# Three-Point Cross-Linking: Potential Red Cell Substitutes from the Reaction of Trimesoyl Tris(methyl phosphate) with Hemoglobin†

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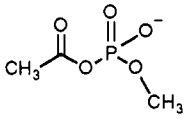
ABSTRACT: The symmetrical trifunctional cross-linking reagent trimesoyl tris(methyl phosphate) (**3**), reacts selectively with amino groups ($\beta$1Val and $\beta$82Lys) in the diphosphoglycerate binding site of human hemoglobin A, producing cross-linked tetrameric species in good yield. A major species is triply linked, $\alpha\alpha\beta^{1}{}_{82}$>B$\beta$82, where B symbolizes benzene-1,3,5-tricarbonyl. Both this triply linked species and the doubly linked species, $\alpha\alpha\beta$1B$\beta$82, produced from deoxyhemoglobin have a considerably lower oxygen affinity than does native hemoglobin while maintaining a high degree of cooperativity ($n_{50}$ = 2.4), making them potentially useful as red cell substitutes, in principle delivering twice as much oxygen as whole blood between $p_{O_2}$ = 100 and = 40 Torr. The yield of products indicates that triply and doubly linked species form in parallel so that there are independent routes to each. It is proposed that differences in routes are due to stereoisomerism about the amide bonds which form from reaction of the reagent with the protein.

The need for the development of red cell substitutes (Vandegriff & Winslow, 1991) and for chemicals for the treatment of sickle cell disease (Manning, 1991) has led to the production of anionic reagents which target groups in cationic sites of hemoglobin (Manning, 1991; Walder et al., 1980; Shibayama et al., 1991; Kavanaugh et al., 1988). Methyl acetyl phosphate (**1**) is a site-directed acylating agent for amino groups in cationic regions of proteins (Kluger & Tsui, 1980, 1985).



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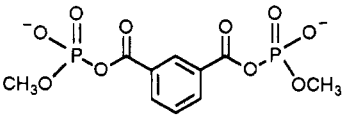
Manning and co-workers showed that **1** reacts selectively with amino groups of the $\beta$ chain of hemoglobin in the site that normally binds 2,3-diphosphoglycerate in human hemoglobins (Ueno et al., 1986a,b 1989) and in the site that binds chloride ion in bovine hemoglobin (Ueno et al., 1989). The high degree of selectivity compares favorably with that observed with other anionic acylating agents (Manning, 1991). Since modification of amino groups in these sites causes a decrease in the affinity of hemoglobin for oxygen (Ueno et al., 1989), the functional group appeared to introduce a modification that produces an oxygen binding property necessary for the development of a red cell substitute (Vandegriff & Winslow, 1991).

Hemoglobin is a tetramer, and outside the red cell it dissociates into dimers which pass through the kidney. This can be overcome by cross-linking the subunits (Vandegriff & Winslow, 1991). Combining modification of the DPG site and cross-linking in a single chemical step provides a potentially efficient route to a blood substitute (Kavanaugh et al., 1988; Walder et al., 1979; Benesch & Benesch, 1981; Benesch & Kwong, 1988; Shibayama et al., 1991).

The development of procedures for making a wide variety of acyl phosphate monoesters permits the preparation of multifunctional acyl phosphate monoesters (Kluger et al., 1990), and the systematic evaluation of the materials and their reactions has been an ongoing collaborative effort of the laboratories of Kluger, Shih, and Jones. The reaction of dicarboxylic acid bis(methyl phosphates) with hemoglobin produces a number of cross-linked hemoglobins with desirable properties for use as red cell substitutes (R. T. Jones, R. Kluger, T. Fujita, C. Head, D. T.-B. Shih, and J. Wodzinska, 1992; unpublished results). In particular, the reaction of isophthaloyl bis(methyl phosphate; **2**, IPMP) with deoxygenated hemoglobin produces cross-linked materials with desirable properties along with some materials that are not cross-linked.



2, IPMP

On the basis of those results and structural analysis, we prepared trimesoyl tris(methyl phosphate) (**3**, hereafter referred to as TMMP), to obtain a higher yield of desirably cross-linked species. In particular, we expected that triply linked hemoglobin would result from reactions which create amide linkages from three amino groups within the diphosphoglycerate binding site of human hemoglobin.

The reaction of TMMP with hemoglobin occurs readily. The anticipated triply linked material is efficiently produced, and the material has properties which make it desirable as a red cell substitute. In addition, the rate and patterns of product

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[1] Abbreviations: TMMP, trimesoyl tris(methyl phosphate); IPMP, isophthaloyl bis(methyl phosphate).

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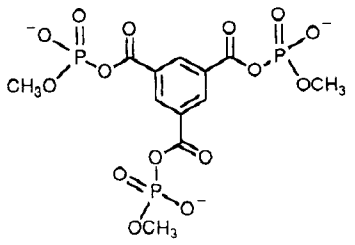


3, TMMP

formation provide new insights into the structural and stereochemical consequences of protein modifications which produce amides and illustrate the principles and utility of using a site-directed trifunctional reagent.

## EXPERIMENTAL PROCEDURES

*Chemical Synthesis and Analysis of Cross-Linking Reagents.* Commercial reagents were utilized without further purification. Solvents were dried prior to use. Acetone was dried over magnesium sulfate for 5 min and then filtered immediately before solutions of sodium iodide were made. Tetrahydrofuran was dried by distillation from sodium wire. Sodium dimethyl phosphate was prepared by the reaction of trimethyl phosphate with sodium iodide in acetone (Zervas & Dilaris, 1953). Deuterated solvents were from MSD Isotopes Ltd. and Aldrich Chemical Co. Organic reagents and solvents were purchased from BDH Canada Ltd. and Caledon Laboratories Ltd. Inorganic materials were purchased from Fisher Scientific.

*Purity of Synthesized Materials.* The purity of samples of newly synthesized materials was assessed by a combination of NMR spectroscopy and analytical thin-layer chromatography. Elemental analyses were conducted by Galbraith Laboratories, Knoxville, TN.

*Spectra.* NMR spectra were recorded on a Varian Gemini (200 MHz) spectrometer. Phosphorus spectra were recorded on a Varian XL-200 spectrometer.

*Synthesis of Trimesoyl Tris(dimethyl phosphate).* This was prepared by the method described previously (Kluger et al., 1990). A suspension of sodium dimethyl phosphate (3.25 g, 22 mmol) in dry tetrahydrofuran (25 mL) was cooled to 0 °C. A cold solution of 1,3,5-benzenetricarboxylic acid trichloride (1.86 g, 7 mmol) in tetrahydrofuran (25 mL) was added, and the mixture was stirred for 1 h at 0 °C. The mixture was filtered through a fine sintered glass funnel, and the solvent was evaporated, leaving a white solid product (3.7 g). Recrystallization from benzene/diethyl ether gave white needles (3.2 g, 86%): mp 92.2–92.5 °C; IR (KBr) C=O 1760 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 8.94 (3 H, s), 4.03 (18 H, d, $J$ = 11.8 Hz); $^{13}C$ NMR ($CDCl_3$) $\delta$ 158.66 (d, $J_{P-C}$ = 7.8 Hz), 137.01, 130.08 (d, $J_{P-C}$ = 8.8 Hz), 55.33 (d, $J_{P-C}$ = 5.7 Hz); $^{31}P$ NMR ($CDCl_3$) $\delta$ –4.92 (decoupled). Anal. Calcd for $C_{15}H_{21}O_{15}P_3$: C, 33.72; H, 3.96; P, 17.39. Found: C, 33.74; H, 3.98; P, 17.18.

*Synthesis of Trimesoyl Tris(sodium methyl phosphate) (3, TMMP).* A solution of sodium iodide (2.5 g, 17 mmol) in dry acetone (17 mL) was added to an acetone solution (15 mL) of trimesoyl tris(dimethyl phosphate) (2.7 g, 5 mmol). The solution was shaken and left overnight protected from light. The product precipitated as a yellow powder. It was filtered, washed with acetone, and dried under vacuum: mp >250 °C; yield, 2.6 g, 93%; IR(KBr) C=O 1728 $cm^{-1}$; $^1H$ NMR ($D_2O$) $\delta$ 8.76 (3 H, s), 3.58 (9 H, d, $J$ = 11.5 Hz); $^{13}C$ NMR ($D_2O$) $\delta$ 163.29 (d, $J_{P-C}$ = 8.0 Hz), 137.51, 131.87 (d, $J_{P-C}$ = 7.7 Hz), 55.07 (d, $J_{P-C}$ = 6.5 Hz); $^{31}P$ NMR ($D_2O$) $\delta$ –4.96 (decoupled).

*Kinetics of Hydrolysis of TMMP.* TMPP (0.005 M in 0.10 M Bis-Tris buffer, pH 7.2) was kept at 22 °C. Periodically, 1-mL aliquots were removed, brought to pH 2.5 with 0.1 M HCl, and titrated with 0.5 M NaOH. The volume needed to bring the sample from pH 2.5 to pH 6 indicates the phosphoric and carboxylic acids generated in the sample, with constant buffer interference at the high pH end of the titration. Kinetics were plotted according to the integrated first-order rate law and fitted by nonlinear regression using GraFit (Erithacus Software Ltd.). A detailed kinetic study is currently in progress.

*Molecular Modeling.* Structures of cross-linking reagents were analyzed using an MS-DOS-based computer with the program Alchemy II from Tripos Associates.

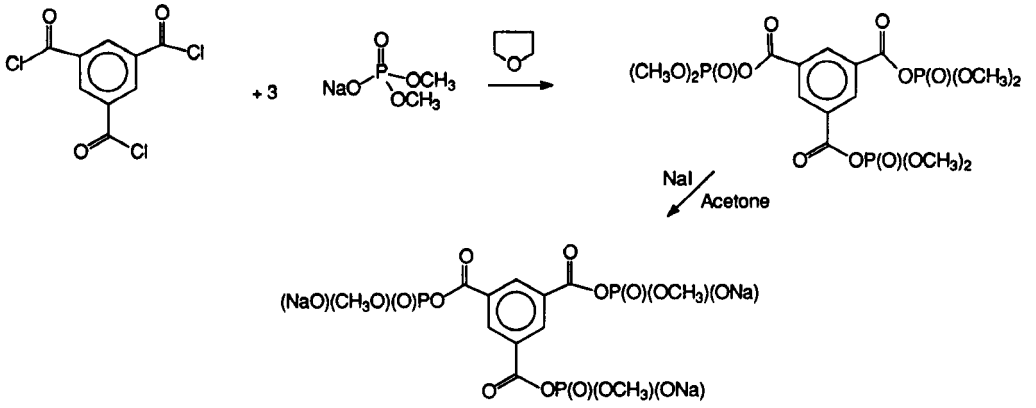
*Biochemical Materials Analysis.* HPLC grade acetonitrile and water were obtained from Mallinckrodt, and trifluoroacetic acid (TFA) was from Pierce Chemical Co. (Rockford, IL). Trypsin from Worthington Biochemical Co. (Freehold, NJ) and the *Staphylococcus aureus* V8 endoproteinase Glu-C from Boehringer Mannheim Biochemical (Indianapolis, IN) were used for enzyme hydrolysis. Reagents for amino acid analysis were from Pierce and Aldrich Chemical Co. Inc. Sephadex G-25, DEAE-Sephacel, and CM-Sephadex were from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Other reagents for preparation of buffers and developers for the modifications of hemoglobin and for chromatography were all of analytical grade or better.

*Hemoglobin.* Human hemoglobin solutions were prepared by a modification of the method of Drabkin (Drabkin, 1949; Kavanaugh et al., 1988).

*Reaction of Hemoglobin with Acyl Phosphate Methyl Esters.* Chemical modifications of hemoglobin were done using hemolysate diluted with 0.1 M Bis-Tris-HCl buffer at pH 7.2 to a final concentration of hemoglobin tetramer of 1 mM Hb. The final concentration of cross-linking reagent was 2 mM in 0.1 M buffer. During the initial phases of this study, the reactions were kept at 35 °C for 2–3 h with hemoglobin in either the deoxygenated (deoxyHb) or carbon monoxide (COHb) form. Later, to improve yield and to destroy any viral contaminants, the reactions were carried out at 60 °C. Reagent was infused from a motor-driven syringe kept at room temperature into the 60 °C hemoglobin solution over a period of 30–60 min with a total reaction time of up to 3 h. Reagents and low molecular weight byproducts were then removed by gel filtration with Sephadex G-25 columns. Stock solutions of COHb were converted to oxyHb by photoirradiation under a stream of humidified oxygen for 60 min at 0 °C in a rotating flask (Shih et al., 1982). Oxygen was then removed to produce a solution of deoxyHb by passing a stream of humidified nitrogen over the oxyHb solution for 2 h at 35 °C, in a rotating flask.

*Cross-Linking Reactions.* Cross-linking reagents were dissolved in water and deoxygenated under vacuum followed by addition of nitrogen three times. In the case of reactions with deoxyHb, the reagents were introduced into the reaction vessel anaerobically to the desired concentration. Nitrogen flowed continuously during the reaction through the rotating flask to maintain the hemoglobin in the deoxy state. In the case of COHb, the hemoglobin solution was saturated with carbon monoxide and the reaction run without further addition of gas. At the end of the reaction period, the flasks containing deoxyHb were flushed with carbon monoxide, cooled to 4 °C, and the solution was filterd first through a 0.45-$\mu$m pore size

Scheme I: Synthesis of TMMP




nylon 66 filter (Rainin Instruments) and then through a Sephadex G-25 column. For measurements of rate of modification of hemoglobin, 2.0 mL of solution of 0.75 mM deoxyhemoglobin (96 mg) and 0.15 M Bis-Tris-HCl buffer (pH 7.2) was placed in the rotary reaction apparatus at 60 °C and flushed with nitrogen saturated with water. To this was added 1 mL of a 3 mM solution of TMMP. The reagents were added from a 1-mL syringe driven by a Sage infusion pump over a period of 30 min. The reaction was allowed to continue for an additional 2 h after the completion of the infusion of the reagents. The final concentrations of reagents were 0.5 mM deoxyHb, 1.0 mM reagent, and 0.1 M buffer. Samples of 0.1 mL were removed at intervals during and after the infusion of reagents. The hemoglobin in each sample was stripped of unreacted reagent by passing through a Pharmacia PD-10 Sephadex G-25M column at 4 °C and then converted to COHb by addition of carbon monoxide. At the end of the reaction, chromatographic and electrophoretic analyses indicate that less than 5% unmodified hemoglobin remains and up to 80% is interchain cross-linked.

*Chromatography of Proteins and Peptides.* Analytical separations of hemoglobins were done by high-performance liquid chromatography (HPLC) with a SynChropak AX300 column (250 × 4.1 mm) using 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). The effluent was monitored at 420 nm. The isolation and purification of single hemoglobin components was done by standard ion-exchange chromatography using DEAE-Sephacel (Huisman & Dozy, 1965) and CM-Sephadex (Schroeder & Huisman, 1980). 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).

Heme and the globin chains were separated by reversed-phase HPLC using 330-Å pore size C-4 Vydac columns (250 × 4.6 mm for analytical and 250 × 12 mm for preparative; The Separations Group, Hesperia, CA) and developers containing 0.1% TFA and various gradients of acetonitrile starting at 20% and ending at 60% modified after the procedure of Shelton et al. (1985). The effluent was monitored at 220 nm, and the globin chains were recovered from the effluent by lyophilization.

Peptide fragments were separated for both analytical and preparative purposes by HPLC procedures modified after that of Shelton (Shelton et al., 1985) using reversed-phase C-18 columns (25 × 0.46 cm Vydac, The Separations Group, cat. no. 218TP54.6). Separations were made using developers of 0.1% TFA and gradients of acetonitrile starting at 0% and ending at 100% generated over a period of up to 100 min. In some cases rechromatography was done with a second developer system consisting of 10 mM, pH 6.0, ammonium acetate buffer and acetonitrile gradients modified after the procedures of Schroeder et al. (1979) and Wilson et al. (1979). Analyses were also conducted with a Waters 810A Maxima system using a microcomputer (MS-DOS).

*Enzyme Hydrolysis of Globin Chains.* Globin chains were first dissolved in 8 M urea (to increase susceptibility to hydrolysis) and kept at room temperature for 2–4 h. The solution was then diluted to 2 M urea with 80 mM ammonium bicarbonate buffer at pH 8.5. Trypsin (2% of total protein) was added, and the solution was digested for 18–20 h at room temperature. The tryptic hydrolysate was then heated in boiling water for 2 min, diluted to 1 M urea with 80 mM ammonium bicarbonate buffer, and digested with endoproteinase Glu-C (1% of total protein) for another 18–72 h at room temperature. The hydrolysates were centrifuged or filtered through a 0.45-fm filter before injection onto the HPLC column.

*Amino Acid Analysis.* Peptides were hydrolyzed in evacuated tubes using 6 M HCl vapor at 110 °C for 22 or more h. The amino acids were derivatized with phenyl isothiocyanate, and the resultant phenylthiocarbamyl amino acid derivatives were separated by reversed-phase HPLC using a Supelcosil LC-18-DB column (Supelco, Inc., Bellefonte, PA; Heinrikson & Meredith, 1984). Effluent was monitored at 254 nm, and the signal was recorded and integrated with an interfaced computer.

*Polyacrylamide Gel Electrophoresis.* The extent of cross-linking of globin chains was determined by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS–PAGE) according to the procedure of Laemmli (1970). A 15% polyacrylamide gel with 2.7% cross-linking was used. The hemoglobins and globins from HPLC separations were prepared by heat denaturation in a buffer containing 65 mM Tris-HCl, pH 6.8, 2% SDS, 10% v/v glycerol, and 5% v/v 2-mercaptoethanol. Approximately 5–20 μg of protein was applied to each lane of the gel and processed at 20 mA for about 4–5 hours (Kavanaugh, 1987).

*Measurement of Functional Properties of Isolated Hemoglobins.* The hemoglobin–oxygen equilibrium properties of modified hemoglobins were measured by the automatic recording method of Imai et al. (1970). The data were analyzed according to the Adair stepwise oxygenation scheme (Imai, 1973) using the apparatus described by Shih and Jones (1986). The conditions for comparing the oxygen affinities of the modified hemoglobins for the present studies were 50 mM Bis-Tris, pH 7.4, 0.1 M $Cl^-$, 2.5 °C, and 55 μM heme.

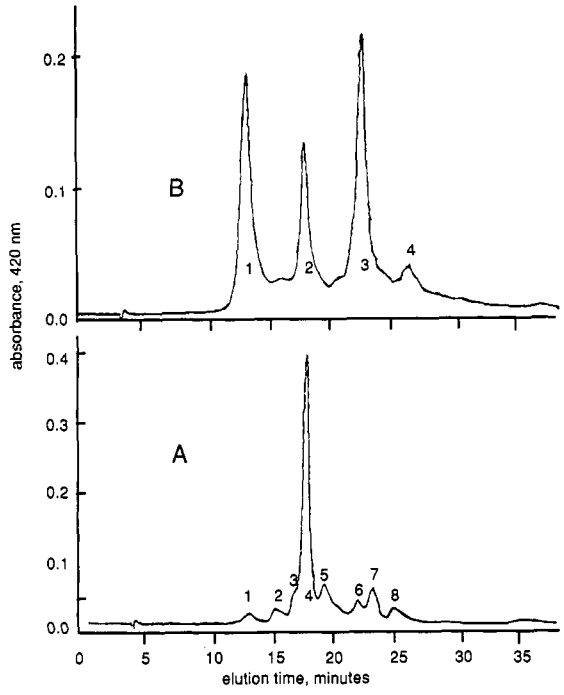



FIGURE 1: (A) Anion-exchange chromatography of intact tetramers after reaction of deoxyHb (0.5 mM) with TMMP (1.0 mM) at 60 °C (reaction time 30 min after 30-min infusion of TMMP). (B) Anion-exchange chromatography of intact tetramers after reaction of COHb with TMMP for 125 min at 60 °C. Unmodified Hb is peak 1.

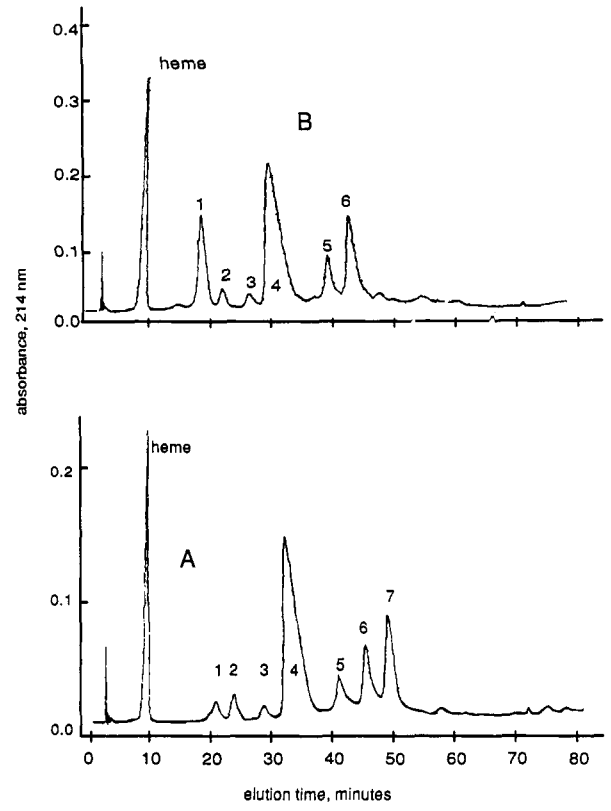



FIGURE 2: (A) Globin chain separation on C-4 reversed-phase column after reaction of deoxyHb with TMMP as in Figure 1. Peak 4 is unmodified $\alpha$ subunit. (B) Globin chain separation on C-4 reversed-phase column after reaction of COHb with TMMP as in Figure 1.

The parameters measured were the oxygen pressure for half-saturation ($P_{50}$) and Hill's coefficient of cooperativity at half saturation ($n$). The magnitude of the alkaline Bohr effect was determined by measurements in the range between pH 7.0 and 7.9. Equilibrium measurements were also made with an Aminco Hemoscan unit under simulated physiological conditions (37 °C, pH 7.4, 0.1 M potassium phosphate, 5% $CO_2$).

## RESULTS

*Synthesis and Structure of TMMP.* Trimesoyl trichloride is readily available commercially and provides a convenient and low-cost source of starting material. The coupling reaction with sodium dimethyl phosphate occurs readily and in high yield (Scheme I).

*Stability of TMMP in Water.* The hydrolysis reaction is slow under the conditions in which TMMP reacts rapidly with hemoglobin. At 22 °C, pH 7.2 (the conditions in the addition syringe), the half-life is about 5 h, while reaction with amines, including those in hemoglobin, is very rapid. The reagent does not undergo a significant amount of hydrolysis prior to reaction with hemoglobin.

*Product Analysis.* The materials eluted in each of the major peaks of the HPLC separation of the reacted hemoglobin were analyzed as described previously (Kavanaugh et al., 1988). Tryptic digestion and analysis of the resulting peptides by comparison with peptide patterns of unmodified hemoglobin confirmed and extended analyses based on patterns and known retention times of hemoglobin and its derivatives. Specific sequences were confirmed by amino acid analysis of each peptide after acid hydrolysis.

*Effect of Ligation of Hemoglobin on Reaction Product Distribution.* The reaction products obtained from treating hemoglobin with TMMP depend upon the ligand state of the hemoglobin during the reaction. This is demonstrated by the anion HPLC separations of the products of deoxyHb compared to those of COHb illustrated in Figures 1 and 2. Figure 1A shows one major and at least six minor hemoglobin components. Rechromatography of the major zone isolated from the anion-exchange column (zone 4, Figure 1A) on a cation-exchange column gives three major components and several more minor components (data not shown). In contrast, in Figure 1B, the COHb reaction mixture shows three main zones and only three minor zones. The first hemoglobin component is unreacted Hb A. The second elutes near the position of the major zone in the deoxyHb reaction mixture but consists of a modified hemoglobin that could only be demonstrated in the COHb reaction mixture by structural studies presented below. The third and major component from COHb is also present in the deoxyHb reaction mixture but only in trace amounts.

*Globin Chain Separation.* The kinds and relative amounts of modified globin chains present in the reaction mixtures resulting from treating deoxyHb and COHb with TMMP were examined by separation of globin chains by C-4 reversed-phase HPLC as shown in Figure 2. The structures of globin components shown were determined as described below. The globin chain separations confirm that major differences in product formation occur depending on the ligand state of the hemoglobin.

As described in the analysis of reaction products of hemoglobin treated with dicarboxylic acid bis(methyl phosphates) (Jones et al., unpublished results), single hemoglobin components were isolated from the complex reaction mixtures by preparative chromatography and rechromatography. These purified hemoglobin components were used for oxygen–hemoglobin equilibrium studies and for structural characterization. The globin chains were also isolated from these components by preparative, reversed-phase HPLC using C-4

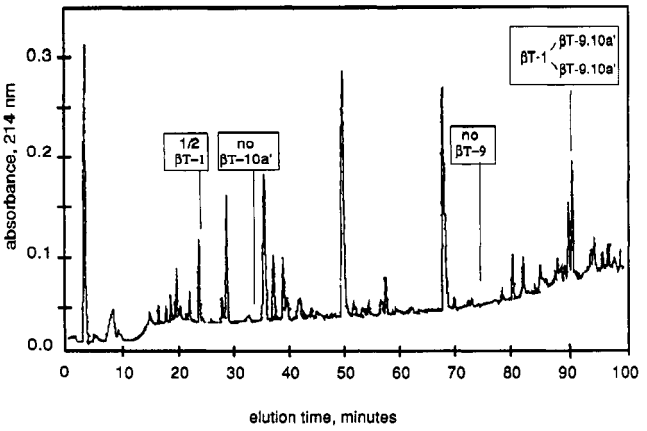



FIGURE 3: Tryptic-Glu-C peptides of the $\beta$ chains of the major modified hemoglobin component produced from the reaction of deoxyHb with TMMP. The peak for tryptic peptide $\beta$T-1 is half the normal relative height (at 24 min). Peaks for peptides $\beta$T9 (74 min) and $\beta$T-10a′ (34 min) are absent, and a new peak appears at 90 min. Amino acid analysis is consistent with the peak accounting for three linked peptides, two from one $\beta$ chain ($\beta$T-1 and $\beta$T-9,10a′) and one from the other $\beta$ chain ($\beta$T-9,10a′). The UV spectrum of the peptide shows the absorption of the trimesoyl group.

Table I: Amino Acid Composition of Modified Peptide from Tryptic + Glu-C Hydrolysis of $\beta$ Chains of Major Species in Trimesoyl-Modified Hemoglobin

| amino acid | modified peptide | normal peptides | | composite of $\beta$T-1, $(\beta$T-9,10a′$)_2$ |
|---|---|---|---|---|
| | | $\beta$T-1 | $\beta$T-9,10a′ | |
| Asp | 5.8 | | 3 | 6 |
| Glu | 4.4 | 2 | 1 | 4 |
| Ser | 4.0 | | 2 | 4 |
| Gly | 6.4 | | 3 | 6 |
| His | 3.0 | 1 | 1 | 3 |
| Thr | 4.9 | 1 | 2 | 5 |
| Ala | 6.2 | | 3 | 6 |
| Pro | 1.2 | 1 | | 1 |
| Val | 2.9 | 1 | 1 | 3 |
| Leu | 10.7 | 1 | 5 | 11 |
| Phe | 3.9 | | 2 | 4 |
| Lys | 2.9 | 1 | 1 | 3 |

columns. Their molecular weights were estimated by SDS–PAGE and, in one case, by electrospray ionization mass spectroscopy. The structural modifications were determined by enzyme hydrolysis, reversed-phase HPLC peptide analysis, and amino acid composition analysis of modified peptides.

*Peptide Patterns.* Figure 3 shows the peptide pattern of the modified $\beta$ chains of the major (and novel) hemoglobin product obtained from the reaction of deoxyHb with TMMP. This peptide pattern is characteristic of the $\beta$ chain of human hemoglobin with the exception of the presence of a new peptide eluting at about 90.6 min, the absence of $\beta$T-9 and $\beta$T-10a′ peptide fragments, and the presence of only half the normal amount of $\beta$T-1 tryptic peptide. The new peptide has UV absorption of the trimesoyl group and an amino acid composition equivalent to 1 mol of $\beta$T-1 to 2 mol of $\beta$T9,10a′, as shown in Table I. The molecular weight estimated from SDS–PAGE of the isolated $\beta$ chains of this modified hemoglobin is that of a dimer of the $\beta$ subunit. The $\alpha$ chain of this hemoglobin component was unmodified.

From these data we see that the modification by TMMP produces protein that is triply linked by one trimesoyl group with amide bonds between the $\alpha$-amino group of the N-terminal valyl residue and the $\epsilon$-amino group of lysyl residue 82 of one $\beta$ chain and the $\epsilon$-amino group of the lysyl residue 82 of the other $\beta$ chain. The presence of these amide bonds involving the two $\beta$-82 lysyl groups prevents trypsin from hydrolyzing the peptide bond between $\beta$T-9 and $\beta$T-10a′. ($\beta$T-10a′ is used to designate the N-terminal part of the $\beta$T-10 tryptic peptide that has been hydrolyzed by the Glu-C endoproteinase at the C-terminal side of $\beta$90Glu). The molecular formula for this modified hemoglobin can be represented as $\alpha_2\beta^1{}_{82}$>B-82$\beta$.

The structures of other modified hemoglobins from the deoxyHb and COHb reaction mixtures were determined in a similar way to that described above. All were found to have normal, unmodified $\alpha$ chains and modified $\beta$ chains. A summary of the data obtained and the formulas that have been deduced are presented in Table II. Under deoxyHb conditions one other cross-linked hemoglobin is also formed in significant amounts, namely $\alpha_2\beta$1-B-82$\beta$. In addition to the two interchain cross-linked products, an intrachain linked product is formed with each $\beta$ chain being bridged internally between the $\beta$1Val residue and the $\beta$82Lys residue of the same chain with a formula of $\alpha_2(\beta^1{}_{82}$>B$)_2$. One of the minor components that was characterized has a trimesoyl group on each $\beta$82Lys residue without either intra- or interchain cross-linking [$\alpha_2(\beta$82-B$)_2$]. This was also formed as a minor component in the COHb reaction mixture.

The two major modified hemoglobins found in the COHb reaction mixture, zones 2 and 3 of Figure 1B, were found to have modified $\beta$ chains that elute in two different positions from the C-4 reversed-phase column, zones 5 and 6, respectively, of Figure 2B. The peptide patterns of each of these modified $\beta$ chains are identical to one another. They each have the same modified peptides eluting at 91.6 and 92.5 min. Analyses of these peptides indicate that modification to be a cross-linking between the $\beta$82Lys residues of the two $\beta$ chains. The difference between these two modified hemoglobins is due to the difference between their two modified $\beta$ chains. This appears to be due to the presence of a bis-tris ester in the case of hemoglobin in zone 2 (Figure 1B) and its absence in the case of the hemoglobin of zone 3 (Figure 1B). This ester group appears to be relatively resistant to hydrolysis in both hemoglobin and globin after the reaction of COHb with the trimesoyl reagent is completed. However, it must be unstable under the conditions of enzyme hydrolysis of the globin and/or preparation of the peptide mixture for reversed-phase HPLC separation. Similar differences appear to exist for the $\alpha_2(\beta$82-B$)_2$ modified hemoglobins.

Only trace amounts of $\alpha_2\beta$82-B-82$\beta$ hemoglobin could be detected in the deoxyHb reaction mixture. In contrast to this, small but easily detected amounts of the $\alpha_2\beta$82-X-82$\beta$ cross-linked hemoglobins have been found in the reaction mixtures resulting from treating deoxyHb with all of the dicarboxy bis(methyl phosphate) reagents (Jones et al., unpublished results).

*Methemoglobin Formation.* Methemoglobin content was measured before, during, and at the end of several reactions of hemoglobin with TMMP at 60 °C. In one typical case, starting with a hemolysate containing less than 1% metHb, a preparation of deoxy Hb to which TMMP was infused over 27 min had 1.6% metHb at 14 min from the start of addition of the reagent and only 0.4% at 120 min after addition. In an experiment using COHb, the metHb content was 0.5% before addition of TMMP and 0.6% 120 min after addition.

*Kinetics of Product Formation.* The reagent modifies only amino groups within the DPG binding cleft of the $\beta$ subunits. Analysis of the amount of each $\beta$-chain product present in the reaction mixture was performed by separating the globin chains

Table II: Summary of Structural Characterization of β Globin Subunits and Molecular Formulas of Hemoglobins Isolated from Reaction Mixtures[a]

| anion zone[a] | C-4 zone[b] | modified peptides: elution times[c] | modified peptides: peptide comp[d] | molar ratios of unmodified peptides[e] βT1:βT9:βT10a′ | SDS–PAGE mobility[f] | formula of modified hemoglobin |
|---|---|---|---|---|---|---|
| | | | Deoxyhemoglobin Reaction Products | | | |
| 1 | 1 | none | | 1:1:1 | m | $\alpha_2\beta_2$ |
| 4a | 5 | 84.6 | βT1,9,10a′ | 0:0:0 | m | $\alpha_2(\beta^1{}_{82}>B)_2$ |
| 4b | 6 | 84.6 | βT1,9,10a′ | 0.5:0.5:0.5 | d | $\alpha_2\beta$1-B-82$\beta$ |
| 4c | 7 | 90.6 | βT1,(9,10a′)$_2$ | 0.5:0:0 | d | $\alpha_2\beta^1{}_{82}>$B-82$\beta$ |
| 8 | 2 | 85.7 | βT9,10a′ | 1:0:0 | m | $\alpha_2(\beta$82-B$)_2$ |
| | | | (Carbonmonoxy)hemoglobin Reaction Products | | | |
| 1 | 1 | none | | 1:1:1 | m | $\alpha_2\beta_2$ |
| 2 | 5 | 92.5 | βT9,10a′ | 1:0:0 | d | $\alpha_2\beta$82-B-82$\beta^g$ |
| 3 | 6 | 92.5 | βT9,10a′ | 1:0:0 | d | $\alpha_2\beta$82-B-82$\beta$ |
| 4 | 2 | 85.6 | βT9,10a′ | 1:0:0 | m | $\alpha_2(\beta$82-B$)_2$ |

[a] See Figure 1 for zone numbers for hemoglobin components separated by anion-exchange HPLC. Zone 4 was separated further by rechromatography to isolate three different components designated 4a, 4b, and 4c. [b] See Figure 2 for zone numbers of globin chains separated by C-4 reversed phase HPLC. [c] The elution times of peptides containing the UV absorption of the trimesoyl group were obtained from chromatograms as shown in Figure 3. The times listed are those of the predominant modified peptides. In most cases lesser amounts of other modified peptides are detected that result from incomplete enzymatic hydrolysis of the βT8 lysyl residue from the N terminus of the βT9,10a′ peptides. [d] The amino acid compositions of the modified peptides were determined and are expressed here in terms of molar ratios of normal βT1, βT9, and βT10a′. The latter represents β chain residues 83–90 and results from the Glu-C endoproteinase hydrolysis of the β90 glutamyl residue. [e] Molar ratios of normal, unmodified βT1, βT9, and βT10a′ to the other tryptic peptides were estimated by comparing heights of peaks on the peptide patterns like those in Figure 3. [f] The mobilities of the modified globin chains in SDS–PAGE were compared to those of unmodified globins to classify each as either single-chain monomers (m) of approximately 16 000 molecular weight or two-chain dimers (d) of approximately 32 000 molecular weight. [g] Bis-Tris is esterified to the cross-linker in this hemoglobin component.

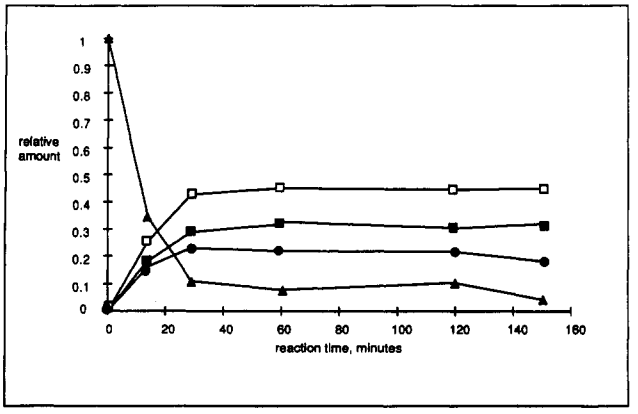



FIGURE 4: Relative concentration of the major β chain species present in the mixture of hemoglobins resulting from reaction of deoxyHb with TMMP as a function of time. Yield of modified hemoglobin β chains is based on analytical C-4 reversed-phase HPLC with chain separation. Yields shown are normalized to 100% for the sum of the products identified in the graph. Minor products in the reaction mixture account for about 20% of the material present and are not shown. (▲) Unmodified protein; (●) $\beta^1{}_{82}>$B; (■) β1-B-82β; (□) $\alpha_2\beta^1{}_{82}>$B-82β.

present in samples taken at noted times from the start of reaction by reversed-phase HPLC and integration of HPLC peaks. In Figure 4, the relative concentrations of the β-chain species are plotted as a function of time.

*Functional Properties of Modified Hemoglobins.* The oxygen binding properties of several of the trimesoyl-modified hemoglobins are listed in Table III. Two of the hemoglobins that have a single cross-link between the β1Val residue of one chain and the β82Lys residue of the other chain have $P_{50}$s of about 17–18. These are the same as that which is found for the β1-I-82β hemoglobin cross-linked with an isophthaloyl group (Jones et al., unpublished results). The internally linked $\beta^1{}_{82}>$ has a $P_{50}$ of 10.8, which is similar to the 9.2 observed for the isophthaloyl derivative. As also shown in Table III, all of the trimesoyl-modified hemoglobins studied have reduced alkaline Bohr effects. Like their isophthaloyl analogue (Jones et al., unpublished results), the β1 to β82 cross-linked trimesoyl hemoglobins have oxygen binding properties in a potentially useful range.

Table III: Functional Properties of Trimesoyl-Modified Hemoglobins[a]

| hemoglobin structure | $P_{50}$ ($n_{50}$)[b] | % $\Delta H^+$ | homogeneity[c] (%) |
|---|---|---|---|
| $\alpha_2\beta_2$ | 5.0 (3.0) | 100 | >99 |
| $\alpha_2\beta$82-B-82$\beta$ | 4.8 (2.4) | 70 | >90 |
| $\alpha_2\beta$82-B-82$\beta^d$ | 2.5 biphasic | 85 | >80 |
| $\alpha_2\beta$1-B-82$\beta$ | 17.1 (2.7) | 65 | >85 |
| $\alpha_2(\beta^1{}_{82}>B)_2$ | 10.8 (2.3) | | |
| $\alpha_2\beta^1{}_{82}>$B-82$\beta$ | 18.1 (2.6) | 67 | >85 |

[a] Conditions: 50 mM Bis-Tris, 0.1 M $Cl^-$, pH 7.4, 25 °C, 55 μM heme used for oxygen equilibrium measurements. Alkaline Bohr effect was measured at pH 7.0, 7.4, and 7.9. [b] Apparent Hill coefficient measured at $P_{O_2} = P_{50}$ (Torr). [c] Estimates indicate lower limit based on chromatographic resolution. [d] The third acyl group of the cross-linker is esterified to the buffer (see text).

The oxygen binding curve of triply linked trimesylHb at 37 °C, pH 7.4, has a $P_{50}$ of 36 Torr and a Hill coefficient at half-saturation ($n_{50}$) of 2.4. The values for whole blood measured under the same conditions are $P_{50} \sim 26$ Torr and $n_{50} = 3.1$. Since the oxygen binding curve of trimesylHb is right-shifted from that of whole blood, i.e., has a higher $P_{50}$, the percent of oxygen that can be delivered by trimesylHb between partial pressures of 100 and 40 Torr is almost twice that of whole blood (Vandegriff & Winslow, 1991).

## DISCUSSION

*Mechanism of Formation of Triple and Double Links.* The hemoglobin molecule contains a twofold symmetry axis which interchanges the β subunits and the groups within the DPG binding site (Dickerson & Geis, 1983). On the basis of reaction patterns of **1** and **2**, we expected TMMP to react with the lysine 82 and valine 1 amino groups of the two β subunits in the DPG binding site. X-ray crystallographic studies of deoxyhemoglobin (Fermi & Perutz, 1984) show the lysine 82 groups to be farther apart than the span of our reagent [we expect the reagent to span amino groups about 7 Å apart (Kluger et al., 1990)], but the amino groups are on flexible side chains

which can move into close proximity. The terminal valine 1 groups are also farther apart from one another than the span of the reagent (Walder et al., 1980) and are not as free to move since they are attached directly to the protein's primary chain (Schulz & Schirmer, 1979). Therefore, reactions linking lysine 82 and valine 1 are possible with TMMP, while reactions between the reagent and two valine 1 groups are not. This has also been demonstrated by our studies of modifying deoxy-Hb with dicarboxy bis(methyl phosphate) reagents (Jones et al., unpublished results).

*Analysis of Reaction Patterns.* The addition of an amine to an acyl phosphate monoester is a rapid second-order process (Di Sabatao & Jencks, 1961). Internal reaction of an amino group with an acyl phosphate monoester, which is remotely attached to an amide to form a cross-link, is expected to be extremely rapid on the basis of the entropic advantage of reactions of bound species (Kluger, 1990; Page & Jencks, 1971). It is unlikely that immediate precursors to doubly and triply linked hemoglobins will accumulate since once the initial amide forms from reaction of TMMP with an amino group of the protein, further acylation reactions should be rapid as noted above (Westheimer, 1956; Page & Jencks, 1971). The data in Figure 4 confirm this since the concentrations of the $\beta^1{}_{82}$>B-82$\beta$ and $\beta$1-B-82$\beta$ species rise in proportion in an apparently simple first-order manner directly to their final amounts. Precursors would be expected to behave as typical reaction intermediates, rising early and declining as products result.

*Further Considerations for Mechanism of Cross-Linking.* A mechanism of formation of all of the species found must also account for the species not found. In particular, why does the $\alpha_2\beta$1-B-82$\beta$ species appear not to convert to the triply linked species? Structural and mechanistic principles can explain these results and give insight into the details of the reaction process. The routes to the triply linked product should include the following routes symbolized by the sites of acylation. The cross-linker is designated B:P,P,P prior to reaction, and remaining acyl phosphate residues are indicated as P:

$$\alpha_2\beta 82\text{-(B:P,P)}\beta \rightarrow \alpha_2\beta 82\text{-(B,P)-}82\beta \rightarrow \alpha_2\beta 82\text{-B-(1,82B)} \quad \text{(a)}$$

$$\alpha_2\beta 82\text{-(B:P,P)}\beta \rightarrow \alpha_2\beta 82\text{-(B:P)-}1\beta \rightarrow \alpha_2\beta 82\text{-B-(1,82}\beta\text{)} \quad \text{(b)}$$

$$\alpha_2\beta 82\text{-(B:P,P)}\beta \rightarrow \alpha_2\beta(1,82)\text{-(B:P)-}\beta \rightarrow \alpha_2\beta 82\text{-B-(1,82}\beta\text{)} \quad \text{(c)}$$

$$\alpha_2\beta 1\text{-(B:P,P)}\beta \rightarrow \alpha_2\beta 1\text{-(B:P)-}82\beta \rightarrow \alpha_2\beta 82\text{-B-(1,82}\beta\text{)} \quad \text{(d)}$$

$$\alpha_2\beta 1\text{-(B:P,P)}\beta \rightarrow \alpha_2\beta(1,82)\text{-(B:P)-}\beta \rightarrow \alpha_2\beta 82\text{-B-(1,82}\beta\text{)} \quad \text{(e)}$$

In routes b and d, species cross-linked between valine and lysine of different $\beta$ subunits are intermediates. Why do they appear only as products? Stereochemical principles can provide a reasonable explanation. Formation of the $\beta$82-(B:P)-1$\beta$ cross-linked species in principle leads to stereoisomeric structures due to the formation of amide bonds in the cross-linking reaction. Since the $\epsilon$-amino group of lysine is connected to the peptide backbone by a flexible four-carbon chain, it is unlikely that isomerism will occur at the amide formed with this group. However, the terminal valine amino group is connected directly to the peptide backbone and will have a high kinetic barrier to rotation, about 20 kcal/mol (Schulz & Schirmer, 1979), while the thermodynamic difference in conformations should be small. The bis amides, which form initially under kinetic control from the reaction of an acyl phosphate ester with the terminal valine amino group and the $\epsilon$-amino group of lysine, will not isomerize about the valyl amide bond which connects to the cross-linker. The unreacted (third) acyl phosphate moiety will have two stereochemically distinct orientations as a result of the cis–trans isomerism.

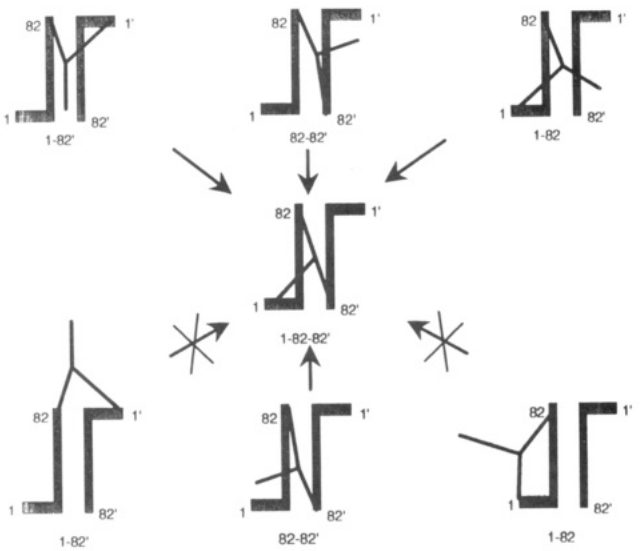



FIGURE 5: Symbolic representation of proposed mechanism of reaction of TMMP with Hb.

A symbolic representation of the structure of the hemoglobin $\beta$ subunits and TMMP is presented in Figure 5. Hemoglobin dimers are represented by two symmetrical L-shaped polygons, and the reagent is shown as three joined lines. Twofold rotation axes perpendicular to the plane of the paper interchange the subunits in each dimer. After reaction with the reagent, there are only three unique product structures: the triply linked material $\alpha_2\beta^1{}_{82}$>B-82$\beta$, the anti isomer of $\alpha_2\beta$1-B-82$\beta$, and the anti isomer of $\alpha_2(\beta^1{}_{82}$>B)$\beta$. The two Val-1 amino groups are too far apart to be spanned by one molecule of the reagent. Reaction of TMMP between Val-1 and Lys-82 leads to two isomers, only one of which may react further. This applies whether the reaction is between two chains or is within the same chain. Reaction between Lys-82 residues in both chains necessarily leads to the triply linked product, as shown. Figure 6 is a structural model for peptide isomerism that might give such an outcome.

*Functional Properties of Trimesoyl-Modified Hemoglobins.* The oxygen affinities (characterized by $P_{50}$ values) of the trimesoyl hemoglobins range from about half that of normal to over 3 times normal depending upon the specific modification. The $P_{50}$ values of the two hemoglobins cross-linked between $\beta$1Val and $\beta$82Lys, $\alpha_2\beta$1-B-82$\beta$ and $\alpha_2\beta^1{}_{82}$>B-82$\beta$, are very similar to one another and to the affinity found for the $\alpha_2\beta$1-I-82$\beta$ isophthalyl analogue. This indicates that fixing the distance between the $\beta$1Val residue of one chain and the $\beta$82Lys residue of the other chain influences the oxygen affinity more than does either altering the charges further in the DPG binding site or coupling the $\beta$82Lys residue of the first chain with its $\beta$1Val residue. It also demonstrates that the effect of linking the $\beta$1Val residue to the $\beta$82Lys residue of the same chain is not additive to the effect of linking this $\beta$1Val residue to the $\beta$82Lys of the other chain. The Hill coefficient ($n$) remains close to that of normal hemoglobin.

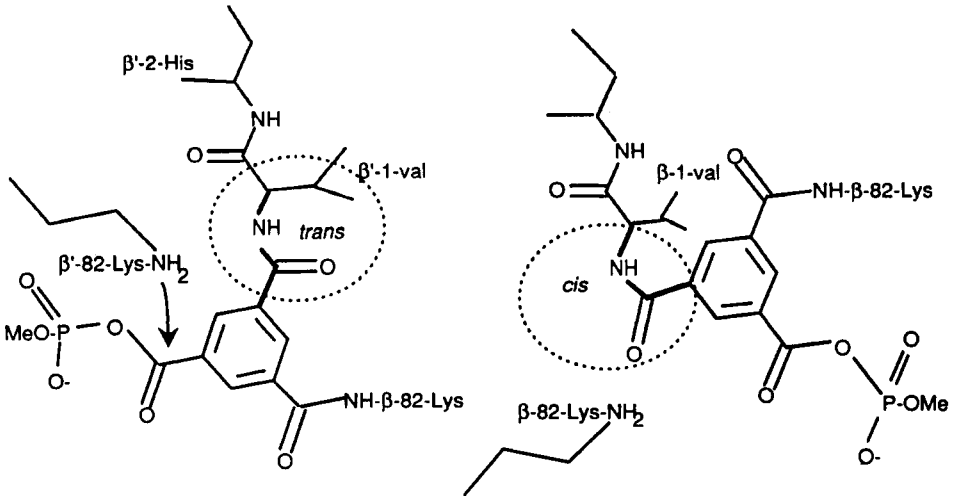



FIGURE 6: Structural model for possible isomerism in reaction of TMMP with hemoglobin.

Since the cross-linker does not directly interact with the heme binding sites, cooperativity resulting from interactions of the subunits can be retained.

Two different forms of $\alpha_2\beta$82-B-82$\beta$ were isolated from reactions of TMMP with COHb, corresponding to zones 2 and 3 of Figure 1A. The one from zone 2 had a biphasic oxygen equilibrium curve (due to impurity or instability) and an overall $P_{50}$ of about 2.5. On the basis of mass spectrometric analysis, we find that this is due to a modified hemoglobin in which a molecule of bis-tris (the reaction buffer) has reacted with the third carbonyl group of the cross-linker. In later work, we have used an alternative buffer (MOPS) which does not react with the cross-linker. The $P_{50}$ of the hemoglobin of zone 3 is 4.5, indicating a high oxygen affinity. Mass spectral studies are consistent with this being a species $\alpha_2\beta$82-B-82$\beta$ in which the third carbonyl group of the cross-linker is a free carboxylate.

The decreases in alkaline Bohr effect observed for the trimesoyl hemoglobins are likely to be due to the loss of the contribution of the protonation of amino groups of $\beta$1Val and $\beta$82Lys residues to the normal anion effect (Imai, 1982). The chloride ion effect on these modified hemoglobins is presently being examined to test this hypothesis.

*Utility of Triply Linked Hemoglobins and Reagents for Their Production.* TMMP is the first trifunctional acylating reagent that has been used to cross-link hemoglobin in which the major reaction products have been structurally characterized. Like other cross-linking reagents, the major products this reagent forms with hemoglobin are dependent upon the ligand state of the heme groups. As predicted, TMMP reacts with deoxyHb more readily than any of the bifunctional acyl methyl phosphate reagents (Jones et al., unpublished results). With deoxyHb it forms two major cross-linked hemoglobin components, both of which involve $\beta$1Val of one chain and $\beta$82Lys of the other chain. TMMP has the additional advantage over the isophthaloyl bis(methyl phosphate) reagent (**2**) in that any $\alpha_2\beta$82-B82$\beta$ which may result serves as an intermediate in the formation of the triply linked $\alpha_2\beta^1{}_{82}$>B-82$\beta$. Thus, the relatively high-affinity $\alpha_2\beta$82-B-82$\beta$ cross-linked hemoglobin is not left in the reaction mixture.

The oxygen binding properties of the two $\beta$1-B-82$\beta$ trimesoyl cross-linked hemoglobins are in a physiologically useful range for transfusion and perfusion applications. Although the equilibrium curve is shifted to the right, or low-affinity side of normal blood, the retention of a high degree of cooperativity makes the $\alpha_2\beta^1{}_{82}$>B-82 modified hemoglobin a physiologically effective transporter of oxygen.

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