both the T and R conformations. These reagents can be used to "trap" or "report" some of these less common structures and may be useful in identifying structural intermediates in the conformational equilibrium between the T and R conformations of hemoglobin. However, because these modified hemoglobins retain significant cooperativity, these new structures must not be inflexible or rigid. Although the mechanisms by which these different types of chemical modifications affect the oxygen affinity of hemoglobin must be different from one another, the correlations between bridging distances and P_{50} values we have observed for hemoglobins with the same type of modifications can be used to predict changes in oxygen affinities of newly designed hemoglobins. Several of the modified hemoglobins have oxygen transport properties that make them potential candidates as blood transfusion substitutes, namely, $\alpha_2\beta$1-I-82β, $\alpha_2\beta$1-T-82β, and $\alpha_2\beta$82-S-82β. Studies of the structure and functional properties of the isophthalyl-β1Val to β82Lys cross-linked hemoglobin lead to the design and use of the trimesoyl tris(methyl phosphate) reagent to modify hemoglobin as reported by Kluger et al. (1992).

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