PEGylation of Val-1(α) Destabilizes the Tetrameric Structure of Hemoglobin[†]

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ABSTRACT: A hexaPEGylated hemoglobin (Hb), (Propyl-PEG5K)$_6$-Hb, is essentially in $\alpha\beta$ dimers (Hu et al. (2007) *Biochem. J. 402*, 143−151). In order to provide a biochemical insight into the tetramer−dimer dissociation of this PEGylated Hb, we prepared and characterized two PEGylated Hbs site-specifically modified at Val-1(α) and at Val-1(β), respectively. PEGylation at Val-1(α) and at Val-1(β) increase the tetramer−dimer dissociation constant (K_d) of Hb by 2 and 1 order of magnitude, respectively. Accordingly, the sites of PEGylation can determine the tetramer stability of the PEGylated Hb. In order to determine the role of the polyethylene glycol (PEG) chains on the tetramer stability of Hb, we prepared a propylated Hb site-specifically modified at Val-1(α). Interestingly, site-specific propylation of Hb at Val-l(α) stabilizes the Hb tetramer by 1 order of magnitude. Therefore, conjugation of the PEG chains at Val-1(α) can greatly destabilize the tetramer stability of Hb. On the structural aspects, the PEG chains conjugated at Va-1(α) unfavorably alter the heme environment and quaternary structure and destabilize the $\alpha 1\beta 2$ interface of Hb. On the functional aspects, the PEG chains conjugated at Val-1(α) decrease the Hill coefficient, the Bohr effect of Hb and the sensitization to the presence of the allosteric effectors. In contrast, PEGylation of Hb at Val-1(β) gives rise to less pronounced structural alteration and different functional change.

Development of hemoglobin (Hb[1]) based oxygen carriers (HBOCs) has been a field of intensive investigation in the past (*1, 2*). As a potential HBOC, the PEGylated Hb is vasoinactive (*3, 4*) and non-nephrotoxic (*5*). For example, MP4 (Sangart Inc.), a hexaPEGylated Hb, is a prototype of (SP-PEG5K)$_6$-Hb and currently under phase III clinical trial (*5*).

Typically, Hb undergoes dissociation from its native tetrameric state ($\alpha_2\beta_2$) to dimeric state ($\alpha\beta$ dimer) (*6*). The *in vivo* rapid dissociation of acellular Hb to $\alpha\beta$ dimers leads to nephrotoxicity (*7*). Due to its unusually high molecular volume that can avoid kidney filtration, the tetramer stability of the PEGylated Hb has been ignored. However, the PEGylation induced extensive tetramer−dimer dissociation led to the significant structural and functional perturbation of Hb (*8*). For example, (Propyl-PEG5K)$_6$-Hb is essentially in $\alpha\beta$ dimers (*8*). However, (Propyl-PEG5K)$_6$-Hb is a heterogeneous mixture and not an ideal model protein to investigate the tetramer−dimer dissociation of the PEGylated Hb. (Propyl-PEG5K)$_6$-Hb is a hexaPEGylated Hb where four polyethylene glycol (PEG) chains are conjugated at Val-1(α) and Val-1(β) and the other two at the Lys residues (*9*). Presumably, PEGylation at Val-1(α) and Val-1(β) may play a stronger influence than that at the Lys residues on the tetramer stability of Hb.

Val-1(α) and Val-1(β) are located topologically at the αα end and the $\beta\beta$ end of the central cavity of HbA, respectively (*10*). They are involved in a number of well-known functions, such as the transition from R state to T state and the cooperative release or uptake of oxygen (*2*). Variants of Hb with amino acid substitutions (e.g., glutamic acid (*11*)) or chemical modification (e.g., carbamylation (*12*), carboxymethylation (*13*) and reductive hydroxyethylation (*14*)) at the amino terminus have been used to investigate the structure and the function of Hb. The substitution of Ala or acetyl Ser for Val-1(α) by site-directed mutagenesis increases the tetramer stability of Hb. The same substitution for Val-1(β) displays an insignificant role in the tetramer stability of Hb (*15*). Therefore, chemical modification at Val-1(α) and Val-1(β) may alter the tetramer stability of Hb.

Association of Hb dimers into tetramers is driven primarily by the interactions between residues at the $\alpha 1\beta 2$ interface (*16*). The structural changes at the $\alpha 1\beta 1$ interface (e.g., the

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[1] Abbreviations: Hb, hemoglobin; PEG, polyethylene glycol; PEGylated, conjugated with PEG; PEGylation, conjugation with PEG; PBS, phosphate buffered saline; IHP, inositol hexaphosphate; TFA, trifluoroacetic acid; K_d, the dissociation constant; HBOC, hemoglobin based oxygen carrier; IEF, isoelectric focusing; P_{50}, partial oxygen pressure at half-saturation; *S*, sedimentation coefficient; SEC, size exclusion chromatography; RP-HPLC, reverse phase high performance liquid chromatography; CD, circular dichroism; ΔH^+, the number of protons per heme released upon full oxygenation; *n*, Hill coefficient; DPG, 2,3-diphosphoglycerate; L35, 2-[4-(3,5-dichlorophenylureido)phenoxy]-2-methylpropionic acid; 4-PDS, 4,4′-dithiopyridine.

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substitution β112 Cys → Gly) appear to propagate to the $\alpha1\beta2$ interface (*17*). The tetramer−dimer equilibrium of Hb is influenced by external factors (e.g., ion strength and pH) or by allosteric effectors (e.g., Cl^- and organic phosphate) (*18, 19*). The PEG chain provides a unique balance of hydrophobicity/hydrophilicity and the propensity to occupy a large volume in an aqueous environment (*20*). This is partially due to the chain flexibility as well as an extensive degree of its hydration (*20*). Accordingly, conjugation of the PEG chains to Hb may perturb the $\alpha1\beta2$ interface and alter the tetramer stability of Hb by weakening the hydrogen bond and the hydrophobic interactions of Hb.

The extensive tetramer−dimer dissociation of (Propyl-PEG5K)$_6$-Hb may be a consequence of the sites of PEGylation [e.g., Val-1(α) or Val-1(β)] or alternatively a cumulative effect of PEGylation. Accordingly, we prepared and characterized two PEGylated Hbs site-specifically modified at Val-1(α) and at Val-1(β) and one propylated Hb site-specifically modified at Val-1(α). Size exclusion chromatography (SEC), sedimentation equilibrium and sedimentation velocity have been used to evaluate the tetramer stability of the PEGylated Hbs. Besides, the effects of the PEG chains and the sites of PEGylation on the structure and the function of the PEGylated Hb have been measured in the present study.

EXPERIMENTAL PROCEDURES

Preparation of the PEGylated Hbs. Human adult hemoglobin (HbA) was purified from human erythrocytes as described previously (*21*). $\alpha\alpha$-Fumaryl Hb was prepared as described previously (*22*). HbA (0.25 mM tetramer) was incubated with 1.0 mM inositol hexaphosphate (IHP) in 50 mM BisTris-Ac buffer (pH 6.5) for 1 h. HbA (0.25 mM) or IHP bound HbA (0.25 mM) was reacted with 1.5 mM PEG5K propionaldehyde (Sunbio Inc., Korea) and 7.5 mM $NaCNBH_3$ (Sigma Chemical Co.) in 50 mM BisTris-Ac buffer (pH 6.5) overnight at 4 °C. The reaction mixture was dialyzed against 50 mM Tris-Ac buffer (pH 8.5) and loaded on a Q Sepharose high performance (HP) column (2.6 × 65 cm^2) equilibrated with 50 mM Tris-Ac buffer (pH 8.5). The column was eluted with 8 column volumes of 50 mM Tris-Ac buffer using a pH gradient of 8.5−7.0 at a flow rate of 2.0 mL/min. The desired fractions were fractionated and concentrated. [Propyl-PEG5K-Val-1(α)]$_2$-$\alpha\alpha$-Hb was prepared and purified essentially as the condition for [Propyl-PEG5K-Val-1(α)]$_2$-Hb, except that $\alpha\alpha$-fumaryl Hb was used for PEGylation.

Preparation of the Propylated Hb at Val-1(α). The IHP bound HbA (0.25 mM) was reacted with 1.5 mM propionaldehyde (Sigma) and 7.5 mM $NaCNBH_3$ in 50 mM BisTris-Ac buffer (pH 6.5) overnight at 4 °C. The reaction mixture was dialyzed against 50 mM Tris-Ac buffer (pH 6.0) and loaded on a Mono S column (0.5 × 5 cm^2). The column was equilibrated by 50 mM Tris-Ac buffer (pH 6.0) and eluted by 40 column volumes of 50 mM Tris-Ac buffer using a pH gradient of 6.0−7.0 at a flow rate of 0.8 mL/min. The desired fractions were fractionated and concentrated.

Analytical Methods. Size exclusion chromatography (SEC) of the Hb samples was carried out at room temperature on two Superose 12 columns (Amersham-Pharmacia Biotech, 1 × 31 cm^2) connected in series. The columns were equilibrated and eluted by PBS buffer (pH 7.4) at a flow rate of 0.5 mL/min. Reverse phase (RP) HPLC analysis of the globin chains was carried out on a Vydac C4 column (0.46 × 25 cm^2) using a linear gradient of 35−50% acetonitrile containing 0.1% trifluoroacetic acid (TFA) in 100 min at a flow rate of 1.0 mL/min. SDS−PAGE analysis was carried out on a precast 14% tris-glycine gel from Invitrogen Corporation. The gel was stained with Coomassie Blue. Isoelectric focusing (IEF) analysis was operated using a precast resolve gel from Isolab and a blend of pH 6−8 resolve ampholytes. The gel was electrofocused for 3 h to resolve the components in the sample completely. Molecular radius measurement of the Hb samples (1 mg/mL) was performed using a dynamic light scattering instrument (DynaPro, Protein Solutions, Lakewood, NJ). The number of reactive sulfhydryl groups in the PEGylated Hb was estimated by titration with 4,4′-dithiopyridine (4-PDS) as described by Ampulski et al. (*23*).

Mass Spectrometry. The intact globins of the modified Hbs were isolated by RP-HPLC on a Vydac C4 column (0.46 × 25 cm^2). The fractions containing the desired globin chains were fractionated and lyophilized for analysis by MALDI-TOF mass spectrometer (Voyger Inc.). The lyophilized globin samples were digested with trypsin at the ratio of 100:1 (w/w) at 37 °C, pH 8.5 for 3 h. Tryptic peptides of the globin chains were analyzed by MALDI-TOF/TOF on an ABI 4700 TOF/TOF mass spectrometer (Applied Biosystem, Foster City, CA). The modified tryptic peptides are selected for fragmentation on the ABI 4700 TOF/TOF mass spectrometer.

Analytical Ultracentrifugation. All experiments were conducted at 25 °C in a Beckman XL-I analytical ultracentrifuge equipped with absorbance optics and an An60-Ti rotor. PBS buffer at pH 6.5, 7.4 or 8.5 was used as indicated in the text. NaOH or HAc was used to adjust the pH of the PBS buffer. Boundary movement or concentration distributions were followed at 405 nm using the centrifuge's absorption optics.

Sedimentation velocity experiments were conducted at 55,000 rpm using double sector cells. Data were collected at three nominal concentrations (A_{405} = 0.1, 0.5 and 1.0) for each sample. The $g(s^*)$ distributions were determined using DCDT+ v2.0.4 (http://www.jphilo.mailway.com) using values of $\bar{\nu}$ of 0.74 mL/g for HbA and [Propyl-Val-1(α)]$_2$-Hb (*24*) and 0.806 mL/g for the PEGylated Hbs (*25*). The $g(s^*)$ was normalized to standard conditions ($S_{20,W}$ and $D_{20,W}$) by correcting for buffer density and viscosity.

Sedimentation equilibrium experiments were conducted at 10,000 and 15,000 rpm using six channel centerpieces. Three protein concentrations (A_{405} = 0.1, 0.4 and 0.8) were analyzed for each sample. The identity of scans taken at 22 and 24 h confirmed that the samples had reached equilibrium. The data was globally analyzed using HeteroAnalysis v1.1.33 (J. L. Cole and J. W. Lary, Analytical Ultracentrifugation Facility, Biotechnology Services Center, University of Connecticut, Storrs, CT 06269). The best-fit values and joint confidence intervals are reported.

Tetramer−dimer Dissociation Constant Measured by SEC. The tetramer−dimer dissociation constant (K_d) of the PEGylated Hbs was measured essentially as described by Manning et al. (*26*). Briefly, Hb samples were diluted to a series of Hb concentrations and subjected to two Superose 12 columns (1 × 31 cm^2) in series. PBS buffer at pH 6.5,

7.4 or 8.5 was used as indicated in the text. NaOH or HAc was used to adjust the pH of the PBS buffer. The columns were equilibrated and eluted at a flow rate of 0.5 mL/min. αα-Fumaryl Hb was set as the tetramer for HbA and [Propyl-Val-1(α)]$_2$-Hb. [Propyl-PEG5K-Val-1(α)]$_2$-αα-Hb was set as the tetramer for the two PEGylated Hbs. The Hb samples in 0.9 M $MgCl_2$ were set as the dimers. The columns were equilibrated and eluted with 0.9 M $MgCl_2$ for establishing the elution position of the dimers.

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra of the Hb samples were recorded on a JASCO-720 spectropolarimeter (Tokyo, Japan) at 25 °C with a 0.2 cm light path cuvette (310 μL). For the CD spectra from 250 to 200 nm, the Hb concentration was 1.3 μM as tetramer. For the CD spectra from 480 to 250 nm, the Hb concentration was 26.0 μM as tetramer. All the Hb samples were in PBS buffer, pH 7.4. The molar ellipticity (θ) is expressed in deg·cm^2/dmol on a heme basis.

Front-Face Fluorescence Measurements. Intrinsic front-face fluorescence measurements were performed using a Shimadzu RF-5301 spectrofluorimeter at room temperature. The emission spectra were recorded from 300 to 400 nm using an excitation wavelength of 280 nm. Excitation and emission slit widths were both 5 nm. All the samples used were at Hb concentration of 6.0 mg/mL in PBS, pH 7.4. A cuvette with 1 cm path length was used.

Oxygen Affinity Measurements. Oxygen equilibrium curves of the Hb samples (0.5 mM) were obtained using Hem-O-Scan (Aminco) at 37 °C in PBS buffer (pH 7.4). In addition, the Hb samples were measured in the presence of allosteric effectors, 2.5 mM 2,3-diphosphoglycerate (DPG), 1.0 M NaCl, 2.5 mM IHP and 2.5 mM 2-[4-(3,5-dichlorophenylureido)phenoxy]-2-methylpropionic acid (L35) (*27*), respectively. The Bohr effect was investigated by measuring the oxygen affinity of the Hb samples in 100 mM sodium phosphate buffer at 37 °C over a wide pH range (6.5−8.5). The number of Bohr protons liberated by oxygenation was estimated using the Wyman equation: $\Delta H^+ = -\Delta \log P_{50}/\Delta pH$, where ΔH^+ is the number of protons per heme released upon full oxygenation (*28*).

RESULTS

Preparation and Purification of the PEGylated Hbs. Val-1(α) and Val-1(β) show an unusually higher reactivity to aldehyde group than Lys residues, due to their lower pK_a (6.95 for Val-1(α) and 7.05 for Val-1(β)) (*29*). IHP, an allosteric effector, can reversibly bind Val-1(β) and efficiently inhibit the modification at Val-1(β). Thus, site-specific PEGylation of Val-1(α) can be achieved at low pH in the presence of IHP. The PEGylated Hb was purified by anion exchange chromatography on a Q Sepharose HP column (Figure 1). Four major peaks (a, b, c and d) were observed for the sample in the absence of IHP (Figure 1A). Peak d elutes as a doublet, and both peaks are unmodified Hb as confirmed by HPLC analysis (data not shown). The unreacted PEG chains in the sample presumably influence the chromatographic behavior of Hb and resulted in its elution as a doublet (peak d). When Hb is PEGylated in the presence of IHP, peaks a and b essentially disappear (Figure 1B). Peaks b and c were fractionated for further characterization.

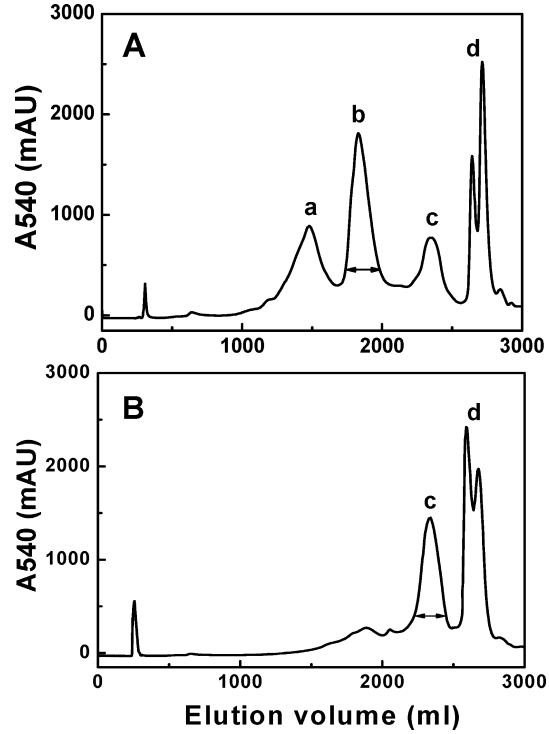

FIGURE 1: Purification of the PEGylated Hbs by ion exchange chromatography. The PEGylated Hb in the absence (A) and in the presence (B) of IHP was purified by a Q Sepharose HP column (2.6 × 65 cm^2). The column was equilibrated with 50 mM Tris-Ac buffer (pH 8.5) and eluted with 8 column volumes of 50 mM Tris-Ac buffer using a pH gradient of 8.5−7.0 at a flow rate of 2 mL/min.

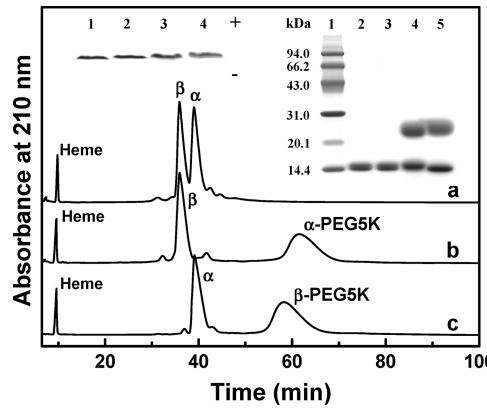

FIGURE 2: Characterization of the PEGylated Hbs. RP-HPLC analysis of HbA (a), fractionated peak b from Figure 1 (b) and fractionated peak c from Figure 1 (c) was carried out on a Vydac C4 column (4.6 × 250 mm^2), using a linear gradient of 35−50% acetonitrile containing 0.1% TFA in 100 min at a flow rate of 1.0 mL/min. The right inset shows the SDS−PAGE analysis of the protein standard (lane 1), HbA (lane 2), [Propyl-Val-1(α)]$_2$-Hb (lane 3), the fractionated peak c (lane 4) and the fractionated peak b (lane 5). The SDS−PAGE analysis was carried out on a precast 14% Tris-glycine gel. The left inset shows the isoelectric focusing of HbA (lane 1), [Propyl-Val-1(α)]$_2$-Hb (lane 2), fractionated peak c (lane 3) and fractionated peak b (lane 4). The + and − signs indicate the anode and the cathode during electrofocusing, respectively.

Characterization of the PEGylated Hbs. (i) RP-HPLC Analysis. RP-HPLC analysis of the PEGylated Hbs is shown in Figure 2. In the fractionated peak b, the peak for α-globin is not shifted and that for β-globin is right-shifted as a broad peak. Thus, α-globin is not modified and β-globin is PEGylated in the fractionated peak b. In the fractionated peak

Table 1: The Mass of Globin Chains of the Modified Hbs[a]

sample	M_w (Da)		no. of PEG chains[b]	
	α-globin	β-globin	α-globin	β-globin
HbA	15127.3[c]	15870.7[c]	0	0
peak b[d]	15127.3	20872.1	0	1
peak c[e]	20127.3	15870.7	1	0
[Propyl-Val-1(α)]$_2$-Hb	15170.0	15868.0	1[f]	0

[a] The PEGylated globin chains isolated by RP-HPLC were analyzed by MALDI-TOF. [b] The number of PEG chains was calculated from the difference between their mass and the unPEGylated globin. [c] The theoretical masses of α- and β-globin are 15126.4 and 15867.2 Da, respectively. [d] The fractionated peak b in Figure 1. [e] The fractionated peak c in Figure 1. [f] α-Globin is modified with one propyl moiety.

c, the β-globin peak is not shifted and that for α-globin is right-shifted as a broad peak. Thus, β-globin is not modified and α-globin is PEGylated in peak c.

(ii) Molecular Mass of the PEGylated Hbs. As measured by MALDI-TOF, the globins of the unmodified Hb exhibit M_w of 15870.7 Da (β-globin) and 15127.3 Da (α-globin), respectively (Table 1). Thus, the fractionated peak b is composed of β-globin that is conjugated with one PEG5K chain and α-globin. Similarly, the fractionated peak c is composed of α-globin that is conjugated with one PEG5K chain and β-globin.

(iii) SDS−PAGE Analysis. The SDS−PAGE pattern of the two PEGylated Hbs exhibits two essentially identical bands (the right inset, Figure 2). One band is at the position of the unmodified globin (~16 kDa). The other band shows lower mobility and corresponds to one globin conjugated with one PEG5K chain.

(iv) Isoelectric Focusing. The IEF pattern of the two PEGylated Hbs shows a single band (the left inset, Figure 2). The anodic mobility of the two diPEGylated Hbs is slightly higher than unmodified Hb. However, the two PEGylated Hbs eluted ahead of HbA on Q Sepharose HP (Figure 1), indicative of an increased basicity. This may reflect the molecular shielding influence of the PEG chains on the surface charge of Hb.

(v) Characterization of the Sites of PEGylation. The sites of PEGylation were characterized by MALDI-TOF/TOF. As compared to the tryptic peptides of α-globin (see Figure S1A, Supporting Information), a broad unimodal peptide distribution with a maximum mass of approximately 6170 Da appeared for the tryptic peptides of the PEGylated α-globin (see Figure S1C, Supporting Information). This peptide corresponds to α-T_{1+2} (1172.4 Da) conjugated with one PEG5K chain. The broad peptide distribution could be due to the polydispersity of the PEG5K chain. MS fragmentation of the peptide at *m*/*z* 6178.3 Da yielded the y ions (C-terminal parts) as normal α-T_{1+2}. The b ions (N-terminal parts) disappeared for conjugation of one PEG5K chain (see Figure S1E, Supporting Information). Thus, peak c is a PEGylated Hb site-specifically modified at Val-1(α) and referred to as [Propyl-PEG5K-Val-1(α)]$_2$-Hb. As compared to the tryptic peptides of β-globin (see Figure S1B, Supporting Information), a broad unimodal peptide distribution with a maximum mass of approximately 5950 Da appeared for the tryptic peptides of the modified β-globin (see Figure S1D, Supporting Information). This peptide corresponds to β-T_1 (953.1 Da) conjugated with one PEG5K chain. MS fragmentation of the peptide at *m*/*z* 5957.3 Da yielded the y ions as normal β-T_1. The b ions disappeared for conjugation of a PEG5K chain (see Figure S1F, Supporting Information). Thus, the fractionated peak b is a PEGylated Hb site-specifically modified at Val-1(β) and referred to as [Propyl-PEG5K-Val-1(β)]$_2$-Hb.

Table 2: Molecular Radii of the PEGylated Hbs[a]

sample	radius (nm)	vol (nm^3)
HbA	3.10	124.8
[Propyl-Val-1(α)]$_2$-Hb	3.12	127.2
[Propyl-PEG5K-Val-1(α)]$_2$-Hb	3.62	198.9
[Propyl-PEG5K-Val-1(β)]$_2$-Hb	4.09	286.6
[Propyl-PEG5K-Val-1(α)]$_2$-αα-Hb	4.22	341.8

[a] The molecular volume of the modified Hbs was calculated from the molecular radius measured by dynamic light scattering at Hb concentration of 1 mg/mL.

SEC Analysis of the PEGylated Hbs. The SEC studies indicated that both diPEGylated Hbs exhibited higher hydrodynamic volume than HbA (see Figure S2, Supporting Information). Thus, the two PEG-5K chains increase the hydrodynamic volume of Hb significantly. However, the PEGylated αα-fumaryl Hb site-specifically modified at Val-1(α), [Propyl-PEG5K-Val-1(α)]$_2$-αα-Hb eluted earlier than that of [Propyl-PEG5K-Val-1(α)]$_2$-Hb and its elution position is very close to the position of [Propyl-PEG5K-Val-1(β)]$_2$-Hb. Thus, PEGylation of Hb at Val-1(α) presumably led to an extensive dissociation of Hb tetramer. However, there is no peak of mass less than that of tetrameric Hb (see Figure S2, Supporting Information), apparently due to the contribution of the PEG chain on the hydrodynamic volume of the Hb dimer. In contrast, PEGylation at Val-1(β) leads to a lesser dissociation level of Hb tetramer.

Molecular Volume of the PEGylated Hbs. As shown in Table 2, the molecular volume of [Propyl-PEG5K-Val-1(β)]$_2$-Hb, calculated from the molecular radius, is much larger than that of [Propyl-PEG5K-Val-1(α)]$_2$-Hb and slightly lower than that of [Propyl-PEG5K-Val-1(α)]$_2$-αα-Hb. Both PEGylated proteins exhibit a molecular volume higher than that of HbA. This result is consistent with the SEC studies.

The Dissociation Constant Measured by SEC. The K_d of HbA at pH 7.4 was increased by nearly 290- and 10-fold upon PEGylation at Val-1(α) and at Val-1(β), respectively (Table 3). As pH increased from 7.4 to 8.5, the K_d of Hb decreased 1.7-fold. However, [Propyl-PEG5K-Val-1(α)]$_2$-Hb and [Propyl-PEG5K-Val-1(β)]$_2$-Hb showed 2.4-fold and 13.6-fold decrease in the K_d, respectively. As pH reduced from 7.4 to 6.5, HbA and [Propyl-PEG5K-Val-1(β)]$_2$-Hb showed 12-fold and 46-fold increase in the K_d, respectively. The K_d of [Propyl-PEG5K-Val-1(α)]$_2$-Hb could not be measured due to its very high K_d at pH 6.5. Thus, the K_d of [Propyl-PEG5K-Val-1(α)]$_2$-Hb is less sensitive to pH than that of [Propyl-PEG5K-Val-1(β)]$_2$-Hb and more sensitive than that of HbA.

Purification and Characterization of the Propylated Hb at Val-1(α). The tetramer−dimer dissociation of Hb upon PEGylation of Val-1(α) is possibly due to propylation of Val-1(α) and/or the PEG chains conjugated through the propyl moiety. Thus, we prepared a propylated Hb site-specifically modified at Val-1(α).

The propylated Hb was purified by cation exchange chromatography on a Mono S column, and three major elution peaks appeared (Figure 3A). Peaks c and a correspond to unmodified Hb and Hb conjugated with multiple propyl

Table 3: The pH Dependent Sedimentation Velocity and the Tetramer−Dimer Dissociation Constant of the Hb Samples

	HbA		PEGylated Hb[a]		PEGylated Hb[b]		propylated Hb[c]	
pH	$S_{20,w}$[d]	K_d[e]	$S_{20,w}$	K_d	$S_{20,w}$	K_d	$S_{20,w}$	K_d
6.5	4.18 ± 0.01	30.8	2.69 ± 0.01	ND[f]	2.80 ± 0.01	1134.0	4.64 ± 0.01	3.12
7.4	4.58 ± 0.01	2.5	2.98 ± 0.01	724.0	3.32 ± 0.01	24.5	4.86 ± 0.01	0.30
8.5	4.67 ± 0.01	1.5	3.02 ± 0.01	309.1	3.58 ± 0.01	1.8	4.86 ± 0.01	0.31

[a] [Propyl-PEG5K-Val-1(α)]$_2$-Hb. [b] [Propyl-PEG5K-Val-1(β)]$_2$-Hb. [c] [Propyl-Val-1(α)]$_2$-Hb. [d] The sedimentation coefficient ($S_{20,w}$) was measured at the nominal concentration of A_{405} = 1.0. [e] The tetramer−dimer dissociation constant measured by size exclusion chromatography, in μM on the tetramer basis. [f] K_d is not detectable by the proposed method.

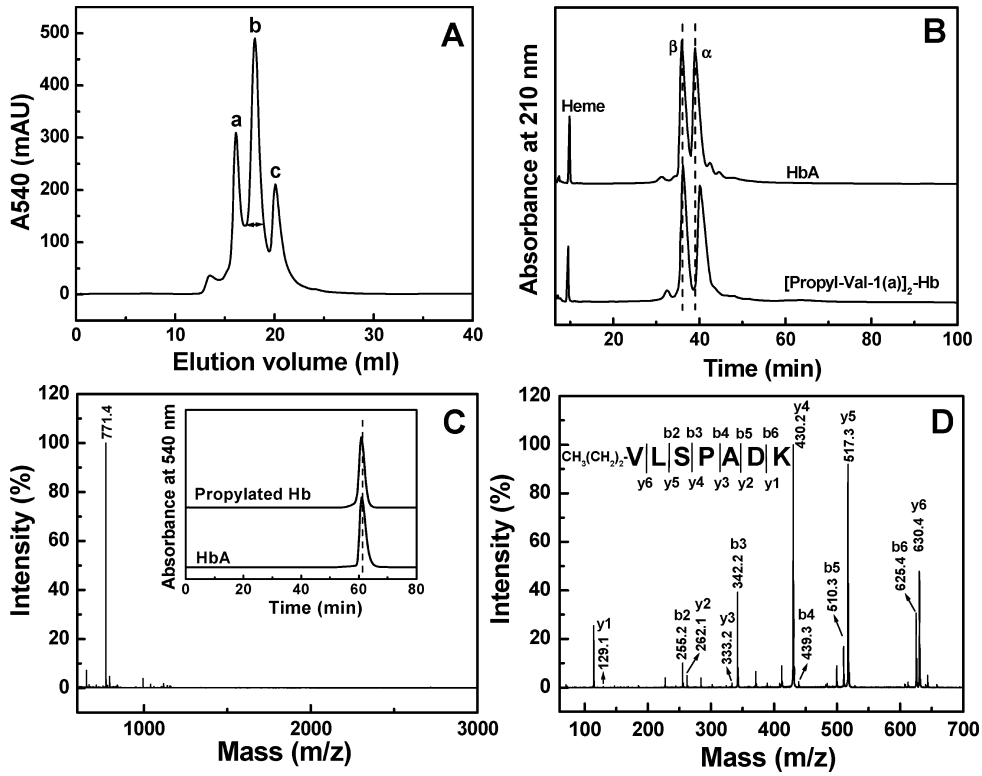

FIGURE 3: Characterization of the propylated Hb site-specifically modified at Val-1(α). The propylated Hb in the presence of IHP was purified by cation exchange chromatography on a Mono S column (0.5 × 5 cm^2) (A). The column was equilibrated with 50 mM Tris-Ac buffer (pH 6.0) and eluted with 40 column volumes of 50 mM Tris-Ac buffer at a pH gradient of 6.0−7.0 at a flow rate of 0.8 mL/min. Globin chain analysis of the propylated Hb was carried out by RP-HPLC (B). MALDI-TOF/TOF spectrum is shown for the propylated α-T1 (VLSPADKT) (C), followed by MS fragmentation of the peptide at *m/z* 771.4 Da (D). Inset (C) shows the size exclusion chromatography analysis of the propylated Hb.

moieties, respectively. Peak b is fractionated and subjected to RP-HPLC analysis. As shown in Figure 3B, the peak of β-globin for peak b is not shifted and the peak of α-globin is slightly right-shifted as compared to that of HbA. MALDI-TOF showed that this peak has a M_w of 15170.0 Da, corresponding to α-globin (15127.3 Da) conjugated with one propyl unit (43.0 Da) (Table 1). IEF analysis showed that the propylated Hb migrated as a single band during IEF, reflecting its homogeneity (the left inset, Figure 2). SDS−PAGE analysis showed that it migrated as a single band comparable to that of HbA (the right inset, Figure 2).

The tryptic peptides of the α-globin of peak b (Figure 3A) were analyzed by RP-HPLC on a C18 column (1 × 25 cm^2). As compared to the tryptic peptides of α-globin of HbA, a tryptic peptide of α-globin of the propylated Hb was right-shifted (data not shown) with a mass of 771.4 Da (Figure 3C). This peptide corresponds to α-T_1 (729.9 Da) conjugated with one propyl moiety (43.0 Da). MS fragmentation of this peptide showed that the y ions appeared as normal α-T_1 and the b ions contain the peptides conjugated with one propyl unit (Figure 3D). This indicated that peak b is a propylated Hb site-specifically modified at Val-1(α) and referred as [Propyl-Val-1(α)]$_2$-Hb.

[Propyl-Val-1(α)]$_2$-Hb was eluted at the same position as HbA in SEC (inset, Figure 3C), indicating that propylation at Val-1(α) does not change the hydrodynamic volume of Hb. Moreover, propylation at Val-l(α) led to an 8-fold decrease in the K_d of Hb at pH 7.4 (Table 3) and thus stabilizes the Hb tetramer. Accordingly, conjugation of the PEG chains at Va-1(α) can greatly destabilize the tetramer stability of Hb.

Analytical Ultracentrifugation of the Modified Hbs. The various modified Hbs were analyzed by both sedimentation velocity and equilibrium analytical ultracentrifugation. In sedimentation velocity experiments, HbA shows its well-documented increase in the sedimentation rate with increasing protein concentration, characteristic of a self-associating system (line a, see Figure S3, Supporting Information). [Propyl-Val-1(α)]$_2$-Hb sediments faster than HbA, independent of protein concentration. This suggests that it is predominantly tetrameric (line b), consistent with the results obtained by SEC (Table 3). The sedimentation of [Propyl-

Table 4: Sedimentation Equilibrium of the Modified Hb Samples[a]

	M_w[b] (Da)	dimer M_w (Da)	K_d[c] (μM)
HbA	48,109 (44,633, 51,585)[d]	32,250	5.92 (2.87, 11.59)[d]
[Propyl-Val-1(α)]$_2$-Hb	50,919 (46,243, 55,675)	32,293	3.11 (1.04, 7.67)
[Propyl-PEG5K-Val-1(α)]$_2$-Hb	42,287 (35,804, 48,828)	37,250	40.65 (17.87, 171.10)

[a] The Hb samples are in PBS buffer at pH 7.4. The concentrations of each protein at the onset of centrifugation were A_{405} = 0.1, 0.4 and 0.8. [b] The best-fit average molecular weight. [c] The best-fit value of K_d determined from the dimer−tetramer association model. [d] The upper and lower parameter bounds calculated from the variance ratio at the 95.46% confidence level.

PEG5K-Val-1(α)]$_2$-αα-Hb shows the decrease in *S* as protein concentration increased, characteristic with stable noninteracting particles (line e); the relatively high *S* value suggests that this protein is tetrameric. In contrast, the sedimentation rates of the two PEGylated Hbs are much lower than that of [Propyl-PEG5K-Val-1(α)]$_2$-αα-Hb and increase with protein concentration. This suggests that the self-association equilibrium is shifted toward dimer (lines c and d). However, [Propyl-PEG5K-Val-1(α)]$_2$-Hb sediments slower and is less sensitive to protein concentration than [Propyl-PEG5K-Val-1(β)]$_2$-Hb. Thus, PEGylation at Val-1(α) shows greater ability than that at Val-1(β) to destabilize the Hb tetramer.

The sedimentation rates of the two PEGylated Hbs and [Propyl-Val-1(α)]$_2$-Hb were compared at three pH values (Table 3). Little to no difference in $S_{20,w}$ was observed upon shifting from neutral to basic pH. In contrast, $S_{20,w}$ consistently decreased upon shifting to acid pH. [Propyl-Val-1(α)]$_2$-Hb displays the least dependence on pH. [Propyl-PEG5K-Val-1(α)]$_2$-Hb is more dependent on pH than HbA. The changes in sedimentation rate correlate well with the K_d determined by SEC as a function of pH (Table 3). Because the pH dependent dissociation of Hb is ascribed to the breaking or to the formation of hydrogen bonds (*18*), the PEG chains at Val-1(α) may break the hydrogen bonds of Hb.

Sedimentation equilibrium measurements were conducted to corroborate the effect of PEGylation on the tetramer−dimer equilibrium of Hb. The values of M_w calculated from the data show that propylation at Val-1(α) stabilizes and PEGylation at Val-1(α) destabilizes the Hb tetramer (Table 4). Fitting the concentration distributions to the dimer−tetramer association model confirms this conclusion. The best-fit K_d determined for [Propyl-Val-1(α)]$_2$-Hb is 2-fold lower than that for HbA. The best-fit K_d determined for [Propyl-PEG5K-Val-1(α)]$_2$-Hb is 7-fold greater than that for HbA (Table 4). Thus, the PEG chains conjugated at Val-1(α) clearly destabilize the Hb tetramer.

It should be mentioned that biomolecules were characterized during sedimentation equilibrium in their native state. In contrast, there are complications caused by interactions between matrices and the PEG chains that can possibly obscure interpretation of the SEC method (*30*). This structural aspect may lead to the observed differences in K_d values between the SEC and the sedimentation equilibrium methods.

Structural Studies of the Modified Hbs. Structural studies of the three modified Hbs were carried out by the CD spectroscopy and the intrinsic front-face fluorescence spectroscopy.

(i) CD Spectroscopy. As shown in the inset (Figure 4A), the two PEGylated Hbs and the propylated Hb showed an ellipticity at 222 nm comparable to HbA. This reflects that the overall secondary structure of Hb is not altered in these modified Hbs.

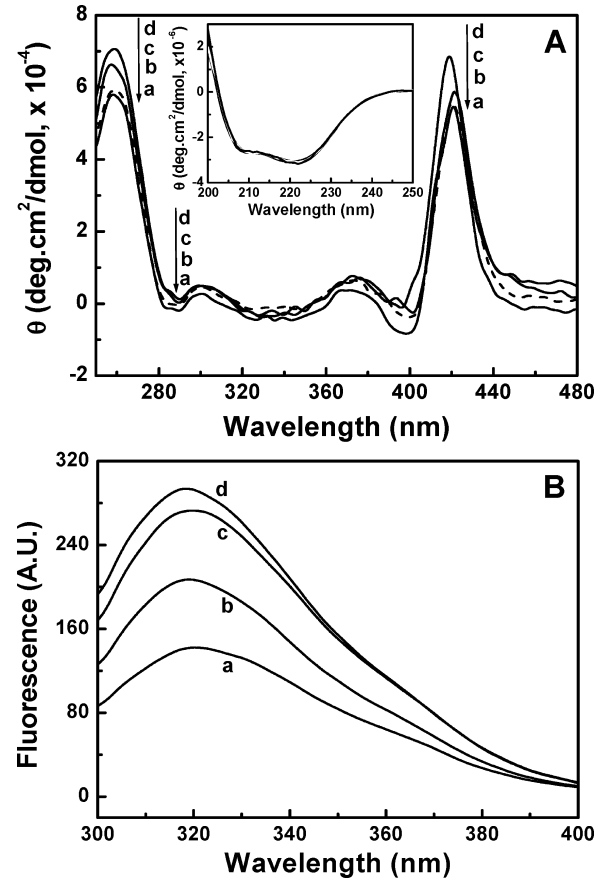

FIGURE 4: Structural studies of the modified Hb. The circular dichroism spectra (A) of HbA (a), [Propyl-Val-1(α)]$_2$-Hb (b), [Propyl-PEG5K-Val-1(β)]$_2$-Hb (c) and [Propyl-PEG5K-Val-1(α)]$_2$-Hb (d) were recorded at 25 °C with a 0.2 cm light path cuvette (310 μL). The molar ellipticity (θ) is expressed in deg·cm^2/dmol on a heme basis. Intrinsic fluorescence emission spectra (B) of HbA (a), [Propyl-Val-1(α)]$_2$-Hb (b), [Propyl-PEG5K-Val-1(β)]$_2$-Hb (c) and [Propyl-PEG5K-Val-1(α)]$_2$-Hb (d) were recorded at room temperature. The excitation wavelength was 280 nm.

The intensity of the L band (in the vicinity of 260 nm) of HbA was not altered upon propylation at Val-1(α). However, it was increased by PEGylation at Val-1(α) (Figure 4A). Since the L band is very sensitive to the attached ligand of the heme (*31*), the PEG chains at Val-1(α) may alter the Hb structure and make the heme strongly bind the oxygen. In contrast, PEGylation at Val-1(β) increased the intensity of the L band to a lesser extent than that at Val-1(α).

The region around 285 nm is considered to be indicative of the transition from the R state to the T state (*32*). The ellipticity at 285 nm was increased by propylation at Val-1(α) and by PEGylation at Val-1(α) or at Val-1(β). This indicated that propylation and PEGylation of Hb may alter the quaternary structure of Hb.

The Soret band of Hb is informative on the interactions of the heme prosthetic group with the surrounding aromatic

Table 5: Oxygen Affinity of the Modified Hbs and Its Modulation by the Allosteric Effectors[a]

effector	HbA	PEGylated Hb[b]	PEGylated Hb[c]	Propyl-Hb[d]
none	14.0 (2.8)	7.4 (1.9)	5.1 (2.4)	7.6 (2.7)
2.5 mM DPG	21.9 (2.4)	12.7 (1.7)	6.2 (2.2)	13.8 (2.2)
1 M NaCl	29.4 (2.5)	13.0 (1.8)	8.6 (2.0)	19.7 (2.4)
2.5 mM IHP	44.8 (2.3)	24.3 (1.6)	5.5 (2.3)	22.3 (2.0)
2.5 mM L35	68.3 (2.0)	18.2 (1.4)	16.6 (1.7)	45.0 (1.7)

[a] Oxygen equilibrium curves of the samples were measured at 37°C in PBS (pH 7.4) at a Hb tetramer concentration of 0.5 mM. The Hill coefficient is given in the parentheses. P_{50} is the partial oxygen pressure at 50% saturation and is expressed in mmHg on the left of the Hill coefficient. [b] [Propyl-PEG5K-Val-1(α)]$_2$-Hb. [c] [Propyl-PEG5K-Val-1(β)]$_2$-Hb. [d] [Propyl-Val-1(α)]$_2$-Hb.

residues (*33*). In the Soret region, the ellipticity of HbA was not altered by propylation at Val-1(α). However, it was increased by PEGylation at Val-1(α) and its maximal ellipticity at 421 nm was blue-shifted to 419 nm. Thus, the PEG chains at Val-1(α) significantly altered the heme environment of Hb. In contrast, the ellipticity of Hb was slightly increased by PEGylation at Val-1(β).

(ii) Intrinsic Front-Face Fluorescence. Intrinsic front-face fluorescence of Hb could be used to monitor the quaternary conformational changes. This sensitivity of the signal was assigned mostly to Trp-37(β) (*34*). When excited at 280 nm, the fluorescence intensity of HbA slightly increased upon propylation at Val-1(α) and increased further by conjugation of the PEG chains with a peak position at 320 nm (Figure 4B). Trp-37(β) is close to the neighboring α subunit heme, which is liable to become perturbed by changes at the α1β2 interface (*35*). Thus, the increase in the fluorescence of Trp-37(β) results partially from the tetramer−dimer dissociation of Hb. PEGylation at Val-1(β) showed a lower fluorescence intensity than that at Val-1(α). This suggests that the α1β2 interface of Hb is perturbed to lesser degree by PEGylation at Val-1(β) than PEGylation of Val-1(α).

Functional Studies of the Modified Hbs. (i) Oxygen Affinity of the Modified Hbs. The oxygen affinity of the three modified Hbs was investigated in PBS (pH 7.4) at 37 °C. As shown in Table 5, the oxygen affinity of HbA increased upon propylation at Val-1(α) and did not increase further by conjugation of the PEG5K chains. The Hill coefficient (*n*) of HbA was slightly decreased upon propylation at Val-1(α) and significantly decreased upon PEGylation at Val-1(α). This indicated that the PEG chains at Val-1(α) can decrease the *n* of HbA, presumably due to the tetramer−dimer dissociation of Hb upon PEGylation. [Propyl-PEG5K-Val-1(β)]$_2$-Hb exhibits a slightly lower P_{50} and a higher *n* than that of [Propyl-PEG5K-Val-1(α)]$_2$-Hb. Thus, the sites of PEGylation can influence the P_{50} of HbA. It should be noted here that Cys-93(β) of [Propyl-PEG5K-Val-1(β)]$_2$-Hb is not PEGylated, as reflected by the presence of two free thiol groups on titration of the diPEGylated Hb with 4-PDS (inset, Figure 5). Thus, the high oxygen affinity of [Propyl-PEG5K-Val-1(β)]$_2$-Hb does not indicate involvement of Cys-93(β).

(ii) Influence of the Allosteric Effectors on Oxygen Affinity of the Modified Hbs. Chloride and L35 can bind at the αα end of the central cavity and are quite opposite to DPG and IHP that bind at the $\beta\beta$ end. Propylation at Val-1(α) did not alter the sensitivity of HbA to DPG and IHP but increased the sensitivity to NaCl and L35 (Table 5). In contrast, PEGylation at Val-1(α) did not alter the sensitivity of HbA to DPG and IHP but decreased the sensitivity to NaCl and L35. Thus, the PEG chains at Val-1(α) can partially inhibit the binding of chloride and L35 at the αα end of Hb. On the other hand, PEGylation at Val-1(β) did not alter the sensitivity to NaCl and L35 but essentially diminished the sensitivity to DPG and IHP.

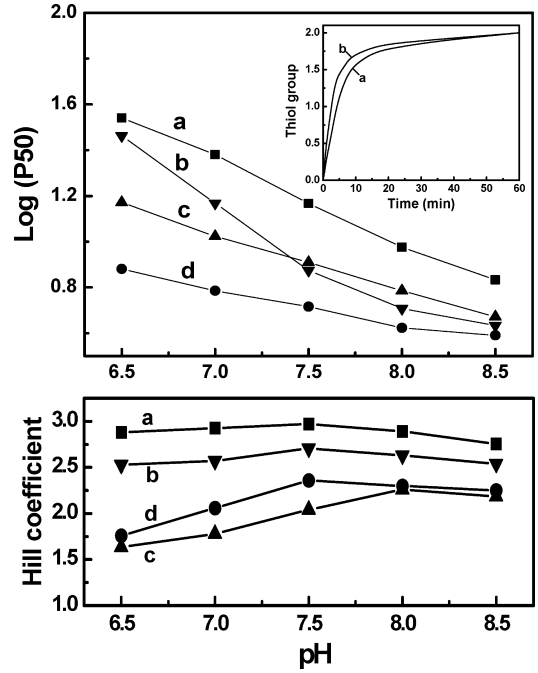

FIGURE 5: The pH dependent oxygen affinity (A, top panel) and the Hill coefficients (B, bottom panel) of the modified Hbs. Oxygen affinity (P_{50}) of HbA (a), [Propyl-Val-1(α)]$_2$-Hb (b), [Propyl-PEG5K-Val-1(α)]$_2$-Hb (c) and [Propyl-PEG5K-Val-1(β)]$_2$-Hb (d) was measured using Hem-O-Scan (Aminco) in 100 mM sodium phosphate buffer as a function of pH at 37 °C. Each point presents an average of two duplicate results. The inset shows reactivity of the -SH groups of Hb (a) and [Propyl-PEG5K-Val-1(β)]$_2$-Hb (b) as measured by titration with 4,4′-dithiopyridine.

(iii) Bohr Effect of the Modified Hbs. The Bohr effect (the decrease in oxygen affinity as function of a decrease in pH) is expressed as the number of Bohr protons released upon oxygenation. Figure 5 shows the oxygen affinity of the modified Hbs as a function of pH. The Bohr effect estimated from the slopes (ΔH^+) between pH 6.5 and 8.0 is 0.38 proton/heme for HbA. Propylation at Val-1(α) leads to an increase in the H^+ release (0.52 proton/heme) and enhances the Bohr effect of HbA. However, PEGylation at Val-1(α) results in a decrease in the H^+ release (0.25 proton/heme). Thus, the PEG chains conjugated at Val-1(α) can significantly lower the Bohr effect of HbA. In contrast, PEGylation at Val-1(β) leads to a lower H^+ release (0.17 proton/heme) than PEGylation at Val-1(α). Thus, the Bohr effect of the PEGylated Hb is dependent on the sites of PEGylation. In addition, the *n* of HbA and [Propyl-Val-1(α)]$_2$-Hb slightly changes as a function of pH. However, the *n* of the two PEGylated Hbs significantly increases as pH increased from 6.5 to 8.0. This is presumably due to the pH-dependent tetramer−dimer dissociation of the PEGylated Hb.

DISCUSSION

The present study is aimed at providing a biochemical insight into the influence of PEGylation (the sites of PEGylation and the PEG chain) on the tetramer−dimer

dissociation of Hb. Our present results show that the PEG chains conjugated at Val-1(α) of Hb may lead to the extensive tetramer−dimer dissociation of Hb.

The tetramer stability of Hb depends on the polar interactions (e.g., hydrogen bonds) and the hydrophobic interactions (*18*). The pH dependent dissociation of Hb is ascribed to the breaking or to the formation of hydrogen bonds upon protonation/deprotonation of one of the residues involved in their proximity (*18*). The PEG chains conjugated at Val-1(α) enhance the breaking of the hydrogen bonds (Table 3). The quaternary structure of oxyHb generates a central cavity that can accommodate at least 80 water molecules. The PEG chains perturb the hydration layer of Hb and the organization of the water molecules around Hb. This presumably attenuates the number and the strength of the hydrogen bond. The PEG chains at Val-1(α) also lead to the greater hydration of Hb, force the exposure of a more extensive hydrophobic surface to solvent and decrease the hydrophobic interactions of Hb.

The increased tetramer stability of Hb by propylation at Val-1(α) is presumably due to the increased hydrophobicity of the surrounding region. Similar results have been advanced for the higher tetramer stability of HbF (whose dimer−tetramer K_d is close to 2 orders of magnitude lower than HbA) relative to that of HbA. This is due to the increased electrostatic interactions and hydrophobicity upon conversion of the T to the R state (*36, 37*). Besides, the substitution of Val-1(α) by Ala and acetyl Ser has been shown to increase the K_d by 5.5- and 11.3-fold, respectively (*20*). However, the PEG chain on Val-1(α) extensively destabilized the Hb tetramer. Thus, the tetramer−dimer equilibrium of Hb depends on the hydrophobicity of the amino acid residue at the Val-1(α) position. Consistently, a recent study showed that there is a direct correlation between the association constant and the hydrophobic surface buried at the α1β2 interfaces (*38*).

Association of Hb dimers into tetrameric structure is driven primarily by the interactions between residues at the α1β2 interface (*16*). The α1β2 interface is a highly polar and dynamic region of the molecule, undergoing large structural changes as the Hb molecule transitions from the R to T state. Variants with substitutions at the α1β2 interface, e.g., Hb Rothschild (β37 Trp → Arg) (*39, 40*) and Hb Kansas (β102 Asn → Thr) (*41, 42*) may destabilize the α1β2 interface and lead to the extensive dissociation of Hb tetramer. Since Val-1(α) is very close to the α1β2 interface, the PEG chains at Val-1(α) may alter the polarity, hydrophobicity and spatial orientation of residues at the α1β2 interface. This alteration may destabilize the α1β2 interface and unfavorably influence the structural and functional properties of Hb (e.g., dissociation of Hb tetramer). As revealed by the CD data in Figure 4A, the heme environment and quaternary structure of Hb were significantly perturbed upon PEGylation of Val-1(α). These structural alterations may be related to the destabilization of the α1β2 interface and the tetramer−dimer dissociation of Hb.

Several salt bridges present in the R structure are not present in the T structure because the rotation of dimers moves the charged group apart (*43*). At the αα end, two anion binding sites are associated with the bridging of Val-1(α) of each α chain to Ser-131(α) of the same chain, and to the guanidinium group of Arg-141(α) of the opposite α chain. The propyl chain on Val-1(α) can disrupt these interchain anion bridges by changing the hydrophobicity of the region. This can destabilize the T structure and thus increase the oxygen affinity. Consistently, reductive hydroxyethylation (*14*) and carbamoylation (*12*) of Val-1(α) increase the oxygen affinity of Hb.

The Bohr effect of Hb was increased by propylation at Val-1(α) and decreased by the PEG chains at Val-1(α). Since the dimer Bohr effect is about 20% as large as the tetramer Bohr effect (*19*), this result suggests the linkage between the tetramer−dimer equilibrium and proton binding. Moreover, the salt bridge between the protonated α-amino group of Val-1(α) and the terminal carboxyl of Arg-141(α) has been theorized to contribute to about 25% of the Bohr effect (*44*). Arnone et al. observed the presence of unidentified anion between carboxyl of Arg-141(α) and the α-amino of Val-1(α) in deoxy-Hb (*45*). Presumably, the PEG chains conjugated at Val-1(α) may shield the anion, interrupt the formation of this salt bridge and weaken the Bohr Effect contributed by this salt bridge.

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SUPPORTING INFORMATION AVAILABLE

Identification of the sites of PEGylation for the two PEGylated Hbs by MALDI-TOF/TOF (Figure S1), size exclusion chromatography analysis of the PEGylated Hbs (Figure S2), and sedimentation velocity analysis of the modified Hbs a function of the protein concentration (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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