An ether-linked tetrafunctional acylating reagent and its cross-linking reactions with hemoglobin

Ronald Kluger, Krisztina Paal, and J. Gordon Adamson

Abstract: A new type of tetrafunctional reagent for cross-linking proteins has been prepared and used to modify human hemoglobin A. DPEE (1,2-bis{2-[3,5-bis(3,5-dibromosalicyloxycarbonyl) phenoxy]ethoxy}ethane)) has two separate pairs of reacting sites connected by a flexible tetraether chain. DPEE is capable of connecting a cross-link within a protein to another cross-link, either within the same protein molecule or between molecules. DPEE was readily prepared by esterification of a tetraether-linked bisphthalate (prepared by coupling of 1,2-bis(2-iodoethoxy)ethane and 5-hydroxyisophthalic acid). DPEE reacts with deoxy hemoglobin to produce a mixture of modified proteins. Ion-exchange HPLC was used to separate the modified proteins in the mixture. The most abundant products were selected for structural analysis, which used data from reverse-phase chromatography and tryptic peptide mapping. To prevent dissociation of the modified proteins during analysis, the products were further reacted with the bifunctional reagent, bis(3,5-dibromosalicyl) fumarate, which produces fumaryl cross-links between α-subunits. From peptide analysis of the separated products, the major modified protein from DPEE was identified as a novel species with four links within the same $\alpha_2\beta_2$ tetramer. In addition, a minor product that involves cross-links in two different proteins was observed. These results imply that the reagent reacts primarily in a folded state within the protein.

Key words: acylation: cross-linking, multifunctional, hemoglobin, reaction pattern.

Résumé : On a préparé un nouveau type de réactif tétrafonctionnel pour effectuer la réticulation des protéines et on l'a utilisé pour modifier de l'hémoglobine humaine A. Le DPEE (1,2-bis{2-[3,5-bis(3,5-dibromosalicyloxycarbonyl) phénoxy]éthoxy}éthane) possède deux paires séparées de sites réactionnels liés par une chaîne tétraéther flexible. Le DPEE peut relier une réticulation à l'intérieur d'une protéine avec une autre réticulation de la même molécule de protéine ou entre des molécules. On peut facilement préparer le DPEE par estérification d'un bisphtalate relié à un tétraéther (préparé par couplage du 1,2-bis(2-iodoéthoxy)éthane et de l'acide 5-hydroxy-isophtalique). Le DPEE réagit avec la désoxyhémoglobine pour produire un mélange de protéines modifiées. On a fait appel à la CLHP par échange d'ions pour séparer les protéines modifiées présentes dans le mélange. On a choisi les produits les plus abondants pour réaliser une analyse structurale en utilisant des données obtenues par chromatographie en phase inversée et par cartographie d'un peptide tryptique. Dans le but d'empêcher la dissociation des protéines modifiées au cours de l'analyse, on a fait réagir les produits avec le réactif bifonctionnel, fumarate de bis(3,5-dibromosalicyle) qui produit des réticulations fumaryles entre les sous-unités α. Sur la base de l'analyse peptidique des produits séparés, on a déterminé que la protéine principale découlant de la modification par le DPEE est une nouvelle espèce comportant quatre liaisons à l'intérieur du même tétramère $\alpha_2\beta_2$. On a aussi observé la présence d'un produit mineur qui comporte des réticulations dans deux protéines différentes. Ces résultats impliquent que le réactif agit principalement dans un état replié de la protéine.

Mots clés : acylation : réticulation, multifonctionnel, hémoglobine, patron de réaction.

[Traduit par la Rédaction]

Introduction

Human hemoglobin A is an $\alpha_2\beta_2$ tetramer that reversibly dissociates into αβ dimers (1, 2). Cross-linking prevents dissociation into dimers while providing the possibility for systematic alteration of the protein's properties (3-8). Cross-linked hemoglobins are of special interest since they may have clinical significance as red cell substitutes in transfusion medicine (9–11). A minimal reagent that prevents hemoglobin from dissociating into dimers contains two reactive functional groups that create a cross-link (12–14). The properties of the resulting altered protein depend on the sites that are connected and the structure of the link (7, 14–18). Reagents with three reactive functional groups can produce a cross-link between two sites within a protein, leaving a functional group available for bioconjugation (19). Alternatively, all three groups can react within the protein, creating a novel cross-linked structure (7, 20, 21).

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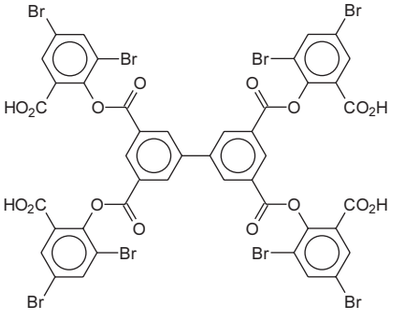
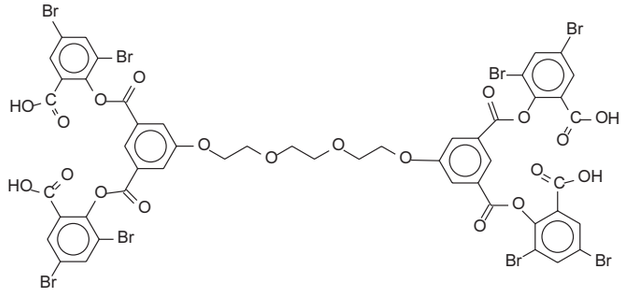
Reagents with four reactive centers present additional possibilities for creating novel modified proteins (22, 23). A reagent that has two pairs of connected reactive sites can produce two cross-links that are connected by the core of the reagent. Depending on the geometry of the core, the two cross-links can form within one protein molecule or in two separate molecules, resulting in modified-connected proteins. If one or two functional groups of the reagent remain intact in the reaction with the protein these sites can be utilized for further conjungation reactions.

We have reported that a relatively small reagent with four reactive groups, BTDS (3,5,3′,5′-biphenyltetracarbonyl tetrakis(3,5-dibromosalicylate)), reacts with deoxy hemoglobin to give principally a product that has a stable cross-link between the β-subunits. The two additional reactive groups of the reagent do not combine with the protein (22). It is likely that once reaction has occurred at two of the groups, the geometric relationship of the remaining groups prevents them from being in proximity to nucleophiles in the same protein or in a second protein.



BTDS

To arrive at new types of altered proteins from a reagent with four reactive groups, we therefore assumed that a greater separation of the groups is necessary. In this paper we report the synthesis of DPEE (1,2-bis{2-[3,5-bis(3,5-dibromosalicyloxycarbonyl) phenoxy]ethoxy}ethane). DPEE contains isophthalyl dibromosalicylate reaction sites, as in BTDS, but they are connected in a longer, more flexible, and more hydrophilic array.



DPEE

Chemically modified hemoglobins are readily amenable to ion spray mass spectral analysis (21, 24, 25). Combining the results of such an analysis for separated materials with results from size-exclusion chromatography and tryptic peptide mapping (26) enables us to propose structures for the major modified proteins that result from reaction of DPEE and hemoglobin.

Experimental

Commercial reagents and chemicals were used without further purification. Solvents were dried prior to use. Reagents for preparation of buffers were analytical grade or better. Purified human hemoglobin A was from Hemosol, Inc. Proton NMR spectra were obtained at 400 MHz or 200 MHz and ^{13}C NMR spectra at 100 MHz.

The structures of the materials associated with the synthesis of the DPEE are shown in Schemes 1 and 2 in the Results section. The synthesis of the linked esters follows the general procedure of Collman et al. (4).

Synthesis of 1,2-bis{2-[3,5-bis(ethoxycarbonyl) phenoxy]-ethoxy}ethane (2)

Diethyl 5-hydroxyisophthalate (0.95 g, 0.0040 mol) and potassium *tert*-butoxide (0.47 g, 0.0042 mol) were stirred in 20 mL anhydrous tetrahydrofuran at room temperature for 30 min. A solution of 0.74 g (0.0020 mol) of 1,2-bis(2-iodoethoxy)ethane (**1**) in 20 mL anhydrous tetrahydrofuran was added and the reaction mixture was refluxed for 48 h under nitrogen. Water was added and the product was extracted with ether. The solution was dried over magnesium sulfate, filtered, and the solvent removed. Separation on a silica column (3 cm × 30 cm, hexanes – ethyl acetate 1:1) produced a white powder (0.60 g, 51%). ^1H NMR ($CDCl_3$) δ: 8.25 (2 H, t, J = 1.2 Hz, ArH), 7.74 (4 H, d, J = 1.2 Hz, ArH), 4.36 (8 H, q, J = 6.9 Hz, CH_2), 4.20 (4 H, t, J = 5.6 Hz, CH_2), 3.87 (4 H, t, J = 5.6 Hz, CH_2), 3.75 (4 H, s, CH_2), 1.36 (12 H, t, J = 6.9 Hz, CH_3).

1,2-Bis[2-(3,5-dicarboxyphenoxy) ethoxy]ethane (3)

The tetraethyl ester (0.60 g, 0.0010 mol, **2**) was dissolved in 30 mL ethanol. The solution was heated to 65°C. Then 0.25 g (0.0060 mol) sodium hydroxide dissolved in 1 mL water was added and the reaction mixture kept at 65°C for 22 h. The resulting white precipitate was filtered and dissolved in 10 mL of water. The tetraacid (**3**) was precipitated by addition of concentrated hydrochloric acid and filtered. The product was dried under vacuum (0.46 g, 95%). ^1H NMR (DMSO-d_6) δ: 8.05 (2 H, s, ArH), 7.62 (4 H, s, ArH), 4.19 (4 H, s, CH_2), 3.75 (4 H, s, CH_2), 3.60 (4 H, s, CH_2); a broad band is also present under the three high-field signals.

1,2-Bis{2-[3,5-bis(chlorocarbonyl) phenoxy]ethoxy}-ethane (4)

The tetraacid (0.23 g, 0.0047 mol, **3**) was dissolved in 15 mL thionyl chloride containing three drops of dimethylformamide. The solution was refluxed under nitrogen for 5 h and then the solvent was evaporated. The product (yellow oil) was dried under vacuum and solidified overnight (0.26 g, 100%). ^1H NMR ($CDCl_3$) δ: 8.43 (2 H, t, J = 1.6 Hz, ArH), 7.90 (4 H, d, J = 1.6 Hz, ArH), 4.24 (4 H, t, J = 5 Hz, CH_2), 3.89 (4 H, t, J = 5 Hz, CH_2), 3.74 (4 H, s, CH_2). This tetraacid tetrachloride was used without further purification.

1,2-Bis{2-[3,5-bis(3,5-dibromosalicyloxycarbonyl) phenoxy]ethoxy}ethane (DPEE, 6)

tert-Butyl 3,5-dibromosalicylate (0.66 g, 0.0019 mol) (27, 28) and potassium *tert*-butoxide (0.21 g, 0.0019 mol) were stirred in 15 mL anhydrous tetrahydrofuran at room temperature for 30 min. Then a solution of 0.26 g (0.00047 mol) of **4** in 15 mL anhydrous tetrahydrofuran was added and the reaction mixture was stirred at room temperature overnight. Water was added and the product was extracted with ether. The extract was dried over magnesium sulfate, filtered, and the solvent removed, leaving the tetra *tert*-butyl ester (**5**). The white solid was dissolved in 15 mL anhydrous trifluoroacetic acid (to convert the *tert*-butyl ester to the free acid) and left at room temperature for 2.5 h. Dry ether was added (15 mL) and a white precipitate formed. The product (**6)**, DPEE, was filtered and dried in vacuum (white powder, 0.55 g, 74%). IR (KBr): 3177 (br s, ν_{OH}), 1736 (s, $\nu_{C=O}$), 1195 (s, $\nu_{C—O}$) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.36 (2 H, t, J = 1.4 Hz, ArH), 8.33 (4 H, d, J = 2.5 Hz, ArH), 8.09 (4 H, d, J = 2.5 Hz, ArH), 7.98 (4 H, d, J = 1.4 Hz, ArH), 4.31 (4 H, br s, CH_2), 3.79 (4 H, br s, CH_2), 3.63 (4 H, s, CH_2); a broad band is also present under the three high-field signals; ^{13}C NMR (acetone-d_6) δ: 163.57, 163.25, 160.59, 148.36, 140.13, 134.68, 131.79, 128.06, 124.54, 122.12, 120.29, 120.06, 71.50, 70.29, 69.47; MS (negative FAB), M – 1 calcd. for $C_{50}H_{30}Br_8O_{20}$: 1589; found: 1589.

Reactions of human hemoglobin A with DPEE were carried out as described previously (22). A typical example is described here. A solution of carbonmonoxy hemoglobin (2.0 mL, 1.25 mM, 0.000025 M) in 50 mM Bis-Tris buffer (pH 6.5) was passed through a Sephadex G-25 column (2.5 × 15 cm) equilibrated with 50 mM sodium borate buffer (pH 8.0). The carbonmonoxy hemoglobin was converted to oxy hemoglobin by irradiation under flowing oxygen at 0°C for 2 h. The solution was then kept under flowing nitrogen at 37°C for 2 h to convert the hemoglobin to the deoxy form. DPEE (0.0080 g, 5.0×10^{-6} mol) was dissolved in 1.0 mL of dioxane, and 3.0 mL of 50 mM sodium borate buffer (pH 8.0) was added. Oxygen was removed from the solution by evacuation followed by addition of nitrogen. This was repeated three times. The material was then added under nitrogen to the deoxy hemoglobin solution. The reaction mixture was kept at 37°C for 20 h with humidified nitrogen flowing through the rotating flask. The flask was then flushed with carbon monoxide. The solution of modified carbonmonoxy hemoglobin was passed through a Sephadex G-25 column (2.5 × 15 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7.5) to remove residual reagent. (The resulting material is referred to as DPEE-Hb.) C-4 reverse-phase HPLC columns were used to separate heme and the various modified and native globin chains under denaturing conditions. Hemoglobins of different molecular weights were resolved using gel filtration chromatography, both with high concentrations of magnesium salt that lead to dissociation into $\alpha\beta$ dimers (29) as well as under nondissociating conditions where the tetramers remain intact.

Analysis of modified hemoglobin

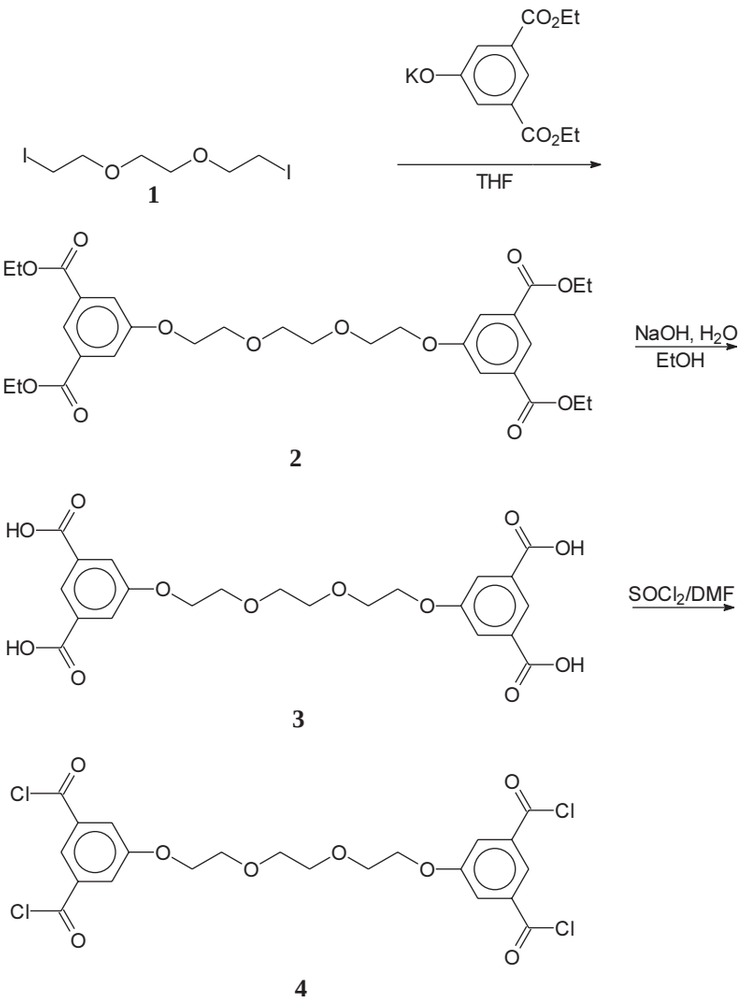
General procedures for structural analysis were as previously reported (22). The product mixture was separated on a C-4 reverse-phase HPLC column. In addition to known peaks corresponding to heme and native globin chains, new peaks appeared that correspond to cross-linked globin chains. The molecular weights of these materials were determined by ion spray mass spectrometry (30, 31). The sites of modification were deduced by comparison of peptide patterns (reverse-phase HPLC) from digestion of native and modified chains with trypsin and endoproteinase Glu-C (14, 22, 32–34) along with ion spray mass spectral results as has been described in detail for other cross-linked hemoglobins (24, 27). Reference data for weights and mass spectra of native globin chains are conveniently summarized in the work of Adamczyk and Gebler (25). Size exclusion chromatography was used to distinguish materials by their approximate molecular weights (35). Columns were calibrated with cross-linked and oligomerized hemoglobin.

The formation of a cross-link between α-subunits of DPEE-Hb with fumaryl bis(3,5-dibromosalicylate) was accomplished by the procedure described for production of doubly cross-linked hemoglobin (14). After the 20 h incubation period of deoxy hemoglobin with DPEE (described above), a deoxygenated solution of fumaryl bis(3,5-dibromosalicylate) (0.0035 g, 5.0×10^{-6} mol) in 4.0 mL 50 mM sodium borate buffer (pH 8.0) was added to the hemoglobin reaction solution under nitrogen. (This reagent should introduce a cross-link between the ε-amino groups of the α-99 lysyl residues of hemoglobin (14, 16). It is effective if the sites in the β-subunits that make up the DPG-binding site (3, 36) are blocked.) The mixture was kept at 37°C for 2 h and then the flask was flushed with carbon monoxide. The solution of twice-modified carbonmonoxy hemoglobin was passed through a Sephadex G-25 column (2.5 × 15 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7.5) in order to remove residual reagent. (The resulting material is referred to as XL2-Hb.) C-4 reverse-phase HPLC columns were used to separate the hemes and the globin chains. In another set of analyses of XL2-Hb, gel filtration chromatography was used to separate modified hemoglobins by their gross molecular weight (size). We used size-exclusion columns under conditions that dissociate the $\alpha\beta$ dimers from hemoglobin that is not cross-linked between these dimers, and also used conditions under which hemoglobin remains tetrameric ("nondissociating conditions"). Elution was under isocratic conditions. The nondissociating eluent was 0.050 M pH 7.0 sodium phosphate with 0.15 M sodium chloride flowing through a PSEC-12 column at 0.5 mL min^{-1}. The dissociating eluent was 0.025 M pH 7.2 Tris with 0.5 M magnesium chloride flowing through a TSK G2000 swxl column at 0.2 mL min^{-1}.

Results

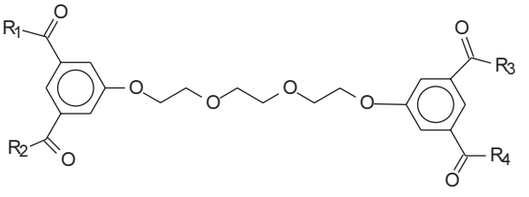
The synthesis of DPEE efficiently introduces the necessary paired reactive functional groups for cross-linking onto a flexible linking chain. The route could be extended to produce a reagent library by varying the length of the polyether linker. The synthesis of the ether is based on Collman's method for producing a tetraether-linked tetraester (4). The reactive groups are conveniently introduced by Klotz's procedure for forming dibromosalicylates (28) (Schemes 1 and 2).

Scheme 1.



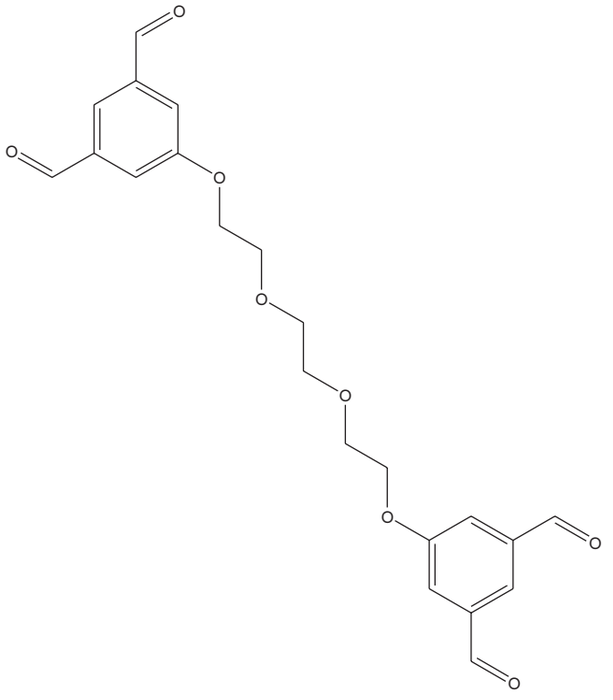
DPEE reacts with deoxy hemoglobin but not with carbonmonoxy hemoglobin, based on analysis of the resulting globin chains by C-4 reverse-phase HPLC. This is consistent with reaction occurring at the binding site for DPG in hemoglobin, which is more accessible in the deoxy form (37). The observed product pattern of the reaction remained constant as the ratio of the reagent to hemoglobin was varied from 1:2 to 2:1 and temperature was changed from 37 to 50°C. The reaction mixture appears to contain a considerable variety of altered protein products. The relative amounts were estimated based on peak areas in the chromatograms. We chose the materials in the largest peaks for structural analysis.

The heme and globin chains were separated on a C-4 reverse-phase HPLC column (see Fig. 1). The products in the largest peaks were detected and their molecular weights were determined from the parent peaks of ion spray mass spectra. Table 1 summarizes the ion spray mass spectral assignments associated with major peaks that were observed on reverse-phase HPLC analysis. The product from the largest chromatographic peak (**7**) is a tetraamide formed by acylation at four amino groups of two β-subunits. Products from other peaks retain one or more 3,5-dibromosalicyl (DBS) esters from DPEE or are partially hydrolyzed. Structures consistent with the observed masses are indicated as substituted derivatives of the core structure below:



7–13

The mass of the linked globin chain that is proposed as the structure of the major product (**7**) is consistent with the electrospray mass spectral peak. This is a dimer of two β-subunits linked by four amide connections derived from the core of DPEE (**14**), which has a molecular mass of 410. The molecular mass of a β-subunit is 15 868 and that of the α-subunit is 15 126. Combining the masses of two β-subunits and **14**, less the mass of hydrogens that are dissociated from



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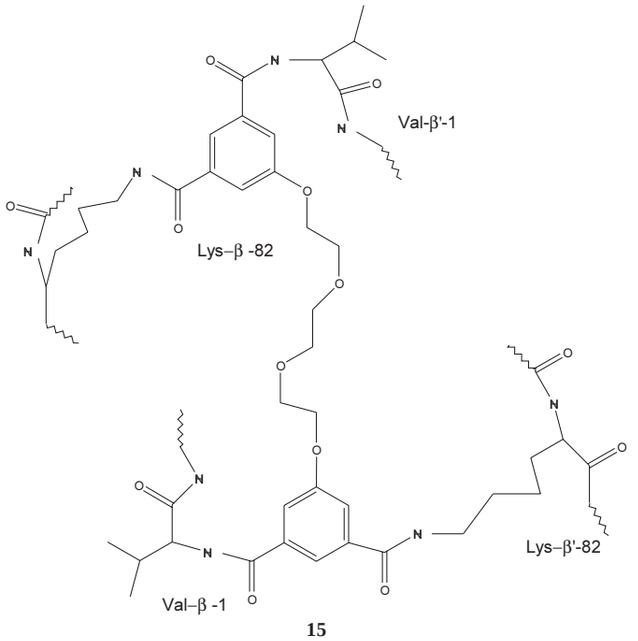

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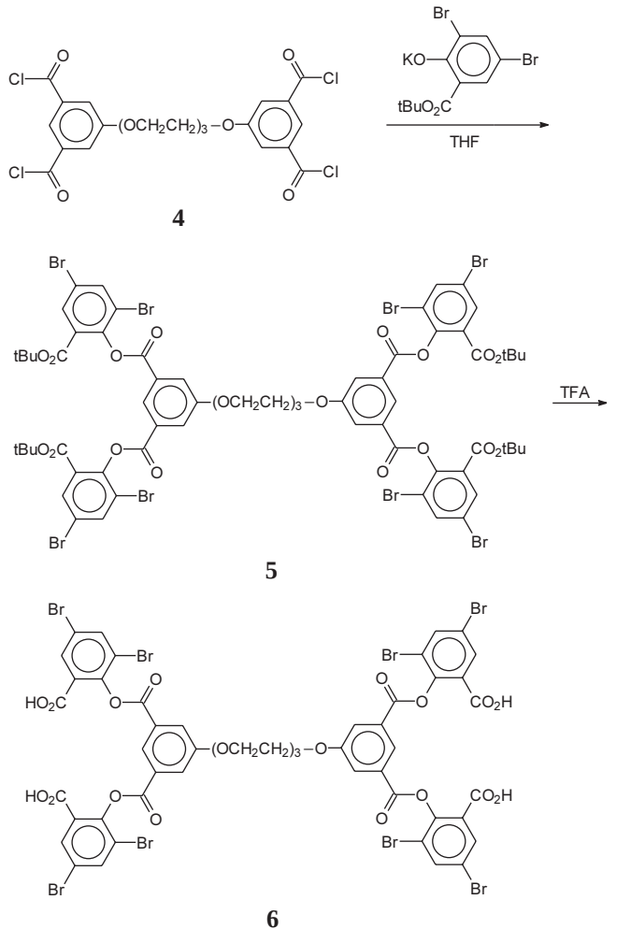
each resulting amide, gives a predicted mass of 32 142 for **7**. This is consistent with what is observed.

Scheme 2.



4

5

6

To determine the residues within hemoglobin that are modified by DPEE in **7**, the material from the peak corresponding to the cross-linked β-subunits was subject to tryptic digestion and peptide mapping. The products of digestion were separated by C-18 reverse-phase HPLC and the peptides were compared to those from digestion of the unmodified β chain (26). Three fragments of the native β digest were absent from that of the modified β-subunits: β-T1, β-T9, and β-T10a′. β-T1 is the amino terminus (Val-β-1) and β-T9 is connected to β-T10a′ through the β-Lys-82 residue, indicating that the amino termini of both β-subunits as well as the ε-amino groups of both β-Lys-82 residues are acylated by DPEE (Fig. 2). (The other peaks listed in Table 1 represent smaller quantities and the structural assignments are based on masses, with attachment sites based on the consistent patterns of modification seen with other reagents with similar functionality. Digests were not performed for these.) The results for **7** indicate that there are two sets of cross-links connecting positions 1 and 82 of β-subunits introduced by a single tetrafunctional reagent (**15**).

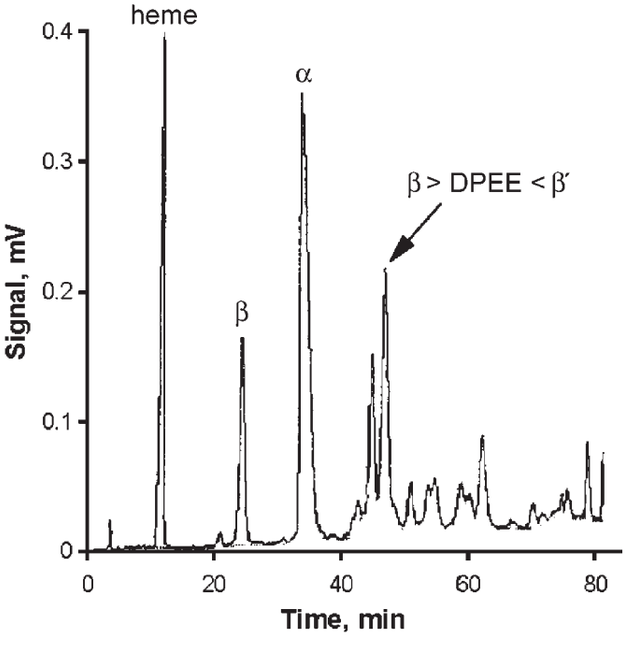
Formation of bis tetramers

The four functional groups of DPEE are arranged so that it is possible for them to produce a connection between tetramers. If we assume that reaction occurs at positions 1 or

Table 1. Ion spray mass spectral assignments for altered globin chains derived from the reaction of DPEE with hemoglobin. The structures are indicated based on the core structure above connected to hemoglobin β-subunits at the indicated amino group. A hydrolyzed site is indicated as "OH" and an intact ester as "DBS". Mass assignments for the native peaks are based on literature summations (25). The structure **12** is listed as two possibilities where either ester group from DPEE has undergone hydrolysis.

Mass (found)	Mass (calcd.)	Label	R_1	R_2	R_3	R_4
32 140	32 142	**7**	NH(ε)-β-Lys-82	NH(α)-β′-Val-1	NH(ε)-β′-Lys-82	NH(α)-β-Val-1
32 163	32 160	**8**	NH(ε)-β-Lys-82	NH(α)-β′-Val-1	NH(ε)-β′-Lys-82	OH
16 421	16 420	**9**	NH(ε)-α-Lys-99	DBS	DBS	DBS
30 971	30 972	**10**	NH(ε)-α-Lys-99	NH(ε)-α′-Lys-99	DBS	OH
31 250	31 250	**11**	NH(ε)-α-Lys-99	NH(ε)-α′-Lys-99	DBS	DBS
31 713	31 714	**12a**	NH(ε)-β-Lys-82	DBS	NH(ε)-α′-Lys-99	OH
31 713	31 714	**12b**	NH(ε)-β-Lys-82	OH	NH(ε)-α′-Lys-99	DBS
31 444	31 436	**13**	NH(ε)-β-Lys-82	OH	NH(ε)-α′-Lys-99	OH

Fig. 1. Globin chain separation after reaction of deoxy hemoglobin with DPEE: 20 h at pH 8.0 and 37°C



82 of β-subunits, and the product is symmetrical, schematic structures **16** or **17** are possible. To determine whether any bis tetramer formed, we analyzed the reaction solutions on size exclusion columns that give an indication of mass based on calibrated globular size. Since hemoglobin tetramers (64 000 Da) are in equilibrium with αβ dimers (32 000 Da) (38), cross-linking between two β-subunits or between two α-subunits prevents dissociation while a link within a subunit does not. Addition of magnesium chloride to the solution influences the equilibrium toward dissociation into dimers (29). Thus, **16** will dissociate into dimers while **17** will not.

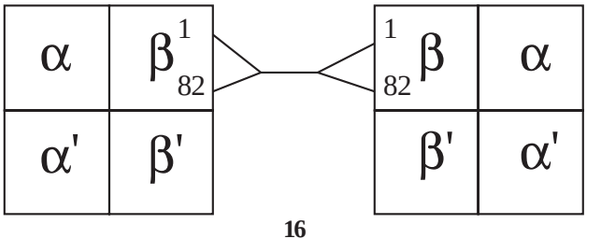
Hemoglobins that are doubly cross-linked, with a link in between α- as well as β-subunits, can be prepared by addition of the reagent bis(3,5-dibromosalicyl)fumarate (DBBF), to a ββ-cross-linked hemoglobin (14). Thus when the α-subunits of **16** are subsequently cross-linked by reaction with DBBF, the tetrameric protein will no longer dissociate into dimers. In the possible cross-linked tetrameric structures, shown as **18** and **19,** reaction with all four sites of DPEE

Table 2. Expected masses (kDa) of peaks of modified hemoglobins. Addition of 0.5 M magnesium chloride causes dimers that are not cross-linked to separate from tetramer. See text for details (indicated as "dissociating").

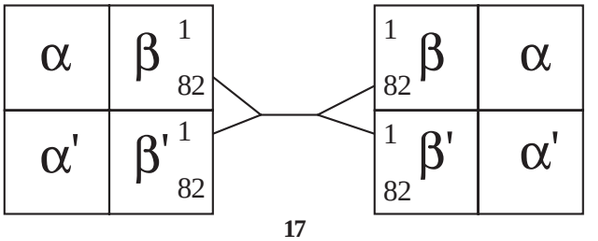
Structure	Mass	Mass (dissociating)	Mass (dissociating) after formation of α–α cross-link
16	128	64, 32	128
17	128	128	128
18	64	64	64
19	64	64	64

will give material that does not dissociate into dimers. Therefore, introducing the α–α cross-link should not change the effect of adding magnesium chloride. These possibilities are summarized in Table 2.

We note that α–α cross-linking permits separations that



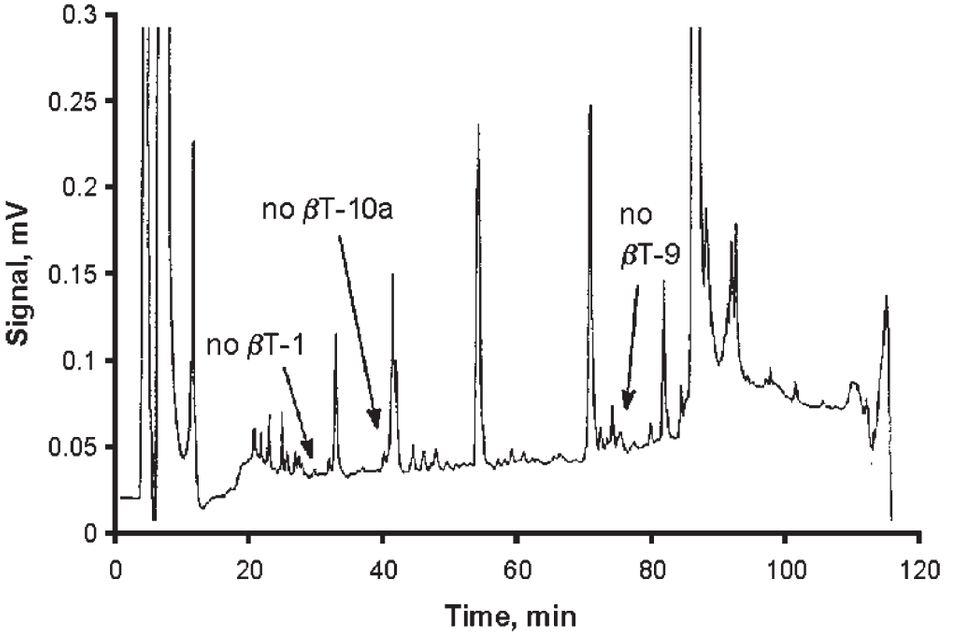
16



17

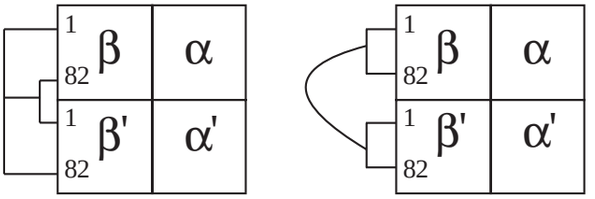
distinguish **16** from **17**. Structure **17** has cross-links between β-subunits, preventing dissociation into dimers. Cross-linking between the α-subunits does not change the results for dissociation. Therefore, formation of **17** should not change the apparent product–mass distribution. However, two of the four αβ dimers of **16** can dissociate. If an α–α cross-link is

Fig. 2. Tryptic-Glu-C peptides of the β-subunits linked at four sites by DPEE. Comparison is noted against native protein digest.



introduced, there will be a decrease in the amount of 32K material present.

Structures **18** and **19** are both capable of generating the major product. Both are fully cross-linked and will not dissociate into dimers. Both have a molecular mass of 64K. Both have all the β-1 and β-82 amino groups converted to amides.



18

19

Separation of the globin chains by C-4 reverse-phase HPLC, before and after treatment of the reaction product with DBBF, shows that the reagent completely converts all native α-subunits in the product solution. The results of analysis by size exclusion chromatography are given in Table 3.

Discussion

DPEE appears to react with amino groups in the β-subunits of deoxyhemoglobin within its DPG-binding site as do other 3,5-dibromosalicylate esters. However, unlike BTDS, which reacts at only two of its four ester sites, major products include species in which all four esters are converted to amide derivatives of hemoglobin. The reaction of DPEE with deoxy hemoglobin gives one major product (>50%) and several minor products, including β–β, α–β, and α–α cross-linked products. Even after a long reaction period, considerable amounts of native protein subunits are found on reverse-phase HPLC analysis. The rates of the reactions between hemoglobin and DPEE are considerably slower than with other reagents that have the same dibromosalicyl leaving groups on aromatic hydrocarbon cores (trimesyl and biphenyl) (22, 27). These cross-linkers readily react with hemoglobin and reactions go to completion within a few hours. It is very likely that the slow reaction of DPEE would be the result of the hydrophobic end groups of DPEE stacking on one another, blocking its accessibility, and controlling its reaction patterns.

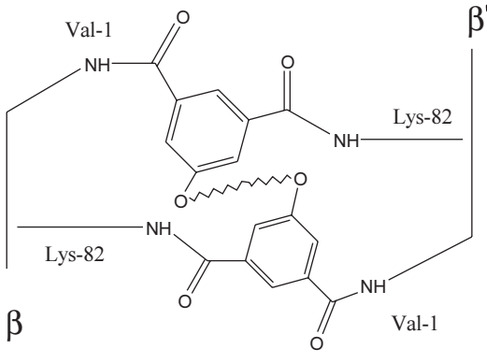
Our data show that the major product is a β–β′ cross-linked tetraamide containing one tetraacyl unit derived from DPEE. Several different modified hemoglobins could give data consistent with our results. From the mass and tryptic digestion analyses we know that cross-links are only between β-subunits, and all four amino groups from β-Val-1 (N-terminal α-amino group) and β-Lys-82 (ε-amino group) of both chains must be acylated. From the reaction patterns of other reagents, we know that the acyl groups on *meta* positions of a substituted benzene can connect ε-amino groups of the lysyl residues at position 82 of each β-subunit. They also can connect the α-amino group of the N-terminal valine of one β-subunit and the ε-amino group of Lys-82 of the other β-subunit. The same amino groups can also be connected within the same subunit. The β-Val-1 amino groups are too far apart to be connected by the *meta*-substituent groups.

If one end of DPEE has reacted between amino groups of Val-1 and Lys-82 on different β-subunits, then the other end must react with the alternative Val-1 and Lys-82 amino groups (**18**). The schematic diagrams for **20** and **21** (for **18** and **19**) are shown in terms of functional groups (following page).

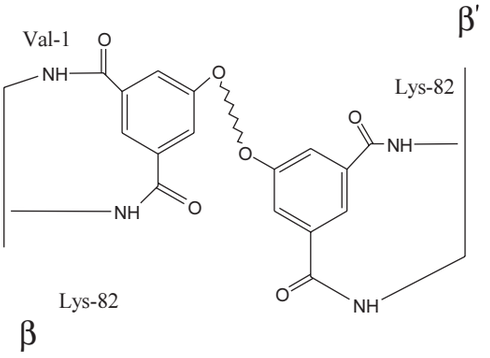
The alternative products (**19** and **21**)) have the *meta*-substituted dicarbonyls from one end of DPEE connected as amides on the same β-subunit to Val-1 and Lys-82. The other end of DPEE is then connected to the other β-subunit in the

Table 3. Size exclusion chromatography analysis of relative amounts of various modified hemoglobins (DPEE-Hb) and those that have been reacted further to cross-link their α-subunits (HBXL2). Results are for distribution of relative amounts, according to their mass. Dissociating conditions refer to the presence of 0.5 M magnesium chloride.

Hemoglobin	Conditions	% ≥128 kDa	% 64 kDa	% 32 kDa
Native (human)	Dissociating	0.7	15.3	82.0
DPEE-Hb	Dissociating	2.5	89.3	8.2
DPEE-Hb	Nondissociating	5.7	87.7	6.6
HBXL2	Dissociating	5.2	82.4	12.4
HBXL2	Nondissociating	6.1	86.3	7.6



20



21

same manner. Based on the reaction patterns of other isophthalyl-DBS esters, we know that the ε-amino group of a β-Lys-82 is most likely to be converted to an amide. Since the α-amino groups of each N-terminal β-Val-1 are also modified in the product, and the *meta*-substituted reagent cannot span the distance between the two N-termini, these must react more quickly than does the other β-Lys-82 ε-amino group with the same end of DPEE. The reagent must arrange itself within the DPG binding site in order to achieve this selectivity, perhaps directed by hydrogen-bonding interactions.

The data in Table 3 show that a portion of the reaction products consists of two connected β-subunits, derived from different tetramers, producing a bis tetramer (mass: 128 kDa). Such a species of connected tetramers should be of interest for studies of cooperativity (1) as well as for analysis of physiological effects. Approximately half of the 128 kDa material found in DPEE-Hb under nondissociating conditions is converted to 64 and 32 kDa species under dissociating conditions (Table 3). This scenario is consistent with structure **16** (Table 2). The portion of 128 kDa species that is present under dissociating conditions could be explained by structure **17**, which should be stable under such conditions.

The introduction of cross-links between α-subunits holds the tetrameric structure together regardless of whether cross-linking has occurred between the β-subunits (14, 18). Stabilization of DPEE-Hb by α–α cross-linking, giving HBXL2, makes the 128 kDa species stable under dissociating conditions. These findings are also consistent with structure **16**. In such a case, the reagent would have had to join Val-1 and Lys-82 on the same subunit within each tetramer. Structure **17** remains intact under dissociating conditions, requiring that the cross-linking of each tetramer be between different β-subunits. The results from size exclusion chromatography confirm that at least one of the minor but significant products is a bis tetramer.

While these assignments are self-consistent, we know that other difunctional *meta*-substituted DBS reagents react between the ε-amino groups of each of the β-Lys-82 residues only (14, 22, 27, 33). The formation of such a product within one tetramer would require that the two β-Val-1 amino groups be connected at *meta* substituents. This is most unlikely since the distance between the amino termini of the two β chains of hemoglobin is too great (18.4 Å in the deoxy and 19.9 Å in the oxy conformation) (37) to be spanned by the isophthalyl group. No reagent that we have examined, even with a larger span (32), connects the N-termini of two β-subunits.

The slow rate of the reaction of DPEE with deoxyhemoglobin is consistent with either of the four-way cross-linked tetramers (**18** and **19**) being the product. Since movement of the bulky reagent inside the DPG binding site is likely to be restricted, it should be in a folded conformation as it enters the cavity. In addition, there is probably a limited orientation by which DPEE can get into the DPG site, once it is in an appropriate overall conformation.

Conclusion

The reaction of DPEE with deoxy hemoglobin is slower than that of smaller reagents, suggesting that the reagent is less accessible to the protein, presumably because it exists in a folded state. This has consequences in the reaction pattern as well. The major product from the reaction of DPEE with hemoglobin has four amino groups of the $\alpha_2\beta_2$ tetramer, two from each β-subunit, modified and cross-linked by a single molecule of reagent. In contrast, the rigid reagent derived from a symmetrical biphenyl tetracarboxylate (BTDS), which is unable to fold onto itself, reacts with hemoglobin primarily at only two sites in any modification process. The "knotted" species from the DPEE reaction may present an

interesting basis for studies of the protein's structural dynamics. Our results show that DPEE is sufficiently long to produce bis tetramers but the product is not present to a large extent, consistent with a predominant folded state of the reagent. A long but rigid reagent will give such products in greater proportion.

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References

1. M. Perutz. Nature (London), **228**, 1 (1970).
2. R.E. Dickerson and I. Geis. Hemoglobin: structure, function, evolution, and pathology. The Benjamin/Cummings Publishing Co., Menlo Park, Calif. 1983. p. 1.
3. A. Arnone, R.E. Benesch, and R. Benesch. J. Mol. Biol. **115**, 627 (1977).
4. J.P. Collman, J.I. Brauman, J.P. Fitzgerald, P.D. Hampton, Y. Naruta, J.W. Sparapany, and J.A. Ibers. J. Am. Chem. Soc. **110**, 3477 (1988).
5. S. Miura and C. Ho. Biochemistry, **23**, 2492 (1984).
6. R. Kluger, R.T. Jones, and D.T. Shih. Artif. Cells Blood Substitutes Immobilization Biotechnol. **22**, 415 (1994).
7. M.A. Schumacher, M.M. Dixon, R. Kluger, R.T. Jones, and R.G. Brennan. Nature (London), **375**, 84 (1995).
8. M.A. Schumacher, E.E. Zheleznova, K.S. Poundstone, R. Kluger, R.T. Jones, and R.G. Brennan. Proc. Natl. Acad. Sci. U.S.A. **94**, 7841 (1997).
9. R.M. Winslow. Hemoglobin-based red cell substitutes. Johns Hopkins University Press, Baltimore, Md. 1995.
10. M. Jing, F.G. Panico, J.L. Panico, M.A. Ledvina, S. Bina, and S.M. Muldoon. Artif. Cells Blood Substitutes Immobilization Biotechnol. **24**, 621 (1996).
11. S.M. Cohn and T.J. Farrell. J. Trauma, **39**, 210 (1995).
12. K. Kikugawa, K. Adachi, H. Kosugi, and T. Asakura. Hemoglobin, **7**, 533 (1983).
13. I.M. Klotz, D.N. Haney, and L.E. Wood. J. Biol. Chem. **260**, 16215 (1985).
14. R.T. Jones, D.T. Shih, T.S. Fujita, Y. Song, H. Xiao, C. Head, and R. Kluger. J. Biol. Chem. **271**, 675 (1996).
15. E. Bucci, A. Razynska, H. Kwansa, B. Matheson-Urbaitis, M. O'Hearne, J.A. Ulatowski, and R.C. Koehler. J. Lab. Clin. Med. **128**, 146 (1996).
16. J.A. Walder, R.H. Zaugg, R.Y. Walder, J.M. Steele, and I.M. Klotz. Biochemistry, **18**, 4265 (1979).
17. Y. Zheng and K.W. Olsen. Artif. Cells Blood Substitutes Immobilization Biotechnol. **24**, 587 (1996).
18. S.R. Snyder, E.V. Welty, R.Y. Walder, L.A. Williams, and J.A. Walder. Proc. Natl. Acad. Sci. U.S.A. **84**, 7280 (1987).
19. R. Kluger and Y. Song. J. Org. Chem. **59**, 733 (1994).
20. K.W. Olsen, Q.Y. Zhang, H. Huang, G.K. Sabaliauskas, and T. Yang. Biomater. Artif. Cells Immobilization Biotechnol. **20**, 283 (1992).
21. R. Kluger, J. Wodzinska, R.T. Jones, C. Head, T.S. Fujita, and D.T. Shih. Biochemistry, **31**, 7551 (1992).
22. K. Paal, R.T. Jones, and R. Kluger, J. Am. Chem. Soc. **118**, 10380 (1996).
23. P.A. Pavlik, M.K. Boyd, and K.W. Olsen. Biopolymers, **39**, 615 (1996).
24. Z. Yu, G. Friso, J.J. Miranda, M.J. Patel, T. Lo-Tseng, E.G. Moore, and A.L. Burlingame. Protein Sci. **6**, 2568 (1997).
25. M. Adamczyk and J.C. Gebler. Bioconjugate Chem. **8**, 400 (1997).
26. R.T. Jones. Methods Enzymol. **231**, 322 (1994).
27. R. Kluger, Y. Song, J. Wodzinska, C. Head, T.S. Fujita, and R.T. Jones. J. Am. Chem. Soc. **114**, 9275 (1992).
28. E.J. Delaney, S.E. Massil, G.Y. Shi, and I.M. Klotz. Arch. Biochem. Biophys. **228**, 627 (1984).
29. G. Guidotti. J. Biol. Chem. **242**, 3685 (1967).
30. J.B. Fenn, M. Mann, C.K. Meng, and S.F. Wong. Mass Spectrom. Rev. **9**, 37 (1990).
31. J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, and C.M. Whitehouse. Science (Washington, D.C.), **246**, 64 (1989).
32. R.T. Jones, C.G. Head, T.S. Fujita, D.T. Shih, J. Wodzinska, and R. Kluger. Biochemistry, **32**, 215 (1993).
33. R. Kluger, L. Shen, H. Xiao, and R.T. Jones. J. Am. Chem. Soc. **118**, 8782 (1996).
34. M.P. Kavanaugh, D.T. Shih, and R.T. Jones. Biochemistry, **27**, 1804 (1988).
35. H. Pötzschke, W.K.R. Barnikol, R.G. Kirste, and M. Rosenbaum. Macromol. Chem. Phys. **197**, 1419 (1996).
36. R. Benesch and R.E. Benesch. Biochem. Biophys. Res. Commun. **26**, 162 (1967).
37. G. Fermi and M.F. Perutz. J. Mol. Biol. **175**, 159 (1984).
38. J.M. Manning, L.R. Manning, S. Morgan, R.C. Beavis, and B. Chait. Proc. Natl. Acad. Sci. U.S.A. **88**, 3329 (1991).