A Doubly Cross-linked Human Hemoglobin

EFFECTS OF CROSS-LINKS BETWEEN DIFFERENT SUBUNITS

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Human deoxyhemoglobin cross-linked with trimesyl tris(3,5-dibromosalicylate) produces the previously reported cross-linked hemoglobin in which the ε amino groups of the two β chain 82 lysyl residues are joined by a trimesyl bridge. Further specific modification of this protein directed to the α subunits with bis(3,5-dibromosalicyl) fumarate gives a doubly cross-linked material in which the ε amino groups of the two α chain 99 lysyl residues are now joined by a fumaryl bridge. The singly cross-linked β chain species binds oxygen cooperatively with a high oxygen affinity (P50 = 4.8 torr at pH 7.4). The addition of the second cross-linking reduces the oxygen affinity to 15.9 torr, which compares with 13.0 torr for the singly cross-linked α chain species. The doubly cross-linked hemoglobin retains significant cooperativity with a Hill coefficient of 2.3 compared with 3.0 for unmodified hemoglobin. Because some of the groups responsible for the Bohr effect are acylated, this doubly cross-linked hemoglobin exhibits 25% of the normal Bohr effect and less than 20% of the normal chloride effect. The use of two distinct cross-links within the same tetramer provides a material for physical and structural analysis as well as for further modifications for specific applications. The results indicate that the cross-link introducing the lowest oxygen affinity in the two singly cross-linked species appears to control the overall affinity in this doubly cross-linked species.

Specifically cross-linked hemoglobins are useful for the investigation of structure-function relationships, (Schumacher et al., 1995) as well as in the development of potential substitutes for red blood cell transfusion (Snyder et al., 1987; Keipert et al., 1989; Bucco et al., 1989; Vandegriff and Winslow, 1991; Kluger et al., 1994). By retaining a reactive site in a cross-linker after modification of hemoglobin, other modifications can be readily prepared by addition of nucleophiles to the modified hemoglobin (Kluger and Song, 1994). This makes it possible to use hemoglobin as a carrier for drugs or other chemicals that may have pharmacologic value (Seymour, 1992) or provide probes for physical and structural analysis. However, hemoglobin containing a trimesyl dibromosalicyl ester as the bisamide of the ε-amino groups of both β82 lysine residues (β82-TDBS-82β)3 schematic structure is shown in Structure 1), has a relatively high affinity for oxygen.

Due to its high affinity for oxygen, this is unlikely to deliver oxygen efficiently. Since combining oxygen delivery and bioinjection is potentially of theoretical as well as medicinal significance, we considered the possibility of further modification to cause a reduced oxygen affinity by introducing a second cross-link.

Walder et al. (1994) developed an efficient hemoglobin-based oxygen carrier that contains a fumaryl bisamide of the ε-amino groups of both α99 lysine residues (α99F-99αβ2; the Baxter Healthcare red cell substitute-derived form of this is called DCLHB) (Walder et al., 1994; Chatterjee et al., 1986; Snyder et al., 1987). If the fumaryl cross-link at the α99 lysines is added to αβ82-TDBS-82β (Kluger et al., 1992b), the oxygen affinity might be altered to make it suitable as an oxygen carrier (schematic structure shown in Structure 2).

Whatever the case, the structure and properties of the doubly cross-linked material would provide an interesting basis for functional studies. We have been able to produce these chemical modifications of the same hemoglobin efficiently. The resulting material has the desirable features of both individual modifications. Olsen et al. (1991) have also reported in abstract form the preparation of multilinked hemoglobins.

MATERIALS AND METHODS

Hemoglobin Preparation and Modification—Hemoglobin solutions were prepared as described previously (Jonas et al., 1994) and stored as COHb at 0 °C on ice. For reaction with TTDS, COHb (0.5 mM in pH 9.0, 0.05 M sodium borate) was converted to deoxy-Hb by photolysis under a stream of humidified oxygen for 60 min at 0 °C in a rotating flask (Shih et al., 1982). Oxygen was removed by passing a steam of 1 The abbreviations used are: αβ82-TDBS-82β; Hb containing a trimesyl bisamide of the ε-amino groups of both Lys99 residues; αβ82-T-82β; Hb containing a fumaryl bisamide of the ε-amino groups of both Lys82 residues; αβ82-F-82β; Hb containing a fumaryl bisamide of the ε-amino groups of both Lys99 residues; α99F-99αβ2; Hb containing a fumaryl bisamide of the ε-amino groups of both Lys82 residues; α99F-99αβ2; Hb containing a fumaryl bisamide of the ε-amino groups of both Lys82 residues. DCLHB, diaspi-rin-cross-linked hemoglobin, another designation for α99F-99αβ2; COHb, carboxyhemoglobin; DBS, 3,5-dibromosalicylate; DPG, 2,3-diphosphoglycerate; HPLC, high performance liquid chromatography; MOPS, 3-(N-morpholinol)propanesulfonic acid; nHb, Hill coefficient at P0.2; P0.2, oxygen pressure (torr) where Hb is 50% oxygenated; TDBS, trimesyl mono(3,5-dibromosalicylate); TTDS, trimesyl tris(3,5-dibromosalicylate).

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humidified N\textsubscript{2} over the oxy-Hb solution for 1.5 h at 35 °C in the rotating flask. For analytical studies, reactions were carried out with 100 mg of protein while preparative studies were conducted on 1–2 g samples.

TTDS was synthesized and analyzed as previously reported (Kluger et al. 1992b). Because TTDS is not readily soluble in water, 1,4-dioxane was used to dissolve the reagent. (We have also added the reagent in water with sonication.) The solution of TTDS was degassed under an atmosphere of CO form by passing a stream of humidified carbon monoxide over the solution for 2–3 min. The COHb mixture was separated from the excess COHb by passing through a Sephadex G-25 column equilibrated with 0.005 M pH 8.0 borate buffer. (We have also added the reagent in water with sonication.) The solution of TTDS was degassed under an atmosphere of CO form by passing a stream of humidified nitrogen at 35 °C in the same rotating flask as used for the deoxygenation.

DBSF was either synthesized as described by Walder et al. (1979) or purchased. Although DBSF can be added as a solid to deoxy-Hb (Walder et al., 1994), we added it as a solution in 1,4-dioxane and 0.1 M pH 9.0 borate. (We have also added the reagent in water with sonication.) Oxygen was removed in the same way for TTDS solutions. An amount of DBSF was added to the deoxy-Hb that had been reacted with TTDS to equal twice the moles of Hb present, i.e. to make the DBSF about 1.0 mM. This reaction was allowed to proceed for the next 2 h under a stream of humidified nitrogen at 35 °C in the same rotating flask as used for the reaction with TTDS. The final reaction mixture was then cooled to 0 °C in an ice bath, and the modified Hb was converted to the CO form by passing a stream of humidified carbon monoxide over the solution for 2–3 min. The COHb mixture was separated from the excess reagents and low molecular weight reaction products by passing through a Sephadex G-25 column equilibrated with 0.005 M pH 8.0 MOPS and concentrated by pressure filtration. Portions of the stripped COHb were stored on ice, while others were flash-frozen with dry ice in order to stop the hydrolysis reaction until the C-4 reversed phase HPLC could be done. The rates of hydrolysis were estimated from the rate of decrease of the CO and then deoxygenated. To this was added 37 mg of TTDS in 7 ml containing 2.5 ml of dioxane, 2.5 ml of 0.1 M pH 9.0 borate buffer, and 2.0 ml of water. After 1.5 h of reaction, 30 mg of DBSF in 3 ml of solution containing 1.0 ml of dioxane, 1.5 ml of 0.1 M pH 9.0 borate, and 0.5 ml of water was added to the reaction mixture under nitrogen. After 2 h the reaction was cooled in ice, converted to COHb, and passed through the gel filtration column.

Characterization of Modified Hemoglobins—The procedures for analytical and preparative separations of hemoglobins by ion exchange chromatography, globin chain separation by reversed phase HPLC on C-4 columns, enzyme hydrolysis with trypsin and Glu-C endoproteinase, and peptide and amino acid analysis are described in detail elsewhere (Jones et al., 1994). The molecular masses of globin chains were measured by electrospray ionization mass spectrometry (Fenn et al., 1989).

Measurement of Functional Properties of Isolated Hemoglobins—The oxygen binding equilibria of modified hemoglobins were measured by the automatic recording method of Imai et al. (1970) using the apparatus and procedure described by Shih and Jones (1986). The conditions for comparing the oxygen affinities of the modified hemoglobins were 50 mM Bis-Tris, pH 7.4, 0.1 mM Cl\textsuperscript{−}, 25 °C, and 55 μM heme. The parameters measured were the oxygen pressure for half-saturation (P\textsubscript{50}) and Hill’s coefficient of cooperativity at half saturation (n\textsubscript{H}). The Bohr (H\textsuperscript{+}) effect was measured between pH 6.0 and 9.0 and calculated for the interval of pH 7.0–8.0. The chloride effect was measured for 0.007–0.5 M NaCl.

Rates of Hydrolysis of DBS from \(\alpha\beta\)-TTDS-82 and \(\alpha\beta\)-TTDS-82—The rates of hydrolysis of DBS from \(\alpha\beta\)-TTDS-82 and \(\alpha\beta\)-TTDS-82 with Hbs at 0.1 mM were followed at pH 5.5 in 0.1 M citrate, pH 7.2 in MOPS, and pH 9.0 in borate at 45 °C by measuring the amounts of \(\beta\)-TTDS-82 and \(\beta\)-TTDS-82 chains relative to total a chains present at various times by C-4 globin chain separation. Rates of the hydrolysis solutions were followed at pH 6.0 and 9.0 with a Cary 17 spectrophotometer.

RESULTS

The reaction of deoxy-Hb with two equivalents of TTDS in borate buffer at pH 9 (producing \(\alpha\beta\)-TTDS-82) followed by reaction with two equivalents of DBSF yields the doubly cross-linked hemoglobin tetramer containing an intact ester linkage (i.e. two cross-links connecting four lysine side chains with a 3,5-dibromosalicylate ester on the trimesyl \(\beta\)-cross-link), \(\alpha\alpha\beta\beta\)-TTDS-82β—The structure of this doubly cross-linked product was determined by C-4 globin chain separation. Samples of modified hemoglobin were removed from the hydrolysis solutions and flash-frozen with dry ice in order to stop the hydrolysis reaction until the C-4 reversed phase HPLC could be done. The rates of hydrolysis were estimated from the rate of decrease of \(\beta\)-TTDS-82β as well as the rate of increase of \(\beta\)-TTDS-82β.

Influence of Reaction Conditions on Extent of Product Formation—Conditions for optimal formation of this doubly cross-linked product were determined through trials under varying conditions. We have shown that the extent of reaction of TTDS with hemoglobin is greater with deoxy-Hb than with COHb at 35 °C (0.1 M pH 7.2 MOPS) (Kluger et al., 1992b). In the present study, three other reaction conditions were tested: 0.1 M pH 7.2 bis-Tris, 0.1 M pH 8.0 MOPS, and 0.05 M pH 9.0 borate. The greatest conversion of Hb A to \(\alpha\beta\)-TTDS-82β was obtained using 0.05 M pH 9.0 borate. Fig. 1 shows the results of a typical C-4 reversed phase HPLC globin chain separation of the Hb mixture after 0.5 h of reacting 0.5 mM deoxy-Hb with 0.75 mM TTDS in 0.05 M pH 9.0 borate at 35 °C. Different conditions
were also tried for the second reaction with DBSF; however, optimal results were obtained with 1.0 mM DBSF under the same conditions as the first reaction, i.e., 0.5 mM deoxy-Hb, 0.05 M borate, pH 9.0, 35°C. Fig. 2 shows the globin chain separation of a typical preparation of the doubly cross-linked Hb. From both Figs. 1 and 2 it is apparent that partial hydrolysis of DBS from the $\beta_82$-TDBS-$\beta_82$ chains occurs under the preparation conditions. This can be decreased by reducing the temperature of the reaction and increased by raising the reaction temperature. Eighty percent or more conversion of $\beta_82$-TDBS-$\beta_82$ to $\beta_82$-T-$\beta_82$ was observed when the reaction mixture was converted to CO and then heated to 60°C for 3 h after an initial 1 h for reaction with DBSF at 35°C as shown in Fig. 3. More than 95% conversion has been observed after storing a stripped reaction mixture for 2½ months in the cold room at 4°C. No hydrolysis was observed when $\alpha_99$-F-$\alpha_99$ was stored at 2°C.

Kluger and Song (1994) have reported that $\alpha_99$-F-$\beta_82$-TDBS-$\beta_82$ will react with a variety of nucleophiles to form derivatives with the third carboxyl of the trimesyl cross-linker. Fig. 4 shows the chain separation results after reacting a sample of stripped reaction mixture containing $\alpha_99$-F-$\beta_82$-TDBS-$\beta_82$ with 0.07 M lysyl-lysine at 35°C in 0.05 M borate, pH 9.0, for 2 h. Other nucleophiles including glycine, lysine, and Tris react under the same conditions to convert the $\alpha_99$-F-$\beta_82$-TDBS-$\beta_82$ to covalently linked derivatives.

Structural Characterization of Modified Globin Chains—A reaction mixture that contained some unreacted Hb A and some partially hydrolyzed $\alpha_99$-F-$\beta_82$-TDBS-$\beta_82$ was separated with a C-4 reversed phase HPLC column interfaced to an electrospray ionization mass spectrometer. The molecular masses of the material eluting in the positions of unmodified $\beta$ and $\alpha$ chains as well as peaks believed to be $\beta_82$-T-$\beta_82$, $\beta_82$-TDBS-$\beta_82$, and $\alpha_99$-F-$\alpha_99$ were determined and are listed in Table I. The observed molecular masses are coincident with the calculated values within the experimental uncertainty limits of the method.

The peaks assigned to the $\beta_82$-T-$\beta_82$ and $\alpha_99$-F-$\alpha_99$ globin chains were isolated by preparative C-4 reverse phase HPLC for further structural characterization. The $\beta_82$-T-$\beta_82$ chains were hydrolyzed to a set of peptides with trypsin followed by Glu-C endoproteinase. The $\alpha_99$-F-$\alpha_99$ chains were oxidized with performic acid and then hydrolyzed with trypsin in the presence of 2 M urea. Fig. 5, A and B, show the C-18 reverse phase HPLC peptide pattern of these globin chains. In Fig. 5A, all of the normal tryptic, Glu-C peptides of the $\beta$ chain are present except for those that occur from tryptic cleavage adjacent to Lys$^{\beta2}$, $\beta$T-9 and $\beta$T-10a. A tryptic peptide not found in native Hb elutes at about 94 min and absorbs at 258 nm,
TABLE I
Molecular mass of globin chains by liquid chromatography mass spectrometry

| Elution time (min) | Observed mass (aDa) | Calculated mass (aDa) | Structure of globin |
|-------------------|---------------------|----------------------|--------------------|
| 31                | 15,869.17 ± 1.96    | 15,868.2             | Unmodified β chain |
| 35                | 15,127.89 ± 1.61    | 15,126.4             | Unmodified α chain |
| 46                | 31,907.81 ± 2.35    | 31,910.4             | β82-T-82β          |
| 57                | 32,185.23 ± 1.62    | 32,188.4             | β82-TDBS-82β       |
| 76                | 30,334.64 ± 2.89    | 30,332.8             | α99-F-99α          |

Fig. 5. A, HPLC peptide pattern of a trypsin-Glu-C hydrolysate of globin corresponding to the material eluting at about 45 min from the C-4 column separations (see Figs. 1-4). The peptides were separated on a C-18 reversed-phase column using a water-acetonitrile gradient in 0.1% trifluoroacetic acid. All of the normal peptides were present except β-9 and β-10a. A new peptide was found eluting at about 94 min, which had the amino acid composition of β-9 and β-10a plus the UV absorption of the trimesic cross-linker. B, HPLC peptide pattern of a trypsin hydrolysate of the oxidized globin corresponding to the material eluting at about 75-77 min from the C-4 column separations (see Figs. 2 and 3). The peptides were separated as described for panel A. All of the normal peptides were present except α-T-11 and α-T-12. A new peptide was found eluting at about 83 min, which had the amino acid composition of oxaT-11 and oxaT-12.

indicative of the presence of trimesyl moiety. Amino acid analysis indicates it has the aminoacyl components of β-9,10a. These results are the same as those reported earlier for α₂β₂-TDBS-82β obtained from the reaction of COHb with trimesoyl triis-(methylphosphate); Kluger et al., 1992a). This peptide pattern and the amino acid composition of the new peptide combined with the molecular mass measurement of the globin chain are the basis for deducing the structure to be β82-T-82β. Similarly, Fig. 5B shows all of the normal tryptic peptides observed for oxidized α chains except for the absence of α-T-11 and oxaT-12. In their place is a new peptide that elutes near the end of the chromatogram that has the amino acid composition of oxaT-11,12. These results are the same as those found for the α99-F-99α present in a preparation of DCLHB from Baxter Healthcare Corp. and reported elsewhere (Iones, 1994).

Rates of Hydrolysis of DBS from α₂β₂-TDBS-82β and α99-F-99αβ₂-TDBS-82β—Table II lists the rate constants of hydrolysis of the ester moiety as followed by the decrease in the relative amount of β82-TDBS-82β and the increase of β82-TDBS-82β chains (by HPLC analysis) in samples containing either single or doubly cross-linked Hbs. The data accurately fit integrated first order rate expressions (collected over at least two half-times). The rate of hydrolysis increases with pH but with a slope of less than unity, indicating a change from an uncatalyzed to base-catalyzed mechanism occurs over the pH range studied. Most of the kinetic studies we report were done using α₂β₂-TDBS-82β because of the ease of estimating the amounts of β82-TDBS-82β and β82-TDBS-82β relative to unmodified α chains. We find comparable hydrolysis rates of DBS in the singly and doubly cross-linked Hb under the same conditions at pH 7.2.

Functional Properties of the Modified Hemoglobins—The oxygen-binding properties of α99-F-99αβ₂-TDBS-82β were measured and compared with those of unmodified Hb A, α99-F-99αβ₂, and α₂β₂-TDBS-82β and are listed in Table III. The P₅₀ of α99-F-99αβ₂-TDBS-82β is about three times larger than that of unmodified Hb A at pH 7.4 but comparable with that of α99-F-99αβ₂, which has potential as a blood substitute (Chatterjee et al. 1986).

A trimesyl cross-link between the two Lys⁸₂ residues does not appear to change P₅₀ appreciably from that of unmodified Hb at this pH. Differences in the Bohr effects do result in different outcomes at other pH levels. It is likely that there is little difference in structure of this species compared with native Hb in the oxy or deoxy forms. However, a fumaryl cross-link between the two Lys⁸₂ residues increases P₅₀ by a factor of 2.5, indicating a marked decrease in oxygen affinity through a higher energy R state, lower energy T state, or a combination of both. Thus, the two types of cross-links in this double cross-linked Hb are manifested very differently in their effects on oxygen affinity. The Hill coefficients at pH 7.4 are somewhat reduced compared with that of unmodified Hb for both α₂β₂-TDBS-82β and α99-F-99αβ₂-TDBS-82β with n₅₀ values of 2.4 and 2.3, respectively, while n₅₀ = 2.7 for α99-F-99αβ₂-TDBS-82β at pH 7.0.² Both the Bohr effect and chloride effect are lower than the normal values for unmodified Hb (in decreasing order: α₂β₂-TDBS-82β to α99-F-99αβ₂ to α99-F-99αβ₂-TDBS-82β).

DISCUSSION

The measurement and interpretation of the effects of one or two cross-links on the properties of hemoglobin depends on being able to produce these species efficiently. The reaction of TDDS with hemoglobin is highly selective for the ε-amino groups of the Lys⁸₂ residues (at pH 7.0). Both the Bohr effect and chloride effect are lower than the normal values for unmodified Hb (in decreasing order: α₂β₂-TDBS-82β to α99-F-99αβ₂ to α99-F-99αβ₂-TDBS-82β).

² R. T. Iones, D. T. Shih, T. S. Fujita, Y. Song, H. Xiao, C. Head, and R. Kluger, unpublished results.
groups of the α subunits proceeds with high efficiency in the species containing the cross-link between β subunits. The conversion of the unmodified α chains of the hemoglobins in the reaction mixture resulting from treatment of deoxy-Hb with TTDS to cross-linked α chains by reacting the mixture with DBSF under nitrogen is about 85% complete at pH 9.0 and 35 °C. Consistent with expectations, this yield is significantly greater than for the cross-linking of only α chains of deoxy-Hb using DBSF in the presence of inositol hexaphosphate (to direct DBSF away from the DPG binding site) under the most favorable conditions reported by Walder et al. (1994). Although these authors state that “the yield of this derivative is markedly increased in the presence of polyani ons that bind within the DPG site” they do not report the extent of reaction. Using their conditions, we have been able to obtain a maximum of about 55% yield of αβ-F-99,6 compared with the starting amount of unmodified Hb. The association of inositol hexaphosphate with Hb is a dynamic process involving association and dissociation, so that the competition remains between the reagents toward other sites. Thus efficient covalent modification directs the reaction of DBSF to the ε-amino groups of the Lys829 residues.

By heating the final reaction mixture used to produce the doubly cross-linked species to 60 °C for 3 h in the presence of CO, most of the DBS will be hydrolyzed from the trimesic cross-link, and the main product is αβ-F-99αβ2-T-82. This approach to the preparation of a Hb with an α99-F-99α cross-link may be an efficient alternative to the use of polyani ons to promote formation of this modification.

Structural Analysis—The identification of the cross-link sites for α99-F-99αβ2-T-82 and α99-F-99αβ82-DBS-82β is based on analysis of the various types of globin chains isolated from the reaction mixtures and purified Hb components, their molecular masses, and their peptide patterns. The globin chain peak that elutes at about 46 min from the C-4 column had a molecular mass of 31,908, in agreement with the calculated mass of 31,910 for 2β chains with one molecule of trimesic acid attacked as the bisamide (see Fig. 2 and Table I). The peptide pattern of the material after enzymatic hydrolysis showed it to be β chains with the Lys829 groups blocked and cross-linked by the trimesyl group. Therefore, this is β82-T-82β. The globin chain peak that elutes at about 57 min has an observed mass of 32,185 in agreement with the calculated mass of 32,188 for 2β chains with one molecule of trimesyl bisamide and as a DBS ester. Upon standing at 0 °C for several weeks or after heating, this globin chain elutes in the position of β82-T-82β with an equimolar amount of DBS eluting just ahead of the heme from the C-4 column. Thus, this modified globin is β82-DBS-82β, i.e. the same as β82-T-82β but with the third DBS still present as an ester of the third carboxyl group of the trimesic acid cross-linker. The globin chain peak that elutes at about 76 min has an observed mass of 30,333 in agreement with the calculated mass of 30,333 for 2α chains connected as a fumaryl bisamide. Its peptide pattern shows this modified globin to contain α chains with the Lys829 residues blocked by the fumaryl cross-link. Therefore, it is α99-F-99α.

As Kluger and Song (1994) observed for αβ-F-99αβ2-T-82β, we find that α99-F-99αβ2-T-82β can act as an acylating reagent toward nucleophiles, forming conjugates of hemoglobin. This presents opportunities to investigate combining oxygen delivery with biocorjugation for a transfused or perfused material. It also permits the attachment of probes and other molecules that may be useful in physical and structural analyses.

Ester Hydrolysis and Environment—The rate of hydrolysis of the dibromosaliclyl ester of the cross-linker on the both cross-linked proteins described above, compared with the rate of hydrolysis of unreacted TTDS in solution gives an indication of the accessibility of the ester. The observed first order rate coefficient for hydrolysis of DBS from both α99-F-99αβ2-T-82β and from αβ-F-99αβ2-T-82β (at 35 °C in 0.1 M phosphate buffer; Table II) is 7 × 10⁻⁵ s⁻¹. The rate coefficient for hydrolysis of the first dibromosaliclyl ester of TTDS in 0.1 M MOPS buffer is 3.2 × 10⁻⁵ s⁻¹, comparable with the rate for the protein derivative. Thus, from this comparison we conclude that in the protein derivatives, the ester is not protected from hydrolysis. This suggests that the ester group is extended toward the solvent rather than into the DPG binding pocket of the protein. We are attempting to obtain crystals of the protein with the intact ester so that the structure may be analyzed by x-ray diffraction.

In alkaline solutions, the rate of hydrolysis of DBS from β82-DBS-82β increases. Thus, by controlling the pH of the solution in which the product is kept, the free acid α99-F-99αβ2-T-82β or the ester, α99-F-99αβ2-T-82β, will predominate. The latter is useful for subsequent reaction with nucleophiles that form stable derivatives by displacement of the ester retained on the trimesyl cross-link.

Relationship of Oxygen Binding and Cross-links—Comparisons of the P₅₀ values of αβ-F-99αβ2-T-82β, α99-F-99αβ2, and α99-
F-99F-82-T-82β indicate that the α99 fumaryl cross-linkage has the dominant effect on the oxygen affinity of the doubly cross-linked Hb. Although the P50 of α99β2-T-82β is similar to that of unmodified Hb A at pH 7.4, the decreases in its cooperativity (n50) Bohr and chloride effects show that this structural modification significantly changes its functional properties from those of Hb A. Therefore, both the cross-linking of the β chains with the trimesyl bridge as well as the cross-linking of the α chains with the fumaryl bridge contribute to the changes in the overall oxygen binding properties of this doubly cross-linked Hb.

It is well documented that nearly 45% of the overall alkaline Bohr effect is due to the releasing of protons from the His146 residues in human Hb A that occurs with the decrease in pK associated with oxygenation (Kilmartin et al., 1980; Shih et al., 1993). The remaining 55% of the Bohr effect is attributed to the release of protons linked to the chloride effect. The positively charged cluster of amino acid residues in the central cavity, release of protons linked to the chloride effect. The positively charged residues in the central cavity, i.e., α-NH₂ of Val11, ε-NH₂ of Lys199, and Lys182, and Arg104, has been identified as important in the functional mechanism of the chloride effect (Perutz et al., 1994). According to Perutz et al. (1994), modifications of any positively charged residue in the central cavity should change the chloride effect and thus the Bohr effect. Therefore, the additive losses of Bohr effect and chloride effect in the order of αββ2-T-82β, α99F-99αβ2, and α99F-99αβ2-T-82β would be expected because of the extent of acylation of the ε-NH₂ groups of Lys182 and Lys199. Our data show that the cross-linking modification of Lys182 reduces the chloride effect by 55% and the cross-linking of Lys199 reduces it by 66%. The accompanied decrease in overall Bohr effect with each modification is 26 and 48%, respectively. In the case of the doubly cross-linked α99F-99αβ2-T-82β Hb in which all of the positively charged residues in the central cavity have been eliminated, the accumulated reduction of chloride effect by 84% and accompanying large reduction of Bohr effect of 73% are consistent with the concept of chloride binding proposed by Perutz et al. (1994).

As noted above, His146 is known to be responsible for the chloride-independent part of the overall alkaline Bohr effect of Hb A. Although the double cross-linking modifications do not chemically alter the histidyl residues of β146, the reduction of the Bohr effect of this hemoglobin to 27% of normal, which is about one-half of the chloride-independent part of the Bohr effect, is probably due to constraints in oxygen-linked structural changes in the doubly cross-linked Hb. The high P50 and low n50 values of the doubly cross-linked Hb, which indicate a reduction in the shift in the allosteric equilibrium toward the R state, support this explanation. The extra structural constraint in the α99-F-99αβ2-T-82β Hb that is manifest in its decreased cooperativity is presumably due to the cross-linking modification of the two Lys199 residues than the Lys182 residues because the modification of the latter two does not influence the n50 value of α99-F-99αβ2 as shown in Table III.

These results show that sequentially introduced site-specific cross-links provide practical means for adjusting physical properties of proteins. The use of a reactive ester on one of the cross-links permits the introduction of probes for further analysis, conjugation, or modification.

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