Combining the Influence of Two Low O_2 Affinity-Inducing Chemical Modifications of the Central Cavity of Hemoglobin†

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ABSTRACT: HexaPEGylated hemoglobin (Hb), a non-hypertensive Hb, exhibits high O_2 affinity, which makes it difficult for it to deliver the desired levels of oxygen to tissues. The PEGylation of very low O_2 affinity Hbs is now contemplated as the strategy to generate PEGylated Hbs with intermediate levels of O_2 affinity. Toward this goal, a doubly modified Hb with very low O_2 affinity has been generated. The amino terminal of the β-chain of HbA is modified by 2-hydroxy, 3-phospho propylation first to generate a low oxygen affinity Hb, HPPr-HbA. The oxygen affinity of this Hb is insensitive to DPG and IHP. Molecular modeling studies indicated potential interactions between the covalently linked phosphate group and Lys-82 of the trans β-chain. To further modulate the oxygen affinity of Hb, the $\alpha\alpha$-fumaryl cross-bridge has been introduced into HPPr-HbA in the mid central cavity. The doubly modified HbA ($\alpha\alpha$-fumaryl-HPPr-HbA) exhibits an O_2 affinity lower than that of either of the singly modified Hbs, with a partial additivity of the two modifications. The geminate recombination and the visible resonance Raman spectra of the photoproduct of $\alpha\alpha$-fumaryl-HPPr-HbA also reflect a degree of additive influence of each of these modifications. The two modifications induced a synergistic influence on the chemical reactivity of Cys-93(β). It is suggested that the doubly modified Hb has accessed the low affinity T-state that is non-responsive to effectors. The doubly modified Hb is considered as a potential candidate for generating PEGylated Hbs with an O_2 affinity comparable to that of erythrocytes for developing blood substitutes.

Developing low O_2 affinity hemoglobin (Hb) has been the subject of considerable interest both in terms of understanding the structure−function correlation of Hb and for the development of Hb based oxygen carriers. Central cavity modifications, such as cross-linking and affinity labeling of the effector binding domains of Hb, have been the prominent approaches to reduce the O_2 affinity of Hb (*1*−*6*). However, interest in such molecules eventually subsides because most of these potential Hb based oxygen carriers turned out to be vasoactive (*7*−*9*). The vasoactivity was considered to be a consequence of the NO-scavenging activity of acellular Hb (*10*−*12*). The design of mutant Hbs with reduced NO-binding activity has been one of the approaches advanced to generate non-hypertensive Hb based oxygen carriers (*11*, *13*−*15*).

An alternate approach to overcome the vasoactivity of Hb advocates the induction of unique molecular properties of plasma volume expanders such as colloid osmotic pressure and viscosity into Hb. Conjugation of polyethylene glycol (PEG[1]) chains to Hb appears to achieve this goal (*16*−*19*). Our recent observation that surface decoration of Hb with six copies of PEG-5000 chains nearly neutralizes the vasoactivity of Hb validates the concept that PEGylation of Hb can be used as a way of generating nonhypertensive Hb (*19*). Accordingly, we have generated PEGylated Hb employing different chemistry, thiolation-mediated maleimide chemistry (*19*−*20*), reductive alkylation (*21*), acylation, and thiocarbamoylation (*22*). All these modifications were directed to the amino groups of Hb. The resultant PEGylated Hbs had an average of six copies of PEG chains conjugated at different sites of the Hb. All of these PEGylated Hbs had an increased O_2 affinity, irrespective of the chemistry of modification and sites of PEG conjugation (*19*−*21*).

Though the high O_2 affinity of the PEGylated Hbs is considered an advantageous factor in achieving the neutralization of the vasoactivity of Hb by reducing the amount of oxygen delivered on the arterial side of the microcirculatory system (*18*, *23*−*25*), the O_2 affinity of the present versions of PEGylated Hbs appears to be too high to deliver adequate levels of oxygen to tissues. Accordingly, we have been

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[1] Abbreviations: ACN, acetonitrile; DBBF, bis-dibromosalicyl fumarate; DPG, 2,3-diphosphoglycerate; GY, geminate yield; Hb-P, Hb-Presbyterian; HFBA, heptafluorobutyric acid; HPPr, 2-hydroxy 3-phospho propyl; IEF, isoelectric focusing; IHP, inositol hexaphosphate; L35, 2-[4-(3,5-dichlorophenylureido)phenoxy]-2-methylpropionic acid; 4-PDS, 4,4′-dithiodipyridine; PEG, polyethylene glycol; RPHPLC, reverse phase high performance liquid chromatography.

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considering the use of low O_2 affinity Hbs instead of using normal adult human Hb as substrates for the generation of PEGylated Hbs using the same protocols discussed above (*19*−*22*).

Our recent studies of hexaPEGylation of αα-fumaryl Hb have generated a PEGylated Hb (*26*) with an O_2 affinity (P50 ∼14 mm of Hg) lower than that of hexaPEGylated Hb (P50 ∼7 mm of Hg). HexaPEGylation of modified Hbs, with an O_2 affinity still lower than that of αα-fumaryl Hb, may be expected to facilitate the generation of very low O_2 affinity that is comparable to that of erythrocytes (P50 ∼28 mm of Hg). The preparation of doubly modified Hbs is an approach to generate very low O_2 affinity Hbs that could be used as substrates for PEGylation to generate low O_2 affinity PEGylated Hbs.

Introduction of negative charges at the amino terminal of β-chain induces low O_2 affinity to Hb (*4*, *27*−*28*). Carboxymethylation (*4*) and galacturonic acid (*27*) modification introduce a carboxyl group at Val-1(β), whereas pyridoxal phosphate modification (*28*) adds a phosphate group at the same site. The influence of pyridoxal phosphate in reducing the O_2 affinity of Hb seems to be higher than carboxymethylation or galacturonic acid modification at Val-1(β), presumably because of the presence of a phosphate group. Glyceraldehyde-3-phosphate is similar to DPG in structure, and the site specific modification of Val-1(β) of Hb by this reagent will introduce two phosphate groups in the DPG binding site of Hb. This can induce low O_2 affinity to Hb, similar to that of DPG. Therefore, in the present study, we have explored the use of glyceraldehyde-3-phosphate in the presence of sodium cyanoborohydride to modify the α-amino group of Val-1(β). This reaction is carried out under oxy conditions as compared to the deoxy conditions used for the modification of Hb by pyridoxal phosphate (*28*).

The αα-fumaryl cross-bridging in the mid central cavity of HbA is another structural modification that reduces the O_2 affinity of HbA. The reagent, bis dibromosalicyl fumarate (DBBF), introduces a cross-link between the ϵ-amino groups of Lys-99(α) of the central cavity only in the deoxy conformation (*5*). Under oxy conditions, the same reagent introduces a cross-link between the ϵ-amino groups of Lys-82(β) residues of the ββ-cleft and induces a high O_2 affinity to HbA (*3*). The high conformational selectivity of the reaction of DBBF with HbA and the resulting distinct influence of the cross-linking on the O_2 affinity has been interpreted as the consequence of freezing in the oxy or deoxy conformation of the protein through cross-linking (*5*, *29*). These cross-linking reactions have been used to stabilize the $\alpha_1\beta_2$ interface that is weakened by structural modifications of Hb (*26*, *30*).

The introduction of more than one low O_2 affinity inducing chemical modifications into Hb, generating a doubly modified Hb, is the approach that we have been considering to develop a very low O_2 affinity Hb. These chemical modifications may act additively or synergistically to generate a very low O_2 affinity Hb. Recently, we have engineered the αα-fumaryl cross-bridge into Hb Presbyterian (Hb-P), a low O_2 affinity mid central cavity mutant Hb (*31*). The αα-fumaryl Hb-P exhibited a very low O_2 affinity. The two structural modifications, that is, the Presbyterian mutation (Asn-108(β) → Lys) and αα-fumaryl cross-bridging, exhibited a synergy in reducing the O_2 affinity of the molecule. Because the two structural modifications in this case were in the mid central cavity, the proximity of the two structural perturbations might have facilitated the synergy of the two modifications of Hb structure.

In an attempt to generate a very low O_2 affinity Hb by chemical modifications, we have now introduced the mid central cavity low O_2 affinity-inducing αα-fumaryl cross-bridge into HPPr-HbA. Characterization of the doubly modified HbA and correlation of its oxygen binding properties, geminate rebinding, conformation of heme pocket in the R-state, and Cys-93(β) reactivity are presented in this study. These results are discussed in light of the fact that the R-state conformation of Hb represents a dynamic equilibrium between multiple R-state conformations. The linkage of the low O_2 affinity-inducing perturbation of the mid central cavity with that of the ββ-cleft is only additive and is distinct from the linkage of two mid central cavity perturbations studied earlier (*31*). The possible application of these very low O_2 affinity Hbs in the generation of nonhypertensive lower O_2 affinity PEGylated Hbs is also discussed.

MATERIALS AND METHODS

Preparation of HPPr-HbA. Purified HbA (0.5 mM) was modified with 5 mM glyceraldehyde-3-phosphate in the presence of 10 mM $NaCNBH_3$ in PBS at pH 7.4, at 37 °C for 30 min. The product, HPPr-HbA, was purified on CM-52 cellulose (2.5 × 50 cm) using a gradient of 10 mM phosphate at pH 6.0 to 15 mM phosphate at pH 8.0. The peak corresponding to HPPr-HbA, as characterized by the isoelectric focusing of the peak, was further purified on the same column, using a shallower gradient.

Cross-Linking of HPPr-HbA by DBBF. HPPr-HbA was modified with DBBF as described previously (*5*). Briefly, HPPr-HbA (1 mM) was incubated overnight with 8 mM sodium tripolyphosphate at 4 °C to prevent the modification of DPG pocket residues by DBBF. This sample was deoxygenated at 37 °C and incubated with 2 mM DBBF at the same temperature for 4 h. The reaction was stopped by adding 20 mM Gly−Gly.

Analysis of the αα-Fumaryl Cross-Linking of HPPr-HbA. This analysis was carried out by reverse phase high performance liquid chromatography (RPHPLC) using a Vydac C4 column (4.6 × 250 mm). The same amount of hemoglobin samples were loaded in 0.3% heptafluorobutyric acid (HFBA) on a C4 column equilibrated with 35% acetonitrile (ACN) containing 0.1% HFBA. The globin chains were eluted with a gradient of 35−45% ACN in the first 10 min and then 45−50% ACN in 90 min at a flow rate of 1 mL/min. HFBA (0.1%) was present in the solvents throughout the gradient.

Purification of αα-Fumaryl-HPPr-HbA. On introducing αα-fumaryl cross-linking into HPPr-HbA, the derivative developed some met-Hb. Therefore, the derivative was reduced with dithionite as described by Roy and Acharya (*32*). The oxy form of αα-fumaryl-HPPr-HbA was purified on a Q-Sepharose High Performance (0.9 × 30 cm) column. The column was equilibrated with 50 mM Tris acetate at pH 8.0 and eluted with a linear gradient consisting of 200 mL each of buffer A (50 mM Tris acetate at pH 7.7) and buffer B (50 mM Tris acetate at pH 6.8 containing 25 mM NaCl).

Table 1: Mass of Globin Chains of Hbs Determined by ESI Mass Spectrometry

	molecular mass (Da)			
	α-component		β-component	
sample	calculated	measured	calculated	measured
HbA	15126.4	15129.0	15867.2	15866.0
HPPr-HbA	15126.4	15129.0	16021.3	16020.0
αα-fumaryl-HbA	30332.8	30330.0	15867.2	15868.0
αα-fumaryl-HPPr-HbA	30332.8	30330.0	16021.3	16020.0

Isoelectric Focusing (IEF) of Modified Hemoglobins. Hemoglobin samples were analyzed on precast IEF agarose gels (Perkin-Elmer) containing resolve ampholytes at pH 6−8. The gel was elctrofocused (Isolab) for 1 h.

Mass Spectrometry. The isolated globins of the modified Hbs were analyzed by ESI-MS on a 9.4 T Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (Varian, Inc.). The tryptic peptides of the globin chains were analyzed by LC/ESI-MS (*33*) using a C8 or C18 column (Vydac 1 × 50 mm). A stepwise gradient using 5% ACN containing 0.1% TFA as solvent A and 95% ACN containing 0.1% TFA as solvent B was generated to separate the peptides.

O_2 Affinity Measurements. The oxygen equilibrium measurements of modified Hbs were made at an Hb concentration of 0.5 mM at 37 °C in 50 mM Bis-Tris/50 mM Tris acetate at pH 7.4, using Hem-O-Scan (AMINCO). The measurements were made in the absence and presence of allosteric effectors at the concentrations indicated in Table 2.

Reactivity of Cys-93(β) of Modified Hbs. The reactivity of Cys-93(β) of modified Hbs was determined by the reaction of Hb with 4,4′-dithiodipyridine (4-PDS) as described by Ampulski et al. (*34*). Typically, the carbonmonoxy form of Hb (5 μM) was added to 50 μM 4-PDS in 50 mM Bis-Tris/Tris acetate at pH 7.4, at 30 °C. The reaction kinetics was followed by monitoring the formation of the reaction product of 4-PDS, 4-thiopyridone, at 324 nm. The number of titrable thiol groups of Hb was determined from the initial concentration of Hb and the concentration of 4-thiopyridone formed at the end of the reaction.

Geminate Binding Studies. Geminate recombination of carbonmonoxide to 10 μs photoproducts of carbonmonoxy forms of HbA and modified Hbs was determined as described by Khan et al. (*35*). All of the samples used for the kinetic measurements were at 0.5 mM in heme in 50 mM Bis-Tris acetate at pH 6.5, at 3.5 °C.

Visible Resonance Raman Studies. Visible RR spectra were generated for the 8 ns photoproducts of the CO derivatives of HbA and modified Hbs at 0.5 mM in heme in 50 mM Bis-Tris acetate at pH 6.5, at 3.5 °C (*35*).

Molecular Modeling. The high-resolution crystal structure of hemoglobin (pdb code 4HHB) (*36*), was chosen for the initial model. The molecular model of αα-fumaryl cross-linked Hb was built as described by Chatterjee et al. (*5*). The fumaryl chain was modeled using the builder module of Insight II computer graphics (Accelrys Software, Inc.). The dihedrals of the side chains of both Lys-99 (α) were modified without affecting the main chain configuration such that a covalent fumaryl linkage is feasible between the two side chains. The backbone was kept intact, and the lysine side chains were extended to accommodate the fumaryl linkage (*5*). The modeling was done to have symmetric linkage without van der Waals overlap between the atoms of the new group with the existing atoms of hemoglobin. The modified dihedral angles were also within reasonable limits.

The low-resolution crystal structure, (pdb code 1B86) (*37*) was chosen for HPPr-HbA modeling. This deoxy structure contains the DPG. This would enable us to position the phosphates of the HPPr group close to the DPG phosphate groups. The HPPr group linkage was modeled using both the builder module and the biopolymer module. Efforts were made to bring the two phosphate groups close to the position of the phosphate groups of DPG bound within the ββ-cleft with no van der Waals overlap. The dihedral angles were also within reasonable limits.

RESULTS

Preparation and Characterization of αα-Fumaryl-HPPr-HbA. The reactivity of HPPr-HbA to undergo αα-fumaryl cross-linking with DBBF under the conditions used for HbA was established by globin chain analysis of the reaction products by RPHPLC. As can be seen in Figure 1, the RPHPLC profiles of the two reaction products are quite comparable and consist of uncross-linked β-globin and cross-linked α-globin as the two major products. These results indicated that the extent of cross-linking of HPPr-HbA by DBBF was comparable to that of HbA as reflected by the formation of αα-fumaryl globin (Figure 1). The HPPr modification of Hb did not alter the reactivity of Lys-99(α) to form αα-fumaryl cross-linking.

αα-fumaryl-HPPr-HbA was purified by chromatography on a Q-Sepharose High Performance column. There were two major peaks in the chromatogram, labeled as Peak 1 and Peak 2 (Figure 2). The IEF analysis of the peaks is shown in the inset in Figure 2. Peak 1 is homogeneous (inset in Figure 2, lane 4), whereas Peak 2 is heterogeneous, containing products that are more acidic than the Peak 1 component (lane 5). Presumably, these are the products modified by DBBF at multiple sites. The more faint bands, in Peak 1, corresponding to minor products accounted for less than 5%. Therefore, Peak 1 was selected for further studies without further purification.

The IEF profile of αα-fumaryl-HPPr-HbA was compared with those of HbA, αα-fumaryl-HbA, and HPPr-HbA (Figure 2, inset). As can be seen, HPPr-HbA (lane 3) exhibited a lower isoelectric point than that of HbA (lane 1). This is primarily due to the introduction of the negatively charged phosphate group at Val-1(β) and also due to the lowered pK_a of the α-amino group of Val-1(β) as a result of its conversion into a secondary amine. In contrast, the isoelectric point of αα-fumaryl-HPPr-HbA (lane 4) is comparable to that of HPPr-HbA (lane 3), despite the loss of the positive charges of two of its ε-amino groups due to the introduction of the αα-fumaryl cross-linking. This phenomenon is similar to that observed with the αα-fumaryl cross-linking of HbA (lanes 1 and 2), a result consistent with the earlier reports (*5*).

The two globin chains of αα-fumaryl-HPPr-HbA were analyzed by ESI mass spectrometry (Table 1). The mass of the β-chain indicated that each β-chain is conjugated to only one HPPr moiety, and no DBBF modification of the β-chain has taken place. However, the mass of the α-component

Table 2: Oxygen Affinity of αα-Fumaryl-HPPr-HbA and Its Modulation by Allosteric Effectors[a]

effector	HbA	HPPr-HbA	αα-fumaryl-HbA	αα-fumaryl-HPPr-HbA
none	7.7(2.6)	24.5(2.1)	24.0(2.5)	48.5(1.8)
2.5 mM DPG	19.5(2.1)	26.0(1.8)	45.0(2.0)	49.0(1.7)
2.5 mM IHP	70.8(1.4)	28.5(1.9)	92.0(1.0)	48.5(1.7)
2.5 mM L35	63.0(1.3)	78.0(1.4)	48.5(1.9)	77.0(1.1)
1.0 M NaCl	24.0(2.3)	36.0(2.0)	39.5(2.0)	51.5(1.8)
0.1 M NaCl	13.0(2.4)	30.0(2.0)	29.5(2.1)	50.0(1.6)

[a] The Hill coefficient is given in parenthesis. In the measurements with P50 higher than 60 mm Hg, the Hb samples were not 100% oxygenated. These are some of the samples with IHP and L35. In these cases, cooperativity is also low. Therefore, the oxygenation values of Hbs at the maximum pO_2 (178 mmHg) were considered as 100% saturation in these experiments to determine the P50 values. These approximations underestimate the P50 values calculated.

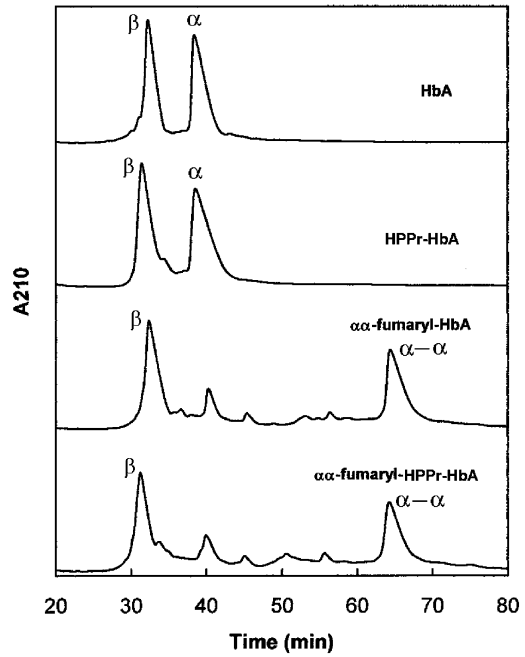

FIGURE 1: RPHPLC of modified hemoglobins. The same amount of hemoglobin samples were loaded in 0.3% heptafluorobutyric acid (HFBA) on a Vydac C4 column (4.6 × 250 mm) equilibrated with 35% acetonitrile (ACN) containing 0.1% HFBA. The globin chains were eluted with a gradient of 35−45% ACN in the first 10 min and then 45−50% ACN in 90 min at a flow rate of 1 mL/min. HFBA (0.1%) was present in the solvents throughout the gradient.

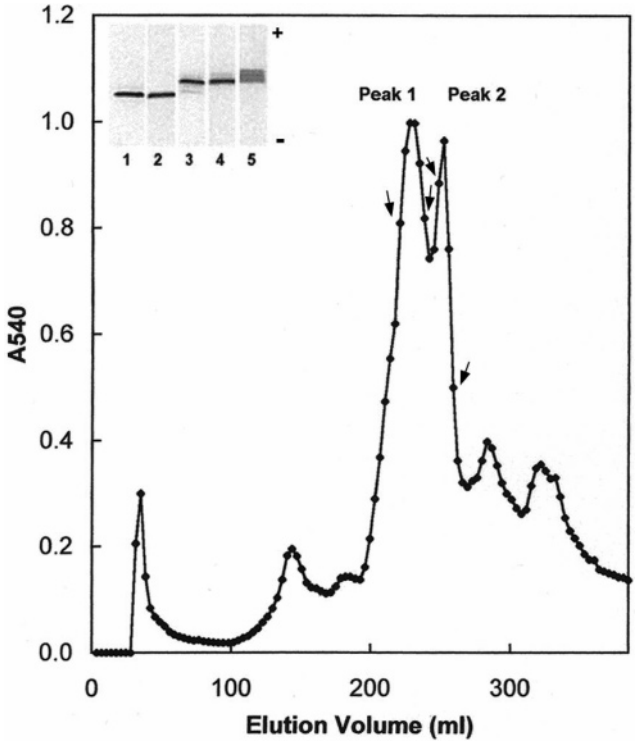

FIGURE 2: Purification of αα-fumaryl-HPPr-HbA on a Q-sepharose High Performance (0.9 × 30 cm) column. The column was equilibrated with 50 mM Tris acetate at pH 8.0 and eluted with a linear gradient consisting of 200 mL each of buffer A (50 mM Tris acetate at pH 7.7) and buffer B (50 mM Tris acetate at pH 6.8 containing 25 mM NaCl). The arrows indicate the fractions pooled from peaks 1 and 2. (Inset) IEF of modified hemoglobins: lane 1, HbA; lane 2, αα-fumaryl-HbA; lane 3, HPPr-HbA; lanes 4 and 5, Peaks 1 and 2 from Q-sepharose chromatography of αα-fumaryl-HPPr-HbA, respectively. The + and − signs indicate the positions of the anode and cathode during electrofocusing.

established the cross-linking of two α-chains by only one fumaryl group, and there were no signs of HPPr conjugation.

To further characterize the sites of modification in the doubly modified Hb, the modified α- and *β*-globins were digested with trypsin, and the tryptic peptides were analyzed by LC/MS. The masses of all of the peptides of the modified *β*-globin matched with that of the control *β*-globin, except for one peptide that corresponded to residues 1−8 of the *β*-chain. The peptide 1−8 carried an excess mass of 154 Da relative to that of the control peptide (Figure 3A). This mass corresponds to the mass of the HPPr moiety that has been conjugated to the *β*-globin. This establishes that G3P has site specifically modified the amino terminal of the *β*-chain in the doubly modified Hb.

A comparison of the masses of the tryptic peptides of the modified α-globin with that of the control revealed the appearance of a new peptide carrying a mass of 7612.056 Da (Figure 3B). This mass matched with the contiguous segment 93−127 of α-globin cross-linked by a fumaryl group. Thus, αα-fumaryl-HPPr-HbA carries an HPPr moiety at the amino terminal of the *β*-chain and a fumaryl cross-link at Lys-99 of the α-chains.

Functional Studies of αα*-Fumaryl-HPPr-HbA.* The O_2 affinity of HbA was lowered nearly to the same degree by both of the modifications studied. The O_2 affinities of HPPr-HbA and αα-fumaryl-HbA were about three times lower than that of HbA. The O_2 affinity of the doubly modified HbA, αα-fumaryl-HPPr-HbA, was about 6-fold lower than that of HbA (Table 2). Thus, αα-fumaryl cross-linking reduced the O_2 affinity of HbA 3-fold and that of HPPr-HbA only 2-fold. Therefore, the influence of HPPr modification and αα-fumaryl cross-linking of HbA on its O_2 affinity appears to be partially additive. The lowering of the O_2 affinity was accompanied by a small reduction in the Hill coefficient.

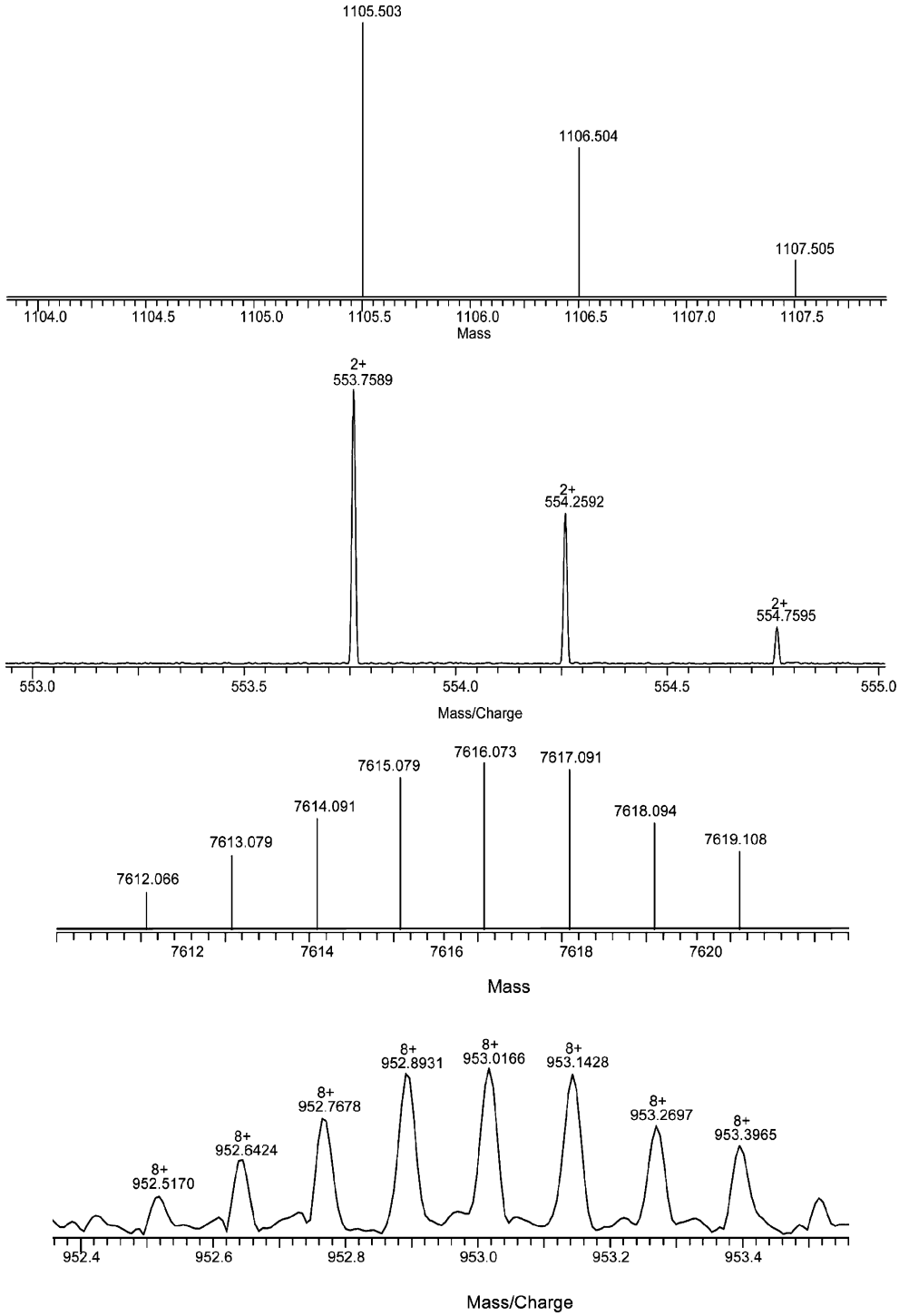

FIGURE 3: (A) Mass spectrum of the G3P modified peptide of β-globin from αα-fumaryl-HPPr-HbA. The mass of this peptide corresponds to the HPPr conjugated to the peptide comprising residues 1−8 of β-globin. (B) Mass spectrum of the fumaryl cross-linked peptide of the cross-linked α-globin from αα-fumaryl-HPPr-HbA. The mass of this peptide corresponds to two copies of the peptide spanning residues 93−127 of the α-globin cross-linked by a fumaryl group. The absence of cleavage at Lys-99(α) by trypsin is apparently a consequence of the covalent attachment of the ϵ-amino group of this residue to the fumaryl moiety.

Modulation of the O_2 Affinity of αα*-Fumaryl-HPPr-HbA by Allosteric Effectors.* The O_2 affinity of the Hb derivatives has been studied in the presence of 0.1 and 1.0 M NaCl. The derivatives with single modification, HPPr-HbA as well as αα-fumaryl-HbA, have retained some sensitivity to the presence of chloride, almost to the same extent (Table 2). However, the O_2 affinity of αα-fumaryl-HPPr-HbA was insensitive to the presence of chloride, reflecting the additivity of chloride mediated reduction in the O_2 affinity of the two modifications. It may also be noted that the O_2 affinity of both HPPr-HbA and αα-fumaryl-HbA in the absence of chloride was comparable to that of HbA in the presence of 1.0 M chloride. The O_2 affinity of the two modified Hbs could be reduced further by 1.0 M chloride. The electrostatic modification of either the $\beta\beta$-cleft or the mid central cavity increases the propensity of Hb to access the lower O_2 affinity conformation in the presence of chloride. The insensitivity of the doubly modified Hb to

Table 3: Percentage of Geminate Yield of Modified Hbs[a]

Hb	no effectors	+IHP	+L35	+IHP + L35
HbA	65	45	60	40
HPPr-HbA	57	57	52	52
αα-fumaryl-HbA	60	53	60	53
αα-fumaryl-HPPr-HbA	52	50	50	50

[a] The Hb concentration was 0.5 mM in the heme. IHP was added in 6-fold excess over the concentration of the tetramer. L35 was added in 4-folds excess over the concentration of the tetramer.

chloride suggests that the modulation of the O_2 affinity by the positive charge density of the central cavity has been completely neutralized by the presence of the two modifications.

The O_2 affinity of αα-fumaryl-HPPr-HbA was not influenced by the presence of 2,3-diphosphoglycerate (DPG) (Table 2). The O_2 affinity of HPPr-HbA was also insensitive to the presence of DPG. HPPr modification of Hb makes the molecule insensitive to the presence of DPG. The intrinsic O_2 affinity of HPPr-HbA was lower than that of HbA in the presence of DPG. Similarly, the intrinsic O_2 affinity of αα-fumaryl-HPPr-HbA was lower than the DPG modulated O_2 affinity of αα-fumaryl-HbA. The covalent attachment of the phosphate group at the DPG pocket seems to stabilize the T-structure of the tetramer better than the physiological modulator DPG.

Although inositol hexaphosphate (IHP) is a stronger modulator of the O_2 affinity of HbA, like DPG, it had negligible effect on the O_2 affinity of HPPr-HbA and αα-fumaryl-HPPr-HbA. In contrast, IHP reduced the O_2 affinity of αα-fumaryl-HbA to a level greater than that observed with HbA. Thus, HPPr modification essentially desensitizes the influence of IHP to modulate the O_2 affinity of HbA as well as αα-fumaryl-HbA.

The effect of the allosteric effector 2-[4-(3,5-dichlorophenylureido)phenoxy]-2-methylpropionic acid (L35) that binds at the αα-end of the central cavity (*38*) is quite opposite to that of DPG and IHP that bind at the *ββ*-cleft. L35 reduced the O_2 affinity of αα-fumaryl-HPPr-HbA to a level lower than that of HbA (Table 2). The intrinsic P50 of αα-fumaryl-HPPr-HbA was comparable to that of HPPr-HbA in the presence of L35. The O_2 affinity-reducing potential of HPPr modification and that of L35 appear to act additively on HbA and on αα-fumaryl-HbA. This additivity is consistent with the report that the O_2 affinity-reducing potential of DPG and/or IHP and that of L35 are additive (*38*). However, the αα-fumaryl cross-linking of HbA reduced the propensity of L35 to lower the O_2 affinity of HbA. The HPPr modification of αα-fumaryl-HbA overcomes the inhibitory activity of αα-fumaryl cross-bridging on the L35 mediated reduction in the O_2 affinity of HbA.

Geminate Recombination Studies. The geminate recombination of CO to photodissociated products of modified Hbs was determined to understand the structure of the initial population of the derivatives in R-state (*39*−*44*). The geminate yield of HPPr-HbA and αα-fumaryl-HbA was about 12 and 8% lower than that of HbA (Table 3). The geminate yield of αα-fumaryl-HPPr-HbA was 20% lower than that of HbA, indicating that the two modifications made an additive impact on the structure of αα-fumaryl-HPPr-HbA. The geminate yield of HPPr-HbA was insensitive to IHP and was lowered by L35. In contrast, the geminate yield of αα-fumaryl-HbA responded to IHP but was not influenced by L35. HPPr modification of αα-fumaryl-HbA neutralized the inhibitory influence of αα-cross-linking on L35 modulation, as was seen with the O_2 affinity.

Table 4: Iron-Proximal Histidine Stretching Frequency of Modified Hbs[a]

Hb	no effectors	+IHP	+L35	+IHP + L35
HbA	230.0	228.0	228.5	225.0
HPPr-HbA	229.0	229.0	227.0	228.0
αα-fumaryl-HbA	227.0	225.5	226.0	224.0
αα-fumaryl-HPPr-HbA	226.0	226.0	225.3	226.0

[a] All of the values are given in wavenumbers. Hb concentration was 0.5 mM in the heme. IHP was added in 6-fold excess over the concentration of the tetramer. L35 was added in 4-fold excess over the concentration of the tetramer.

Table 5: Number of Titrable Thiol Groups of Modified Hbs

Hb	titrable thiol groups
HbA	2.1
HPPr-HbA	2.2
αα-fumaryl-HbA	2.2
αα-fumaryl-HPPr-HbA	1.9

Visible Resonance Raman Spectroscopy. Table 4 shows the influence of the chemical modification on the Fe−His stretching frequency of Hb, ν(Fe−His). It is clear that the correlation between the reduction in the frequency of ν(Fe−His) and that in GY is not operative across the board with respect to all of the listed derivatives of Hb. Most notably, the decrease in frequency was less for the HPPr modification than for the αα-fumaryl modification, and yet the GY was lower for the former. The absence of an absolute one-to-one correspondence between the two parameters is likely to arise from either one or two factors. The frequency of ν-(Fe−His) has been correlated with the contribution to the kinetic barrier at the heme because of the proximal strain (*39*−*40*, *45*−*46*). Proximal effects are claimed to be a bigger factor for the α-subunits, whereas distal effects are supposed to dominate the rebinding for the *β*-subunits (*47*). Different modifications may impact the α- and *β*-subunits differently or have disparate effects on factors contributing to the GY, for example, conformational mobility that facilitates ligand escape. Alternatively, as noted above, the relaxation of structure subsequent to photodissociation can influence the GY, whereas the given Raman frequency is reflective of the unrelaxed or the minimally relaxed conformation. Differences in the GY could arise from differences in the conformational relaxation rates subsequent to photodissociation.

Correlation between the O_2 Affinity of Modified/Mutant Hbs and the Reactivity of Their Cys-93(β) in the Oxy Conformation to Form a Mixed Disulfide with Dithiodipyridine. Alterations in the O_2 affinity of Hb has been suggested to correlate with changes in the reactivity of Cys-93(*β*) (*48*−*53*). In order to determine whether deoxy-like conformational features of the modified Hbs are translated to the reactivity of Cys-93(*β*), the thiol−disulfide exchange reaction of modified Hbs has been studied. The number of titrable thiol groups of the derivatives is listed in Table 5. The kinetics of the reaction of Cys-93(*β*) of these Hbs in their carbonmonoxy form with 4-PDS is shown in Figure 4. The rate of modification of the Cys-93(*β*) of αα-fumaryl-HbA was considerably lower than that of HbA. However, the HPPr

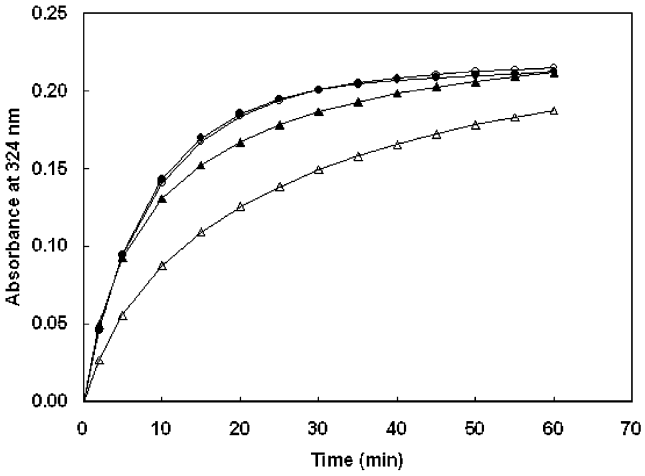

FIGURE 4: Kinetics of the reaction of modified hemoglobins with 4-PDS. The carbonmonoxy form of Hb (5 μM) was reacted with 50 μM 4-PDS in 50 mM Bis-Tris/Tris acetate at pH 7.4, at 30 °C. The reaction kinetics was followed by monitoring the formation of the reaction product of 4-PDS, 4-thiopyridone, at 324 nm. (●) HbA; (○) HPPr-HbA; (▲) αα-fumaryl-HbA; (△) αα-fumaryl-HPPr-HbA.

modification of HbA did not significantly influence the reactivity Cys-93(β). However, the reactivity of Cys-93(β) of αα-fumaryl-HPPr-HbA was even lower than that of αα-fumaryl-HbA. Thus, both modifications together induced a synergistic influence on the reactivity of Cys-93(β).

Molecular Models of αα*-Fumaryl-HPPr-HbA.* Figure 5A depicts the molecular model of doubly modified Hb. In the Figure, the central cavity of the doubly modified Hb is viewed from the $\beta\beta$-end of the central cavity to provide an understanding of the positioning of the two central cavity modifications engineered into Hb to generate the very low O_2 affinity molecule. In the model, the α-chains are shown as red ribbons and the β-chains as blue ribbons. The hemes are depicted in green. The αα-fumaryl cross-bridge is shown in magenta, and the HPPr groups within the $\beta\beta$-cleft are shown in cyan with the phosphate groups in red. The molecular models of singly modified Hbs have also been generated (data not shown), and these models have established that the presence of one modification has very limited influence on the structural changes induced into Hb by the other.

The exploded view of the $\beta\beta$-cleft of doubly modified Hb is shown in Figure 5B. The fumaryl cross-link is not shown for clarity. The model has incorporated the DPG in the background in magenta to depict the location of the phosphate groups of DPG within the positive charge dense DPG binding pocket of the molecule. The carbon chain of the HPPr moiety is shown in cyan with the phosphate group depicted in red. Val-1(β) is shown in purple. The phosphate group of HPPr covalently linked to the amino group of Val-1(β) occupies a position within the $\beta\beta$-cleft, which is very close to the position occupied by the phosphate of DPG that is bound at the $\beta\beta$ cleft. The location of the peptide backbone of six positively charged residues of the DPG binding pocket that interact with DPG (His-2(β), Lys-82(β), and His-143-(β)) are identified in the ribbon diagram in yellow, and the side chains of these residues are also depicted. Internuclear distances between the negatively charged centers of the phosphate and the positively charged centers of the protein

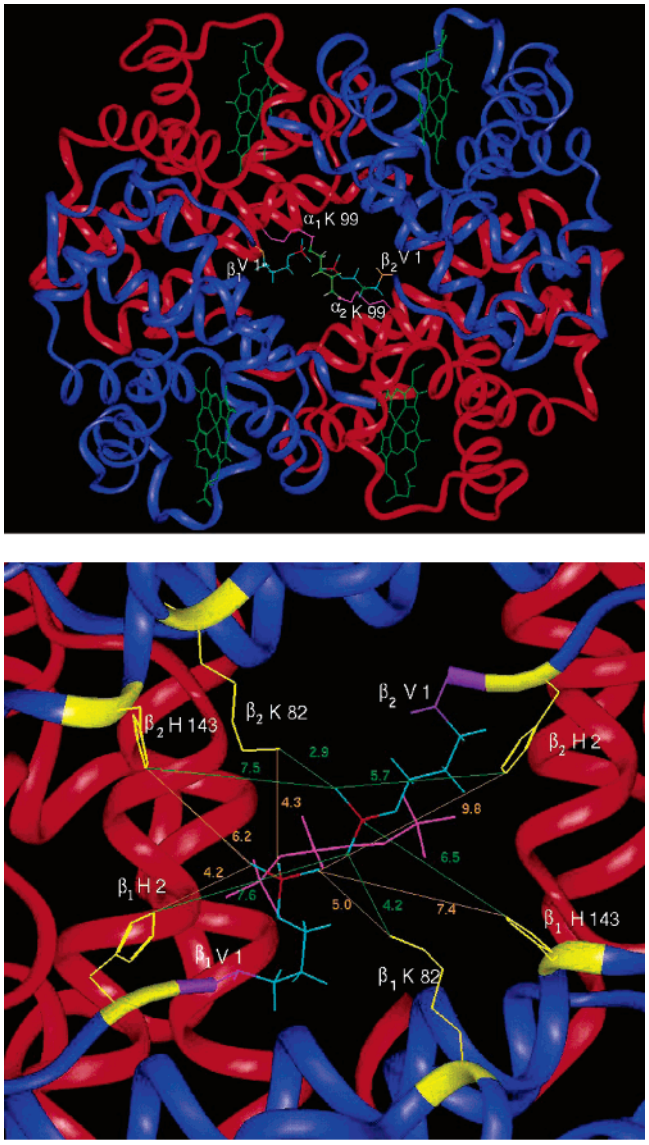

FIGURE 5: Molecular model of αα-fumaryl-HPPr-HbA. (A) The model shows the location of the two covalent modifications in the central cavity viewed from the $\beta\beta$-end of the central cavity. The α-chains are shown in red, the β-chains in blue, and the heme in green. The Val-1(β) side chains are shown in orange, and the HPPr groups are in cyan with their phosphates in red. The Lys-99(α) side chains are in magenta, and the fumaryl group is in green. (B) Exploded view of the DPG binding pocket of αα-fumaryl-HPPr-HbA. The α-chains are shown in red and the β-chains in blue. The fumaryl cross-link is not shown for clarity. The positively charged centers of the DPG pocket are shown in yellow with the side chains projecting out of the peptide backbone, except that Val-1(β) is shown in purple for clarity. The carbon chain of the HPPr moiety is in cyan, and its phosphate is in red. DPG is shown in the background in magenta to provide a feel for the location of the phosphates of the covalently linked HPPr moiety relative to the phosphates of DPG. Internuclear distances from the closest negative charge centers of the phosphates to the positively charged centers of the DPG residues are provided.

in the DPG pocket of Hb that have been implicated to interact with the phosphate groups of DPG have been measured. The closest distances measured are shown in Figure 5B and also summarized in Table 6. It is interesting to note that the negatively charged centers of the phosphate of the HPPr moiety linked to Val-1(β) could interact not only with the positive charge centers of the cis-dimers but also with those of the trans-dimers. Therefore, the HPPr moieties covalently

Table 6: Internuclear Distances within the $\beta\beta$-Cleft of $\alpha\alpha$-Fumaryl-HPPr-HbA[a]

residues of the β1-chain	internuclear distance (Å)	
	phosphate 1	phosphate 2
H2	4.2 (5.7)	9.8 (7.6)
K82	5.0 (2.9)	4.3 (4.2)
H143	7.4 (7.5)	6.2 (6.5)

[a] Only the closest distance between the negatively charged centers of the phosphate of the HPPr group and the positively charged groups of the DPG binding pocket ($\beta\beta$-cleft) are given. Internuclear distances for the opposite β-chain (β2-chain) are given in parentheses.

linked to Val-1(β) may be expected to function as the pseudo cross-link to stabilize the interdimeric interactions of the molecule. Thus, the doubly modified Hb is an intramolecular cross-linked Hb, with a covalent cross-link between the α-chains and a psuedo cross-link between the β-chains.

DISCUSSION

The modification of HbA with G3P generated a low O_2 affinity Hb (HPPr-HbA) that is insensitive to DPG and IHP. The modulation of the O_2 affinity of Hb by the covalently attached HPPr group is comparable to that of pyridoxal phosphate in $\alpha_2(\beta^{PLP})_2$ (*28*) and to a higher level than that by the carboxymethyl group (*4*) or galacturonic acid (*27*) conjugated at Val-1(β). Thus, the introduction of a phosphate group at this site seems to stabilize the low O_2 affinity conformation better than a carboxyl group. However, the addition of a phosphate at Val-1(β) does not seem to be enough to exhibit such an impact on the oxygen affinity of Hb. Affinity labeling of Val-1(β) with glucose-6-phosphate does not reduce the O_2 affinity of Hb (*54*) to the extent that is seen with G3P or pyridoxal phosphate. The structural features of these added groups seem to make a major contribution toward this effect. In $\alpha_2(\beta^{PLP})_2$, the two phosphates of the PLP groups take positions very close to the positions of the phosphates of DPG in deoxy Hb (*55*). Thus, these phosphates can mimic the influence of DPG in stabilizing the deoxy state of Hb. G3P, with similarities in structure with DPG, seems to exhibit a similar impact on the O_2 affinity of Hb.

In order to understand the interactions of the HPPr group with the DPG binding site, we have carried out molecular modeling studies of deoxy HPPr-HbA (Figure 5). These studies indicated that the negative charges of the phosphate of the HPPr group can interact with the positive charge of Lys82 of the cis as well as the trans β-chain of the modified Hbs (Table 6). In addition, interactions between His2 of the cis β-chain and His143 of the trans β-chain may also be possible. These interactions are comparable to the ones reported for pyridoxal phosphate modified Hb (*54*). Although, such interactions are likely to exist in carboxymethylated Hb and galacturonic acid modified Hb (*27*), phosphate mediated interactions in HPPr-HbA and $\alpha_2(\beta^{PLP})_2$ seem to be more intense. These interactions that operate across the $\beta\beta$-cleft, stabilizing a deoxy-like state conformation, are considered to serve as the pseudo cross-links within the DPG pocket (*55*−*56*).

HPPr-HbA reacts with DBBF under deoxy conditions in much the same way as the unmodified HbA in terms of the reactivity of Lys-99(α) to form the cross-bridge. Thus, the electrostatic modification of Val-1(β) of the $\beta\beta$-cleft does not seem to perturb the orientation or reactivity of the ϵ-amino groups of Lys-99(α) in the deoxy state. Besides, $\alpha\alpha$-fumaryl-HPPr-HbA exhibited an isoelectric point comparable to that of HPPr-HbA. The loss of positive charge resulting from $\alpha\alpha$-fumaryl cross-linking is not apparent from the isoelectric focusing pattern. This behavior is consistent with the earlier observation that the isoelectric focusing pattern of $\alpha\alpha$-fumaryl-HbA under oxy conditions is nearly identical to the isoelectric focusing pattern of HbA (*5*). This compensation in the charge of HbA has been suggested to be a result of an increased p*K*a of a neighboring residue, Glu-101(α). This phenomenon seems to be conserved in the doubly modified derivative, $\alpha\alpha$-fumaryl-HPPr-HbA. This behavior is distinct as compared to that seen on the generation of $\alpha\alpha$-fumaryl-Hb-P (*31*). The electrostatic modifications in $\alpha\alpha$-fumaryl-Hb-P are both from the mid central cavity, whereas $\alpha\alpha$-fumaryl-HPPr-HbA has one modification in the mid central cavity and the other in the $\beta\beta$-cleft.

Combining the electrostatic modification of Val-1(β) with $\alpha\alpha$-fumaryl cross-linking results in a partial additive influence in terms of reducing the O_2 affinity of HbA. From the mutant hemoglobin analysis, it has been hypothesized that the number of positive charges in the central cavity determines the O_2 affinity of the molecule (*58*). It is suggested that the stability of the T-structure is inversely proportional to the overall positive charge in the central cavity. Accordingly, the lowered O_2 affinity of $\alpha\alpha$-fumaryl-HPPr-HbA is consistent with the hypothesis that the reduction of the positive charge in the central cavity of Hb generates a more stable T-structure.

$\alpha\alpha$-Fumaryl-HPPr-HbA lacks sensitivity toward allosteric effectors, chloride, DPG, and IHP. The molecular modeling studies of $\alpha\alpha$-fumaryl-HPPr-HbA indicated that the fumaryl cross-link can be introduced into the mid central cavity of HPPr-HbA without altering the positions of the HPPr groups at Val-1(β) (Figure 5). Therefore, the electrostatic interactions between phosphates of the HPPr group and the positive charges of DPG residues that are possible in HPPr-HbA can also operate in $\alpha\alpha$-fumaryl-HPPr-HbA. The presence of the $\alpha\alpha$-fumaryl cross-bridge in the mid central cavity coupled with the pseudo cross-link in the $\beta\beta$-cleft can, therefore, be expected to drastically reduce the plasticity of the molecule in these two domains of the central cavity. It may be noted that HbA that is carboxymethylated at all its four α-amino groups is also insensitive to the presence of these effectors (*4*). Thus, the desensitization of Hb to the presence of chloride, DPG, and IHP can be achieved either by electrostatic modification of the $\alpha\alpha$-end and the $\beta\beta$-cleft of the central cavity or by combining the electrostatic modification of the $\beta\beta$-cleft with the $\alpha\alpha$-fumaryl cross-linking in the mid central cavity of HbA.

$\alpha\alpha$-Fumaryl-HbA exhibited reduced sensitivity to L35 as compared to HbA. This is expected because L35 binds at the $\alpha\alpha$-end with its distal end projecting into the cavity closer to Lys-99 of the α-chain (*38*). Similarly, $\alpha\alpha$-fumaryl-HPPr-HbA also exhibited reduced sensitivity to L35. However, although the extent of modulation of the O_2 affinity of $\alpha\alpha$-fumaryl-HPPr-HbA by L35 was less than that of HbA, the O_2 affinity of this derivative in the presence of L35 was lower than that of HbA and was comparable to that of HPPr-HbA

in the presence of the same effector. The electrostatic modification of the $\beta\beta$-cleft of $\alpha\alpha$-fumaryl-HbA compensates for the structural consequences of the presence of the cross-bridge in the mid central cavity that reduces the modulation of the O_2 affinity of HbA by L35.

The geminate recombination of CO is highly responsive to the conformational properties of HbA (*39*−*44*). The geminate binding of CO to photodissociated COHb occurs within a few hundred nanoseconds. Therefore, the geminate yield indicates the ligand binding affinity of the foremost structure of Hb in the R-to-T transition. Apparently, this initial structure is expected to have the highest geminate yield because other structures in the R-to-T transition attain a lower ligand affinity conformation. The present geminate binding studies of modified Hbs indicated that HPPr modification reduces the ligand binding affinity of the initial population of photodissociated HbA more than $\alpha\alpha$-fumaryl cross-linking. However, the P50 values of HPPr-HbA and $\alpha\alpha$-fumaryl-HbA were comparable. This may imply that the subsequent structures in the R-to-T transition of $\alpha\alpha$-fumaryl-HbA exhibit a larger variation in ligand binding affinity as compared to those of HPPr-HbA. In $\alpha\alpha$-fumaryl-HPPr-HbA, both modifications exerted a combined influence on geminate binding as well as on overall O_2 affinity. The influence of IHP was less on the geminate yield and more on the P50 of $\alpha\alpha$-fumaryl-HbA compared to that of HbA. Low ligand affinity structures of $\alpha\alpha$-fumaryl-HbA seem to respond more to IHP than the initial population. Similarly, the effect of L35 was less on the geminate yield of HPPr-HbA and more on its P50 than that of HbA, indicating the enhanced influence of L35 on the intermediate structures of HPPr-HbA in the R-to-T transition.

The frequency of ν(Fe−His) indicates the conformation of Hb at the heme surroundings (*39*−*46*). This frequency is highest for the fully liganded R-structure and lowest for the unliganded T-structure. Modified hemoglobins with low O_2 affinity have been shown to have reduced ν(Fe−His) frequency in the liganded state. Because the frequency of ν(Fe−His) reflects the structure of the liganded R-state and the geminate yield determines the structure of the initial population for recombination, the comparison of these two parameters of modified Hb may indicate the ease with which one molecule undergoes changes in the tertiary structure at the heme after photodissociation. $\alpha\alpha$-Fumaryl-HbA and HPPr-HbA exhibited reduced frequency compared to that of HbA, indicating conformational changes at the heme. Interestingly, $\alpha\alpha$-cross-linking reduced the frequency more than HPPr modification, whereas the later modification reduced the geminate binding more than the former. This may be interpreted as follows: HPPr modification did not alter the R-state structure of HbA in the heme environment as much as $\alpha\alpha$-cross-linking. However, HPPr-HbA undergoes structural changes at the heme more rapidly than $\alpha\alpha$-fumaryl-HbA, upon photodissociation.

The change in the reactivity of Cys-93(β) in the oxy state can be considered as an indicator of a change at the $\alpha_1\beta_2$ interface in a given mutant or chemically modified Hb (*48*−*53*). The electrostatic modification at the $\beta\beta$-cleft had no influence on the reactivity of Cys-93(β), even though its O_2 affinity was lower than that of HbA. However, the $\alpha\alpha$-fumaryl cross-bridge that lowers the O_2 affinity of HbA also lowered the reactivity of Cys-93(β). The doubly modified Hb, $\alpha\alpha$-fumaryl-HPPr-HbA, exhibited a Cys-93(β) reactivity even lower than that of $\alpha\alpha$-fumaryl-HbA, a synergistic influence of the two modifications.

The fumarate mediated cross-linking of Lys-99(α) of hemoglobin reduces the O_2 affinity of the tetramer without apparent alterations in its deoxy conformation (*5*). The reduction in O_2 affinity is primarily due to the reduction in K_R (*59*). Accordingly, the R-structure of $\alpha\alpha$-fumaryl-HbA has been predicted to be different compared to that of HbA. The environment of Cys-93(β) of $\alpha\alpha$-fumaryl-HbA appears to be perturbed from the one in the R-structure of HbA, reducing the reactivity of its thiol group. The reduced reactivity of Cys-93(β) on the deoxygenation of HbA has been attributed to the conformational changes as well as to the salt bridge formed between His-146(β) and Asp-94(β) (*49*). Des-His-146(β) HbA exhibited an increased reactivity of Cys-93(β) in oxy conformation, indicating that His-146(β) influences the reactivity of Cys-93(β) even in the oxy structure. The FT-IR studies of oxy and met hemoglobins suggested a correlation between the reactivity of Cys-93(β) and the probability of this residue being external to the F−H pocket (*60*). An interaction between Cys-93(β) and Tyr-145(β) that can influence the reactivity of Cys-93(β) has also been suggested. The $\alpha\alpha$-fumaryl cross-linking of HbA has altered one or more of these interactions, resulting in a reduction in the reactivity of Cys-93(β) in the oxy conformation.

The results of the present study along with the earlier results of $\alpha\alpha$-fumaryl-Hb-P and the tetra carboxymethylated Hb demonstrated that electrostatic modification of the $\beta\beta$-end, $\alpha\alpha$-end, and mid central cavity that lower the O_2 affinity can be combined in pairs to generate species of Hb that exhibit O_2 affinity lower than that with either of the modifications. An interesting aspect of the two very low O_2 affinity forms of Hb generated by combining the two chemical perturbations of the central cavity of Hb, namely, $\alpha\alpha$-fumaryl-Hb-P and $\alpha\alpha$-fumaryl-HPPr-Hb, is that the O_2 affinity of both species is insensitive to the presence of allosteric effectors. Presumably, these represent the conformational state of Hb, wherein the protein has accessed the very low affinity T-state. In contrast, the PEGylation of Hb, particularly hexaPEGylation of Hb with PEG-5000, induces a degree of rigidity to the oxy conformational state of Hb, which is apparently a high O_2 affinity R-state, again nonresponsive to allosteric effectors. If the very low O_2 affinity Hbs are subjected to the hexaPEGylation protocol that we have used to generate the current versions of non-hypertensive Hbs, it is conceivable that PEGylated Hbs with very low O_2 affinity are generated.

HexaPEGylation of $\alpha\alpha$-fumaryl cross-linked Hb generated a product that has an O_2 affinity comparable to that of unmodified Hb (*26*). The presence of the $\alpha\alpha$-fumaryl cross-link in the PEGylated Hb has partially compensated the high O_2 affinity-inducing propensity of the PEGylation reaction. The O_2 affinity of the PEGylated $\alpha\alpha$-fumaryl Hb is intermediate to that of PEGylated Hb and $\alpha\alpha$-fumaryl Hb. The G3P modification in HPPr-$\alpha\alpha$-fumaryl-HbA is expected to further neutralize the influence of PEGylation to generate a PEGylated Hb with an O_2 affinity intermediate to that of PEGylated $\alpha\alpha$-fumaryl Hb and HPPr-$\alpha\alpha$-fumaryl-HbA. Availability of a series of PEGylated Hbs with varying O_2 affinities can facilitate the production of non-hypertensive

PEGylated Hbs as blood substitutes for customized clinical applications.

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