Mechanism of Site-Directed Protein Cross-Linking. Protein-Directed Selectivity in Reactions of Hemoglobin with Aryl Trimesates

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Site-directed cross-linking of hemoglobin has become an efficient way to produce a structurally defined altered protein with desirable functional properties. The reagent trimesoyl tris(3,5-dibromosalicylate) (**1**) introduces a bis amide cross-link derived from the ϵ-amino groups of the side chains of the two β-Lys-82 residues in human hemoglobin. The basis of its specificity was investigated using a set of analogues of **1** (**2**−**12**). There are marked differences in the reaction patterns of these compounds with amino groups in hemoglobin compared to reactions with *n*-propylamine. The compounds that effectively modify the protein contain a carboxyl group ortho to the phenolic oxygen of the ester, while materials with meta or para carboxyl groups give little or no reaction. In contrast, the reactions with *n*-propylamine are slowest with the ortho carboxyl materials. Addition of the unreactive compound **5** to a solution containing hemoglobin reduces the ability of **1** to modify the protein, showing that the unreactive compound binds but does not react. On the basis of these observations and the known reaction patterns of salicylates, it is clear that the environment in the protein controls the reaction, regardless of the inherent reactivity of the reagent. We propose that the carboxyl group positions the reagent critically within the protein. Only the ortho arrangement permits transfer of the acyl function to the nucleophile.

Reagents that introduce cross-links into hemoglobin by highly selective reaction processes create structurally defined altered proteins with properties that can be related to structure.[1] Trimesoyl tris(3,5-dibromosalicylate) (**1**) is a remarkably specific and efficient reagent, reacting with hemoglobin to produce a cross-linked product (linked between ϵ-amino groups of lysine-82 side chains in the β subunits) with the third ester site either intact or hydrolyzed.[2] The leaving group in **1** is a salicylic acid derivative, containing a carboxyl group adjacent to the phenolic ester oxygen. The adjacent carboxyl is expected to facilitate the hydrolysis of the ester but not the addition of amines, such as the side chain amino group in a protein.[3] Since the residual ester is useful for making interesting derivatives of cross-linked hemoglobin,[4] hydrolysis is an undesired reaction. Isomers of salicylates, in which the carboxyl is para or meta to the phenolic oxygen are less susceptible to hydrolysis than are salicylates,[3] but we do not know their reactivity with the protein. On the basis of this model, a para-substituted analogue of **1** should be more resistant to hydrolysis and equally reactive toward amines.

The reactivity of **1** with hemoglobin should be significantly affected by its bromine substituents. While bromine atoms as substituents on the aromatic ring of the salicylate leaving group increase the reactivity of acetyl salicylates toward amine nucleophiles in general, the substituent effect is much greater with amino groups of hemoglobin.[5] Klotz suggests that the bromine substituents specifically increase electrostatic interactions between the reagent and protein groups.[5]

We have prepared a set of isomers and analogues of (**1**) to probe the origin of its specificity and reactivity with hemoglobin. How do its selectivity and reactivity relate to the substitution pattern on the salicylate leaving group? Does the ortho carboxyl have a specific effect in the reaction of the ester with amino groups of the protein? Do the halogen substituents in triesters have especially enhanced reactivity? The structures of the compounds we have prepared are summarized below.

The contrasting reaction patterns of the amino groups in the protein and in *n*-propylamine with **1**−**12** reveal fundamental information about the effects of functional group arrays on the leaving group in the reaction of the amino groups in the protein with esters.

Experimental Section

Solvents used in syntheses were dried prior to use. Reagents for buffers and for chromatography were of analytical grade or better. Solutions of human hemoglobin A were obtained from Hemosol, Inc. The structure of newly synthesized materials was assessed by a combination of NMR spectroscopy, mass spectrometry, infrared spectroscopy, and ion spray mass spectroscopy. Proton and carbon NMR spectra were recorded at 200 and 100 MHz, respectively.

We found that elemental analysis did not give reproducible results with the final products (triacids). Thus, in all cases, purity was assessed by HPLC analysis and the structure was analyzed spectroscopically.

Tris(3,5-dibromosalicyl) trimesate (**1**) was prepared by the published procedure.[2] All other tris trimesate esters were

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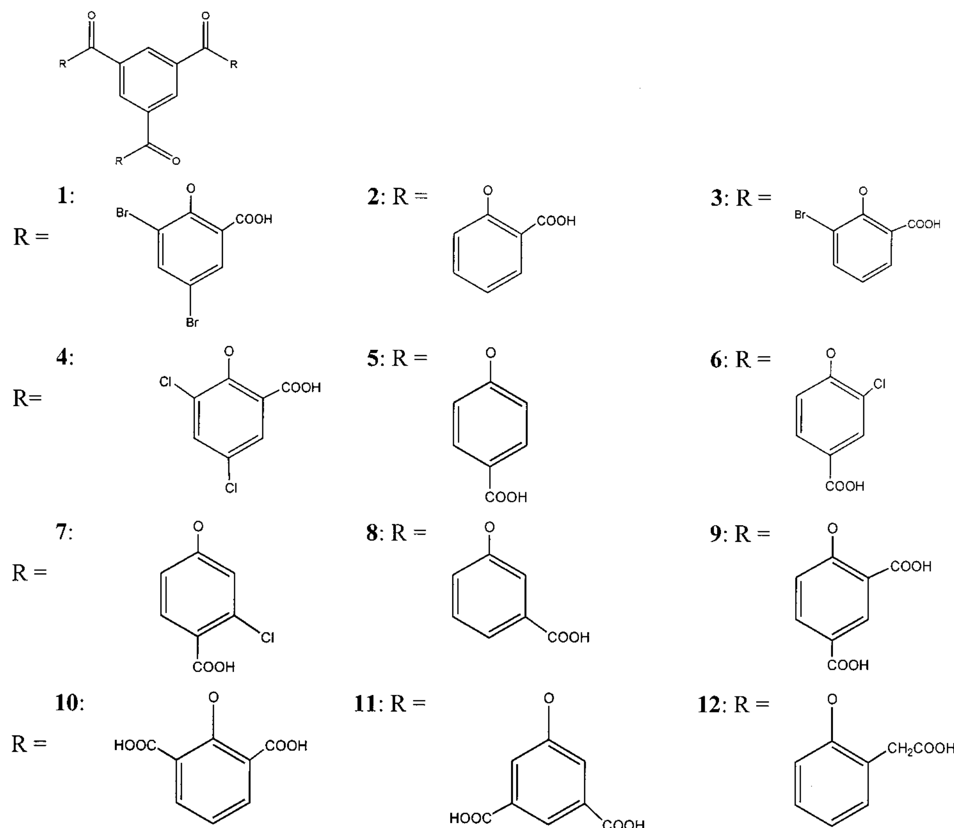

prepared by variations on this route, which involves coupling the *tert*-butyl salicylate ester with an activated form of trimesic acid, followed by removal of the *tert*-butyl group.

tert-Butyl salicylate was prepared as a precursor for tris (salicyl) trimesate (**2**). DCC (8.0 g, 0.039 mol) dissolved in dry THF (50 mL) was added dropwise over 30 min to a stirred suspension of salicylic acid (5.00 g, 36.1 mmol) and *N*,*N*-dimethylaminopyridine (DMAP) (0.17 g, 0.0014 mol) in dry *tert*-butyl alcohol (125 mL). The mixture was stirred at room temperature overnight and then concentrated. The residue was stirred in diethyl ether (50 mL), and oxalic acid (5.3 g, 0.059 mol) was introduced in portions to decompose excess DCC and precipitate DMAP. The mixture was filtered, and the filtrate was washed with an aqueous solution with three 40 mL portions of 0.3 M sodium bicarbonate solution. The organic solution was concentrated to dryness, leaving an oil, which was purified using flash chromatography (silica gel in dichloromethane). Yield: 6.0 g (86%) of *tert*-butyl salicylate. ^{1}H NMR ($CDCl_3$): δ 1.62 (s, 9H, $C(CH_3)_3$), 6.82 (dt, 1H, ArH), 6.95 (dd, 1H, ArH), 7.42 (dt, 1H, ArH), 7.77 (dd, 1H, ArH), 11.05 (s, 1H, ArOH).

Potassium *tert*-butoxide (0.50 g, 0.0025 mol) was added to a solution of *tert*-butyl salicylate (0.28 g, 0.0025 mol) in anhydrous THF (30 mL). The mixture was stirred at room temperature for 15 min. A solution of trimesoyl trichloride (0.22 g, 0.000 85 mol) in anhydrous THF (15 mL) was added dropwise over 15 min. The mixture was stirred overnight at room temperature and then concentrated. The solid was dissolved in diethyl ether (35 mL), and the mixture was washed with water (2 × 20 mL). The organic phase was dried and concentrated to give trimesoyl tris(1-(*tert*-butoxycarboxycarbonyl)salicylate). The white solid was dissolved in anhydrous trifluoroacetic acid (20 mL) and stirred at room temperature for 1 h. Ether (20 mL) was added, and the solution was kept overnight at 4 °C. The product (tris(salicyl) trimesate (**2**)) was collected by filtration as white crystals (0.36 g, 75%; mp >240 °C). ^{1}H NMR (acetone-d_6): δ 7.5 (dt and dd, 6H, ArH), 7.76 (dt, 3H, ArH), 8.15 (dd, 3H, ArH), 9.18 (s, 3H, ArH). IR (KBr): 1757 (m), 1708 (s), 1609 (m), 1209 (s) cm^{-1}. MS (negative FAB): 569 (M − 1). ^{13}C NMR (acetone-d_6): δ 165.50 (s), 164.09 (s), 151.60 (s), 136.14 (s), 134.95 (s), 132.72 (s), 132.40 (s), 127.32 (s), 124.77 (s), 124.39 (s).

Trimesoyl tris(5-bromosalicylate) (**3**) was prepared from *tert*-butyl 5-bromosalicylate, which was prepared from the acid as above in 91% yield. ^{1}H NMR ($CDCl_3$): δ 1.6 (s, 9H, C $(CH_3)_3$), 6.84 (d, 1H, ArH), 7.48 (dd, 1H, ArH), 7.84 (d, 1H, ArH), 11 (s, 1H, ArOH). The protected tris ester, trimesoyl tris(1-(*tert*-butoxycarbonyl)-5-bromosalicylate) was cleaved with TFA, giving **3** (24% yield from monoester, mp 214 °C). ^{1}H NMR (DMSO-d_6): δ 7.48 (dd, 3H, ArH), 7.95 (dd, 3H, ArH), 8.12 (d, 3H, ArH), 9.02 (s, 3H, ArH). IR (KBr): 1750 (s), 1708 (s), 1476 (m), 1202 (s) cm^{-1}. MS (negative FAB): 803 (M − 1). ^{13}C NMR (DMSO-d_6): δ 164.09 (s), 162.83 (s), 149.08 (s), 136.72 (s), 135.09 (s), 133.83 (s), 130.89 (s), 126.30 (s), 125.90 (s), 118.83 (s). Anal. Calcd for $C_{30}H_{15}O_{12}Br$: C, 46.36; H, 1.56. Found: C, 46.09; H, 1.85.

Trimesoyl tris(3,5-dichlorosalicylate) (**4**) was prepared by a similar sequence. *tert*-Butyl(3,5-dichlorosalicylate) was prepared by DCC-DMAP coupling of *tert*-butyl alcohol and 3,5-dichlorosalicylic acid in 64% yield. ^{1}H NMR ($CDCl_3$): δ 1.60 (s, 9H, $C(CH_3)_3$), 7.54 (dd, 1H, ArH), 7.66 (dd, 1H, ArH), 11.48 (s, 1H, ArOH). The material was neutralized with potassium *tert*-butoxide and combined with trimesoyl trichloride in THF (15 mL). Extraction, drying, and concentration were followed by removal of the *tert*-butyl groups with trifluoracetic acid. The product was collected by filtration as a white powder (34% yield; mp 175 °C). ^{1}H NMR (acetone-d_6): δ 8.02 (dd, 3H, ArH), 8.07 (dd, 3H, ArH), 9.40 (s, 3H, ArH). IR (KBr): 1757 (m), 1701 (s), 1455 (m), 1209 (s) cm^{-1}. MS (negative FAB): 773 (M − 1). ^{13}C NMR (acetone-d_6): δ 163.62 (s), 162.60 (s), 146.52 (s), 136.82 (s), 134.58 (s), 132.77 (s), 131.68 (s), 131.15 (s), 130.61 (s), 127.59 (s).

Tris(4-carboxylphenyl) trimesate (**5**) was prepared using *tert*-butyl-4-hydroxybenzoate, which was prepared by DCC coupling of *tert*-butyl alcohol and *p*-hydroxybenzoic in 45% yield: ^{1}H NMR ($CDCl_3$): δ 1.58 (s, 9H, $C(CH_3)_3$), 6.40 (broad, s, 1H, ArOH), 6.83 (dd, 2H, ArH), and 7.90 (dd, 2H, ArH). Using the general procedure described above, the product was obtained in 25% yield (mp >240 °C). ^{1}H NMR (DMSO-d_6): δ 7.52 (d, 6H, ArH), 8.08 (d, 6H, ArH), 9.05 (s, 3H, ArH). IR

(KBr): 1750 (s), 1694 (s), 1602 (m), 1209 (s) cm^{-1}. MS (negative FAB): 569 (M − 1). ^{13}C NMR (DMSO-d_6): δ 166.43 (s), 162.41 (s), 153.59 (s), 135.19 (s), 130.91 (s), 130.54 (s), 128.82 (s), 122.02 (s).

Tris(4-carboxyl-2-chlorophenyl) trimesate (**6**) was prepared by the same procedures.

tert-Butyl 3-chloro-4-hydroxybenzoate was prepared using DCC coupling as a white powder in 75% yield. ^1H NMR ($CDCl_3$): δ 1.60 (s, 9H, $C(CH_3)_3$), 5.95 (broad, s, 1H, ArOH), 7.20 (dd, 1H, ArH), 7.83 (dd, 1H, ArH), 7.97 (d, 1H, ArH). Reaction with trimesoyl trichloride and removal of the *tert*-butyl groups gave the product as a white powder in 43% yield (mp 138 °C). ^1H NMR (acetone-d_6 with sonication): δ 7.76 (dd, 3H, ArH), 8.15 (dd, 3H, ArH), 8.23 (d, 3H, ArH), 9.29 (s, 3H, ArH). IR (KBr): 1757 (m), 1708 (s), 1420 (m), 1202 (s) cm^{-1}. MS (negative FAB): 671 (M − 1). ^{13}C NMR (DMSO-d_6): δ 165.37 (s), 161.41 (s), 149.49 (s), 135.63 (s), 130.85 (s), 130.81 (s), 130.70 (s), 130.16 (s), 129.62 (s), 126.06 (s), 124.59 (s).

Tris(4-carboxyl-3-chlorophenyl) trimesate (**7**) was prepared from *tert*-butyl 2-chloro-4-hydroxybenzoate. The latter was prepared in 15% yield. ^1H NMR (DMSO-d_6): δ 1.60 (s, 9H, $C(CH_3)_3$), 6.78 (dd, 1H, ArH), 6.88 (dd, 1H, ArH), 7.75 (d, 1H, ArH), 10.14 (s, 1H, ArOH). Combination with trimesoyl trichloride and cleavage with trifluoroacetic acid gave the product as white crystals. Yield: 17%, mp >240 °C. ^1H NMR (DMSO-d_6): δ 7.51 (dd, 3H, ArH), 7.75 (d, 3H, ArH), 7.95 (dd, 3H, ArH), 9.02 (s, 3H, ArH). IR (KBr, cm^{-1}): 1750 (s), 1708 (s), 1595 (m), 1195 (s). MS (negative FAB): 671 (M − 1).^{13}C NMR (acetone-d_6): δ 165.83 (s), 162.24 (s), 152.27 (s), 135.32 (s), 132.62 (s), 132.11 (s), 130.37 (s), 129.19 (s), 124.29 (s), 120.95 (s).

Tris(3-carboxylphenyl) trimesate (**8**) was prepared from *tert*-butyl-3-hydroxybenzoate. ^1H NMR ($CDCl_3$): δ 1.60 (s, 9H, $C(CH_3)_3$), 7.09 (m, 1H, ArH), 7.27 (t, 1H, ArH), 7.55 (m, 1H, ArH), 7.64 (m, 1H, ArH), 7.74 (s, 1H, ArOH)) and trimesoyl trichloride followed by reaction with trifluoroacetic acid. The product is a white powder, mp 188−191 °C. Yield: 43%. ^1H NMR (DMSO-d_6): δ 7.66 (m, 6H, ArH), 7.94 (m, 6H, ArH), 9.06 (s, 3H, ArH). HRMS (FAB^+): 571.0851 $(MH)^+$ (theoretical 571.0876). IR (KBr): 3083, 1699, 1222 cm^{-1}. ^{13}C NMR (DMSO-d_6): δ 122.88, 126.57, 127.36, 130.17, 130.89, 132.60, 135.39, 150.52, 163.11, 166.56.

Tris(2,4-dicarboxylphenyl) trimesate (**9**) was prepared as follows. Di-*tert*-butyl-4-hydroxyisophthalate was prepared from 4-hydroxyisophthalic acid (1.0 g) dissolved in a solution of thionyl chloride (30 mL) and oxalyl chloride (1 mL). The solution was refluxed overnight. Solvents were evaporated using a rotary evaporator and the dry product was then dissolved in anhydrous THF (20 mL). The solution was slowly transferred to a stirred suspension of potassium *tert*-butoxide (1.8) in anhydrous THF (20 mL) and stirred overnight. The organic solution was concentrated to dryness. The resulting product was dissolved in ether (30 mL) and washed twice with saturated sodium bicarbonate. The organic solution was concentrated to dryness to give a white powder. Yield: 0.7 g (56%). ^1H NMR ($CDCl_3$): δ 1.58 (s, 9H, $C(CH_3)_3$), 1.62 (s, 9H, $C(CH_3)_3$), 6.90 (d, 1H, ArH), 8.02 (dd, 1H, ArH), 8.45 (d, 1H, ArH), 11.50 (s, 1H, ArOH).

Potassium *tert*-butoxide (0.3 g) was added to a solution of di-*tert*-butyl-4-hydroxyisophthalate (0.7 g) in anhydrous THF (25 mL). The mixture was stirred for 15 min. A solution of trimesoyl trichloride (0.3 g) in anhydrous THF (25 mL) was added dropwise, and the solution was stirred overnight. The mixture was concentrated to dryness, dissolved in ether, and washed with saturated sodium carbonate (2 × 25 mL). The organic phase was dried and concentrated to give tris(2,4-di-*tert*-butoxycarbonylphenyl) trimesate as a solid. The product was dissolved in anhydrous trifluoroacetic acid (30 mL) and stirred for 1 h. Ether was added to induce crystallization and the solution was kept at 4 °C overnight. The product (**9**) was collected by filtration to give yellow crystals, mp 245 °C (dec). Yield: 0.5 g (24%). ^1H NMR (DMSO-d_6): δ 7.64 (d, 3H, ArH), 8.26 (d, 3H, ArH), 8.56 (s, 3H, ArH), 9.05 (s, 3H, ArH). HRMS (FAB^+): 703.0533 $(MH)^+$ (theoretical 703.0571). IR (KBr): 3086, 1711, 1213 cm^{-1}. ^{13}C NMR (DMSO-d_6): δ 124.02, 124.57, 129.28, 130.90, 132.64, 134.70, 135.14, 153.06, 162.75, 164.68, 165.83.

Tris(2,6-dicarboxylphenyl) trimesate (**10**) was prepared as above from di-*tert*-butyl-2-hydroxyisophthalate. 2-Hydroxyisophthalic acid[6] was converted to the *tert*-butyl ester[7] in 56% yield. ^1H NMR ($CDCl_3$): δ 1.80 (s, 18H, $C(CH_3)_3$), 6.82 (t, 1H, ArH), 7.85 (d, 2H, ArH), 11.90 (s, 1H, ArOH). The ester was combined with trimesoyl trichloride and the product converted to the triacid with trifluoroacetic acid. The residue (**10**) is a pale brown powder (mp 225 °C, dec) in 7% yield. ^1H NMR (DMSO-d_6): δ 6.65 (t, 3H, ArH), 7.87 (d, 6H, ArH), 8.64 (s, 3H, ArH). IR (KBr, cm^{-1}): 3450, 1704, 1252. ^{13}C NMR (DMSO-d_6): δ 165.89, 168.41, 169.26, 113.92, 117.43, 131.94, 133.61, 135.50.

Tris(3,5-dicarboxylphenyl) trimesate (**11**) was prepared from di-*tert*-butyl-5-hydroxyisophthalate, which was prepared from 5-hydroxyisophthalic acid as above (white solid, 50% yield). ^1H NMR ($CDCl_3$): δ 1.60 (s, 18H, $C(CH_3)_3$), 7.11 (s, 1H, ArOH), 7.77 (d, 2H, ArH), 8.11 (t, 1H, ArH). This was combined with trimesoyl trichloride and then treated with trifluoroacetic acid to give **11** as white crystals, mp >250 °C (dec). Yield: 30%. ^1H NMR (DMSO-d_6): δ 8.21 (s, 6H, ArH), 8.44 (s, 3H, ArH), 9.80 (s, 3H, ArH). IR (KBr): 3088, 1718, 1229 cm^{-1}. ^{13}C NMR (DMSO-d_6): δ 127.07, 127.80, 130.70, 132.98, 135.59, 150.67, 163.06, 165.88.

Tris(phenyl-2-acetic acid) trimesate (**12**) was prepared from *tert*-butyl-2-hydroxyphenyl acetate. The *tert*-butyl ester was prepared as follows. A mixture of 2-hydroxyphenylacetic acid (5.0 g), liquid isobutylene (100 mL), and concentrated sulfuric acid (2 mL) was shaken in a pressure vessel (a European beer bottle with a glass−rubber seal and wire bail) for 7 h. (Isobutylene was condensed at −78 °C into a graduated cylinder under nitrogen. The condensed liquid was transferred into the reaction bottle, and the stopper was inserted. After 7 h, the mixture was removed, dissolved in ethyl acetate, and washed with saturated sodium bicarbonate. The organic layer was concentrated, and the resulting yellow oil was purified using flash chromatography (neutral alumina, eluted with chloroform): 1.2 g (23%). ^1H NMR ($CDCl_3$): δ 1.45 (s, 9H, $C(CH_3)_3$), 3.50 (s, 2H, CH_2), 6.88 (m, 2H, ArH), 7.12 (m, 2H, ArH), 8.02 (s, H, ArOH).

Reaction of the ester with trimesoyl trichloride and removal of the *tert*-butyl groups gave **12** as a yellow powder, mp 167 °C (dec). Yield: 22%. ^1H NMR (DMSO-d_6): δ 3.61 (s, 1H, CH_2), 6.90 (m, 1H, ArH), 7.45 (m, 3H, ArH), 8.94 (s, 3H, ArH). LRMS (ESMS): 611 $(M - H)^-$ (theoretical 611). IR (KBr, cm^{-1}): 3446, 1744, 1218. ^{13}C NMR (DMSO-d_6): δ 27.83, 122.28, 128.94, 129.58, 130.88, 131.32, 131.52, 153.72, 162.01, 164.29, 170.52.

Cross-Linking of Deoxyhemoglobin. The method described elsewhere for related reagents was used to prepare hemoglobin in the deoxy form.[8] The cross-linking reagent (2 mol/mol of hemoglobin) was dissolved in 1 mL of dioxane and 4 mL of 0.1 M sodium borate (pH 9.0). The flask was flushed with nitrogen three times. The reagent solution was added to the hemoglobin solution and the mixture was left for 24 h. Samples were analyzed after 2 and 12 h. They were saturated with carbon monoxide, passed through a Sephadex G-25 column (0.1 M MOPS, pH 7.2), collected, and stored in a vial at 0 °C. Hemes and the globin (α and β) chains were separated by reversed-phase HPLC using a 330 Å pore C-4 Vydac column and developers containing 0.1% trifluoroacetic acid in a gradient of acetonitrile−water (20−60%). The effluent was monitored at 220 nm. The procedure was repeated three times for each reagent.

Peptide Pattern Analysis. Dilute hemoglobin solutions were concentrated by ultrafiltration. Hemoglobin was separated into its constituent hemes and globin chains (including cross-linked chains) by reversed-phase HPLC (330 Å C-4 Vydac

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column) using developers containing 0.1% trifluoroacetic acid and a gradient of acetonitrile−water (20%−60%). The globin chains were then dissolved in 8 M urea (to increase susceptibility to hydrolysis) and kept at room temperature for 4 h. The solution was diluted to 2 M urea with 0.08 M pH 8.5 ammonium bicarbonate. Trypsin (2% solution) was added, and the mixture was left for 24 h (room temperature). The tryptic hydrolysate was heated in boiling water for 2 min and diluted to 1 M urea. *Staphylococcus aureus* V8 endoproteinase Glu-C (1% solution) was added to this mixture, and the resulting mixture was left for an additional 72 h at room temperature. The hydrolysates were filtered through a 0.45 fm filter prior to injection onto the reversed-phase HPLC using a C-18 Vydac column.[9]

The HPLC cross-linked peaks were identified by peptide pattern analysis.[2] Peptide fragments were separated using HPLC procedures.[10] Separations were made using developers of 0.1% trifluoroacetic acid and a gradient of acetonitrile−water (0%−100% acetonitrile generated over 1 h). In all cases, products were identified by comparison with those obtained from the reaction of hemoglobin with trimesoyl tris(methyl phosphate).[9] The resulting peptide fragments were also analyzed by comparison with peptide patterns of unmodified hemoglobin.[10]

Ion Spray Mass Spectroscopy. The exact masses of cross-linked hemoglobin were determined by electrospray mass spectroscopy (ESMS).[11] ESMS on the cross-linked hemoglobin products were performed at the Medical Sciences Facility, University of Toronto.

Rate of Cross-Linking Reactions. A series of reactions in which aliquots of cross-linked hemoglobins were removed from the reaction vessel at 20 min intervals were performed to track the progress of the reaction. Six samples were taken over 2 h. Each aliquot was quenched with *n*-propylamine, exposed to carbon monoxide for 10 min and stored at pH 7.2. The cross-linking reaction with **1** was complete after 40 min.

Inhibition of Cross-Linking. Do the reagents that do not modify hemoglobin fail to do so because they do not associate with the protein? Reagent **5** is a triester with para carboxyl substituents and no orth*o* carboxyl. It does not alter hemoglobin. This reagent was combined with hemoglobin in the presence of a reagent that is an effective cross-linker (**1**, in pH 9 borate buffer). **5** (1, 2, and 10 equiv) was added to reaction solutions containing deoxyhemoglobin along with 1 equiv of **1,** and the products were measured after 2 h.

Aminolysis of Triesters. Kinetic data for the reaction of **1**−**12** with *n*-propylamine were acquired by recording the UV/vis absorbance at the wavelength of greatest change for the leaving group (267−328 nm). Preparative reactions were run with the same materials and the products identified spectroscopically. Each reagent (**1**−**12**) was combined with an excess of *n*-propylamine (as pH 10 buffer) in solution. The product that is in common from all reagents is benzene-1,3,5-tricarboxylic acid tris-*n*-propylamide ^{1}H NMR: δ 0.09 (t, 9H, $C{-}CH_3$), 2.6 (q, 6H, $C{-}CH_2{-}C$), 3.2 (q, 6H, $C{-}CH_2{-}N$), 8.6 (s, 3H, ArH), 9.0 (s, 3H, C−NH−C). In addition, each reagent releases the corresponding phenol derivative. The second-order rate constant for reaction of each reagent with *n*-propylamine was determined from the dependence of the observed first-order rate coefficient (average of three measurements of k_0) upon the concentration of free *n*-propylamine (total *n*-propylamine concentrations: 0.2, 0.4, 0.6, 0.8, and 1.0 M). Solutions for the reaction were kept at ionic strength 1.0 (with added sodium chloride) and maintained at 25.0 °C. The reagents (∼0.001 g) were dissolved in 2 mL of DMSO, and 0.020 mL of the solution was then transferred to a cell containing 3 mL of the *n*-propylamine buffer. The data for the aminolysis reactions were also used to obtain the first-order rate constant for hydrolysis (k_{hyd}) by extrapolation to the rate in the absence of amine.

Table 1. Modification of Deoxyhemoglobin A[a]

	resulting modified protein			
		relative amount of each class of cross-linked protein (%)		
reagent	fraction β subunits altered	cross-linked with ester intact	three-way cross-linked	cross-linked with ester hydrolyzed
1	1.00	77	5	18
2	0.55	0	62	38
3	0.83	39	27	34
4	0.89	66	21	13
5	0.00	0	0	0
6	0.00	0	0	0
7	0.00	0	0	0
8	0.00	0	0	0
9	0.10	0	57	43
10	0.00	0	0	0
11	0.00	0	0	0
12	0.00	0	0	0

[a] Yields are based on quantitative HPLC analysis of globin chains. Deoxyhemoglobin A was reacted with the reagents in 0.1 M sodium borate (pH 9.0), 37 °C.

Since each reagent contains three esters, the overall aminolysis reaction is kinetically complex. Preliminary measurements over the course of the whole reaction (formation of the tris amide) gave data that do not fit standard rate laws. It is likely that aminolysis of the first ester group gives an amide−diester product whose reactivity is different from that of the triester. Therefore we used initial rates to obtain the rate of the first reaction separately as the basis for analyzing reactivity.[12] Rates were measured for about 2% of the total reaction. The observed rate was statistically corrected to take into account that these reagents have three ester functions.

Results

We prepared substituted phenolic triesters of trimesic acid (**1**−**12**) and have evaluated their reaction patterns with human deoxyhemoglobin. Trimesyl tris(3,5-dibromosalicylate) (**1**) had previously been prepared.[2] It reacts very efficiently and selectively with amino groups in hemoglobin to produce a principal cross-linked product (linked between ϵ-amino groups of lysine-82 side chains in the β-subunits) with the third ester site either intact or hydrolyzed.[2,4] The general synthetic route for the triester analogues of **1** combines trimesoyl trichloride with an excess of the *tert*-butyl ester of a substituted phenol. The *tert*-butyl groups of the esters are then removed with trifluoroacetic acid. (The *tert*-butyl protecting group is necessary for the synthesis of **1**, and we extended the general route[2,13] in all cases.) The *tert*-butyl esters of the phenols are prepared by DCC coupling of the carboxylic acid and *tert*-butyl alcohol.[13] In one case (**8**), the synthesis of the *tert*-butyl ester precursor did not succeed by this procedure and a number of other standard methods also gave no product. In that case we were finally successful with a route in which the carboxylic acid is converted to the acid chloride, followed by reaction with potassium *tert*-butoxide.

The esters in **1** contain a carboxyl group ortho to a phenolic ester oxygen as well as two bromine atoms as aromatic substituents. It is an effective cross-linking reagent. It gives the highest yield of cross-linked hemo-

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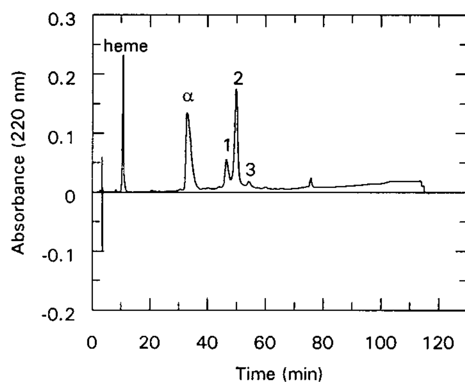

Figure 1. Chromatogram of the product mixture from the reaction between **1** and hemoglobin after 2 h in 0.1 M sodium borate, pH 9.0, 37 °C. Excess *n*-propylamine was added to the solution after 2 h to convert residual esters to amides.

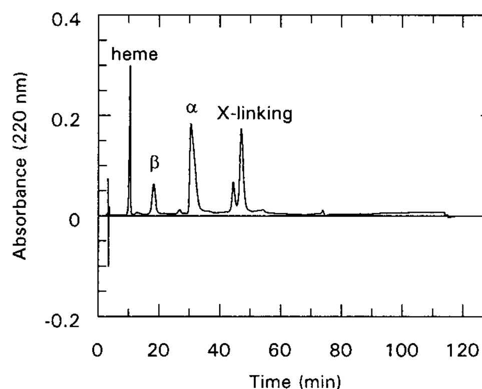

Figure 2. Chromatogram of the product mixture showing the reaction between the mixture of **1** and **5** (1:10 molar ratio, respectively) and hemoglobin after 2 h, in 0.1 M sodium borate, pH 9.0, 37 °C.

globin among the various trimesyl triesters we prepared. Table 1 summarizes the products obtained from reactions of the set of reagents with hemoglobin. Only the reagents with a leaving group containing a carboxyl ortho to the phenolic oxygen (**1**–**4**, **9**) produce a cross-link in hemoglobin. In the one case where there are carboxyls both ortho and para to the ester, cross-links are produced in very low yield (10%). The yield of cross-linked hemoglobin from the ortho-substituted carboxyl reagents is greatest for the reagent having two bromine substituents (**1**). The yield of cross-linked protein is lower with chlorine in place of bromine and lowest in compounds with no halogen present.

The reaction with **1** also gives a small amount of the product in which all three acyl groups react with the protein.[9] The other esters with ortho carboxyl groups give considerably higher proportions of this product. Reagents with carboxyl groups in meta and para positions give no cross-linked hemoglobin products. For these compounds we detect some acylation of amino groups in the β subunits only in cases where the leaving group contains halogens. The product in this instance has the two other esters hydrolyzed.

Figure 1 shows an HPLC analysis of the globin chains resulting from the reaction of hemoglobin with the trifunctional reagent (**1**). The α-globin chains are not modified, while all β chains are altered. The altered chains are identified as peaks: "1" is a combination of cross-linked β chains with the residual ester hydrolyzed. Peak "2" is a combination of cross-linked β chains with the residual ester converted to the *n*-propylamide. Peak "3" is the triply linked material with the cross-linked β chains connected at positions 82, 82, and 1. The nature of the modification was determined as described previously.[9]

Why do some of the reagents not produce a cross-link? Do they enter the reaction site without producing a cross-link, or do they fail to associate with the protein? Since we expect that the positive charge in the effector site of the protein will attract anions,[14] it is reasonable to expect that these anionic materials should be attracted. We tested this by adding **1**, which is an efficient cross-linking reagent, to a solution that contained an excess of the para carboxyl substituted material (**5**), which does not react with the protein. In the presence of excess **5** about 80% of the β subunits react with **1** (Figure 2). In the absence of **5** the β subunits would be completely modified. This suggests that **5** binds but does not react.

Table 2. Rate Constants for the Aminolysis and Hydrolysis of Triesters 1–12[a]

reagents	aminolysis, k_N ($M^{-1}s^{-1}$)	hydrolysis, k_{hyd} (s^{-1})	λ_{max} (nm)
1	3.0 ± 0.2	0.026 ± 0.005	315
2	0.054 ± 0.006	0.016 ± 0.002	300
3	0.12 ± 0.01	0.038 ± 0.003	300
4	1.35 ± 0.08	0.013 ± 0.004	300
5	0.13 ± 0.01	0.054 ± 0.004	300
6	0.07 ± 0.01	0.008 ± 0.004	275
7	0.09 ± 0.01	0.039 ± 0.003	267
8	0.17 ± 0.01	0.014 ± 0.002	312
9	0.16 ± 0.01	0.038 ± 0.002	298
10	not detected[b]	not detected[b]	307
11	0.16 ± 0.01	0.033 ± 0.004	328
12	0.099 ± 0.002	0.0150 ± 0.0007	295

[a] Errors are two standard deviations. [b] No hydrolysis or aminolysis was observed after 20 h.

To what extent are inherent differences in reactivity of **1**–**12** with amines manifested in their reactions with hemoglobin? As a basis for comparison we measured the rate of reaction of each with the small, basic amine, *n*-propylamine. Due to the kinetic complexity of reactions of trifunctional reagents, we measured initial rates of aminolysis so we could obtain the rate constant for amide formation from one ester group in each compound. The rate constants for hydrolysis and aminolysis of the trifunctional reagents, **1**–**12**, are summarized in Table 2. The results were obtained using initial rates to obtain observed first-order rate coefficients in the range 0.2–1.0 M *n*-propylamine (as buffer), pH 10.5, ionic strength 1.0 (with added sodium chloride), 25 °C. (Additional details are in the Experimental Section.)

The rate constants for aminolysis of reagents that have one carboxyl per leaving group are largest for those in which the carboxyl is para to the ester (**5**–**7**), followed by the ortho (**1**–**4**) and meta (**8**) derivatives. This order is consistent with the order of reactivity for aminolysis of substituted phenyl acetates[15] (relative rate constants at 25 °C: *p*-COOH, 1.8; *o*-COOH, 1.1; *m*-COOH, 0.6).

The reagents with an ortho carboxyl and two halogens on the aromatic leaving groups (**1** and **4**) are the most reactive toward *n*-propylamine, and these also react with hemoglobin. Two of the reagents that have two carboxyl

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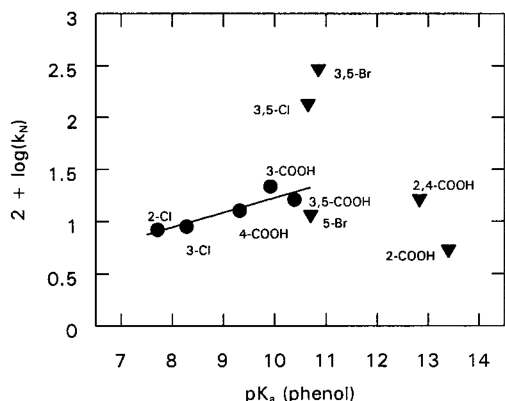


Figure 3. Rate constants for aminolysis vs the pK_a of the acids of the conjugate bases of the leaving groups of the trifunctional reagents, **1**−**10**, where ● are data for the trifunctional reagents with carboxylates meta and para to the ester and ▼ represents the trifunctional reagents with carboxylates ortho to the ester. Reagent **12** is not included in this plot because the necessary pK_a is not available and could not be determined. The acidity constants are from published tables or were estimated from Hammett plots.

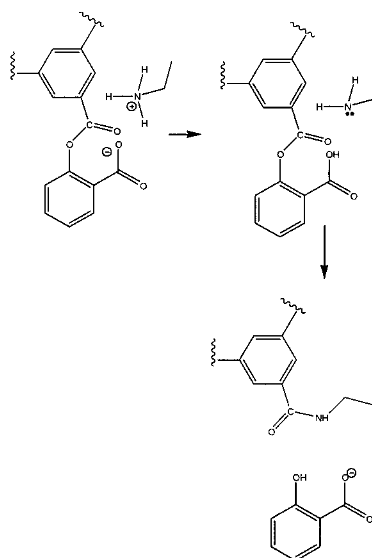
substituents (**9**, **11**) are more reactive toward *n*-propylamine than the reagents that have one carboxyl, but these are unreactive with hemoglobin. The derivative with two carboxyls ortho to the ester (**10**) is unreactive with *n*-propylamine (and does not react with hemoglobin). The low reactivity is consistent with steric effects in ester aminolysis.[16]

Discussion

The yields of cross-linked proteins from the reactions of amino groups in hemoglobin with **1**−**12** indicate the importance of the carboxyl group adjacent to the reacting ester in the reagent (present in **1**−**4**, **9**, and **10**). Acyl phosphate monoesters, which are anionic, acylate hemoglobin at the same sites as do the salicylate derivatives.[9,17] Since the position of the charged group relative to the reaction site is critical, it is possible that the carboxylate participates as a catalyst in the reaction with the protein. However, the reaction pattern is not consistent with what is known about reactions of small amines with salicylates. St. Pierre and Jencks[3] found that the reactions of salicyl esters with basic amines are *not* facilitated by the adjacent carboxyl. (The carboxyl functions as an acid to accelerate aminolysis by *weak* bases, such as hydroxylamine.) The conjugate base also serves as an intramolecular catalyst for the addition of water to the ester.[18]

We expect the reactions of *n*-propylamine with **1**−**12** to follow the normal patterns of ester aminolysis, subject to steric and electronic effects.[19] Figure 3 is a plot of the rate constant for aminolysis against the pK_a of the conjugate acid of the leaving group for 10 of the compounds. There is a linear correlation for five compounds with meta and para carboxyl substituents, giving β = 0.14. Compounds with an ortho carboxyl are *less* reactive than expected from the pK_a of the phenol, a typical steric effect.[19] It is clear that the ortho carboxyl group is not a catalyst in aminolysis. The small value of β for the correlated compounds is reasonable since the carbonyl group is stabilized by its own π system rather than by the phenyl substituents. We note that the rates for the compounds with two halogen substituents are above the correlation, suggesting an alternative mechanism may be involved.

Scheme 1



Our results show that the reactions of the triesters with *n*-propylamine are not sensitive to positions of the substituents on the ester, paralleling the reaction patterns of monosalicylates with amines.[3] This contrasts dramatically with the reactions of the triesters with amino groups in hemoglobin where there is a strong pattern of positional selectivity, with the ortho carboxyl being essential. Therefore, the control of reactions with hemoglobin is not based on the electrophilic reactivity of the reagent. Rather, it must be a consequence of the environment in the protein in relation to the geometry of the reagent. The amino groups of hemoglobin that are modified are in the site that binds the polyanionic effector, 2,3-diphosphoglycerate.[20] The site, between the β subunits, contains a number of basic amino groups, including those that react with the reagents.[2,21] The amino groups will be protonated to a significant extent. However, the amine is not permanently in the form of the ammonium ion, since the proton can be readily transferred to an acceptor.

A mechanistic diagram for the modification reaction is presented in Scheme 1:

A similar mechanism would also apply for modification reactions that use acyl phosphate monoesters, since in those compounds the anionic site and reaction site are adjacent. Since the reaction patterns of these compounds reflect the structure of the protein, it will be interesting to examine results of reactions of this set of materials with other proteins.

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