Autoxidation of the Site-Specifically PEGylated Hemoglobins: Role of the PEG Chains and the Sites of PEGylation in the Autoxidation[†]

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ABSTRACT: The PEGylated hemoglobin (Hb) has been evaluated as a potential blood substitute. In an attempt to understand the autoxidation of the PEGylated Hb, we have studied the autoxidation of the PEGylated Hb site-specifically modified at Cys-93(β) or at Val-1(β). PEGylation of Hb at Cys-93(β) perturbed the heme environment and increased the autoxidation rate of Hb, which is at a higher level than that caused by PEGylation at Val-1(β). The perturbation of the heme environment of Hb is attributed to the maleimide modification at Cys-93(β) and not due to conjugation of the PEG chains. However, the PEG chains enhance the autoxidation and the H_2O_2 mediated oxidation of Hb. Accordingly, the PEG chains are assumed to increase the water molecules in the hydration layer of Hb and enhance the autoxidation by promoting the nucleophilic attack of heme. The autoxidation rate of the PEGylated Hb does not show an inverse correlation with the oxygen affinity. The H_2O_2 mediated structural loss and the heme loss of Hb are increased by maleimide modification at Cys-93(β) and further decreased by conjugation of the PEG chains. The autoxidation of the PEGylated Hbs is attenuated significantly in the plasma, possibly due to the presence of the antioxidant species in the plasma. This result is consistent with the recent suggestion that there is no direct correlation between the *in vitro* and *in vivo* autoxidation of the PEGylated Hb. Therefore, the pattern of PEGylation can be manipulated for the design of the PEGylated Hb with minimal autoxidation.

Hemoglobin (Hb[1]) and its derivatives undergo autoxidation, followed by production of reactive oxygen species (ROS) and nonfunctional metHb. The superoxide ion, a product from the autoxidation of Hb, subsequently dismutates to H_2O_2 and leads to the oxidative modification of Hb (*1*). The metHb undergoes the heme loss and precipitation, giving rise to Heinz body (*2*). The free heme may mediate anti-inflammatory defense mechanisms through stimulation of heme oxygenase-1 and endogenous production of bilirubin (*3*). H_2O_2 may lead to irreversible cell damage and accelerate the autoxidation of Hb (*4*). Thus, the autoxidation of Hb has received considerable attention, especially in the development of Hb based oxygen carriers (HBOC).

The autoxidation of Hb and its derivatives has been investigated extensively. The autoxidation of Hb is mediated by protons and enhanced by anions (*5*). For example, the autoxidation of Hb involves heme deoxygenation prior to electron transfer (*6*) and is markedly enhanced when tetrameric Hb dissociates into $\alpha\beta$-dimers (*7, 8*). The autoxidation of various Hb derivatives, e.g., $\alpha\alpha$-fumaryl cross-linked Hb (*6*), dextran conjugated polymeric Hb (*9*) and glutaraldehyde polymerized Hb (*10*) is different to that of Hb. The autoxidation of Hb also involves the interaction between heme and other oxidants (e.g., H_2O_2). The Hb derivatives are highly susceptible to oxidation of heme iron, heme and heme protein when exposed to the H_2O_2 rich environment (*11*). This may result in an irreversible loss of Hb structure (e.g., the loss of the heme pocket and helical structure of Hb) (*12*).

The PEGylated Hb represents a new class of the Hb derivatives that has emerged as a potential HBOC (*13, 14*). A new hexaPEGylated Hb, $(SP\text{-}PEG5K)_6$-Hb, has been generated using the extension arm facilitated PEGylation (*15*−*17*). $(SP\text{-}PEG5K)_6$-Hb carries two PEG-5K chains conjugated on Cys-93(β) and four PEG-5K chains conjugated on the Lys residues of Hb (*18*). The hexaPEGylated Hb, MP4 (Sangart Inc., CA), is a prototype of $(SP\text{-}PEG5K)_6$-Hb. Recently, Vandegriff et al. (*19*) reported that MP4 exhibits a higher autoxidation rate and a higher rate of the heme loss than Hb *in vitro*. However, MP4 is a heterogeneous mixture and is not an ideal model protein for study of the autoxidation of the PEGylated Hb.

In an attempt to understand the autoxidation of the PEGylated Hb, we investigated the influence of site-specific PEGylation on the autoxidation of Hb. Accordingly, a series

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[1] Abbreviations: Hb, hemoglobin; PEG, polyethylene glycol; HSA, human serum albumin; MHA, met-human serum albumin; k_{ox}, the first-order autoxidation rate constant; HBOC, hemoglobin based oxygen carrier; PEGylation, conjugation of the PEG chains to proteins; CD, circular dichroism; NEM, *N*-ethyl maleimide; IEF, isoelectric focusing; CAT, catalase; SOD, superoxide dismutase; P_{50}, the oxygen pressure at which hemoglobin is half-saturated; E_a, activation energy; ROS, reactive oxygen species; k_{fast}, the fast-phase heme loss rate constant.

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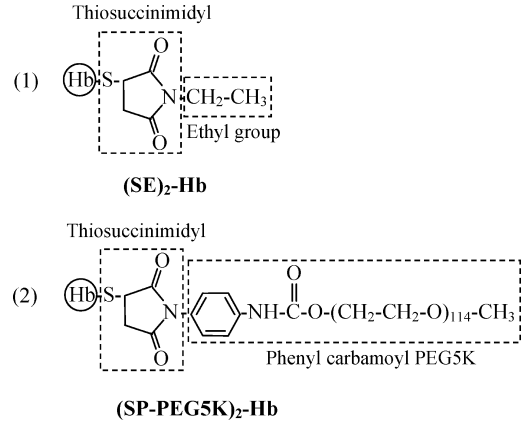

FIGURE 1: Schematic representation comparing the modification of Hb by *N*-ethyl maleimide and PEG maleimide at Cys-93(β).

of site-specifically diPEGylated Hbs were used as the model proteins. The PEGylated Hbs site-specifically modified at Cys-93(β) were prepared by conjugation of Hb with two PEG-3K, 5K, 10K and 20K chains using maleimide chemistry (*20*). In addition, the PEGylated Hb site-specifically modified at Val-1(β) was prepared by conjugation of Hb with two PEG-5K chains using reductive alkylation chemistry (*21, 22*).

The PEGylated Hb can be considered as conjugation of the PEG chains to Hb through a protein modification reagent with small molecular weight. For example, PEGylation at Cys-93(β) can be viewed as modification by *N*-ethyl maleimide (NEM) and the substitution of the ethyl group by a phenyl carbamoyl PEG chain (Figure 1). Thus, the influence of PEGylation on the autoxidation of Hb may result from the NEM modification of Cys-93(β) and conjugation of the PEG chain. Besides, the *in vitro* autoxidation of the PEGylated Hb was studied in the plasma to simulate the *in vivo* exchange transfusion of the blood volume. Accordingly, our present study is aimed to understand the autoxidation of the PEGylated Hb. The results suggest that the pattern of PEGylation can be manipulated to generate the PEGylated Hbs with minimal autoxidation.

EXPERIMENTAL PROCEDURES

Preparation of the PEGylated Hbs. Human adult hemoglobin (HbA) was purified from human erythrocytes (*23*). αα-Fumaryl Hb was prepared as previously described (*24*). $(SE)_2$-Hb, the NEM modified Hb at Cys-93(β), was prepared by incubation of 0.25 mM HbA with 2.5 mM NEM in PBS buffer (pH 7.4) at 4 °C overnight, followed by extensive dialysis against PBS buffer. $(SP\text{-}PEG3K)_2$-Hb, $(SP\text{-}PEG5K)_2$-Hb, $(SP\text{-}PEG10K)_2$-Hb and $(SP\text{-}PEG20K)_2$-Hb are HbA conjugated with two PEG-3K, 5K, 10K and 20K chains at Cys-93(β), respectively. $(SP\text{-}PEG5K)_2$-αα-Hb is αα-fumaryl Hb conjugated with two PEG-5K chains at Cys-93(β). These five PEGylated Hbs were prepared as previously described (*20*). $(Propyl\text{-}PEG5K)_2$-Hb, Hb conjugated with two PEG-5K chains at Val-1(β), was prepared using reductive alkylation chemistry (*21*). Briefly, 0.25 mM Hb was incubated with 1.5 mM PEG-5K propionaldehyde (Sunbio Inc., Korea) and 7.5 mM $NaCNBH_3$ in 50 mM BisTris-Ac buffer (pH 6.5) at 4 °C overnight. The reaction mixture was loaded on a Q Sepharose HP column (2.6 × 65 cm^2) and eluted with 50 mM Tris-Ac buffer at a pH gradient (pH 8.5 to 7.0) in 8

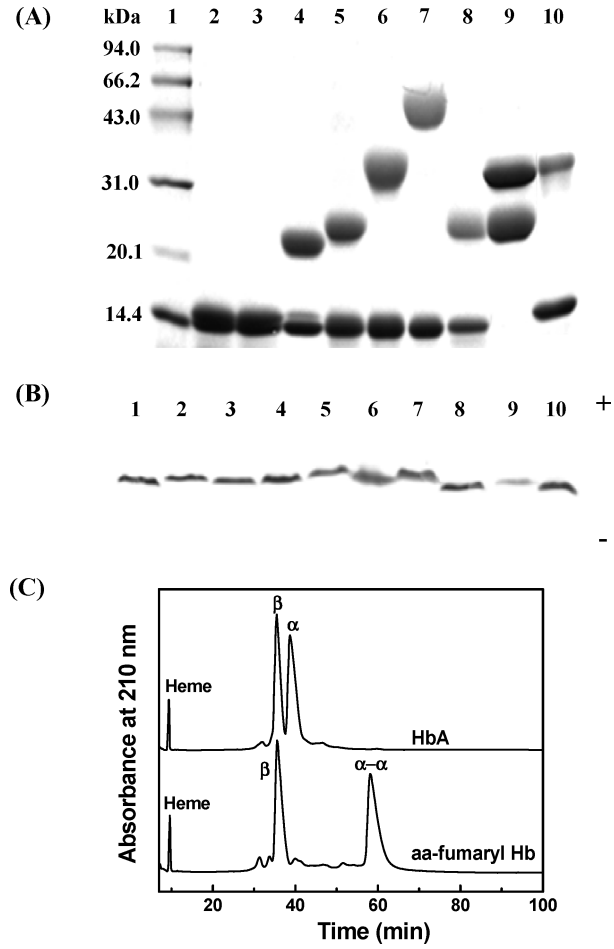

FIGURE 2: Characterization of the PEGylated Hbs. SDS−PAGE (A) was carried out on precast 14% Tris-glycine gel. Lane 1, protein standards; lane 2, HbA; lane 3, $(SE)_2$-Hb; lane 4, $(SP\text{-}PEG3K)_2$-Hb; lane 5, $(SP\text{-}PEG5K)_2$-Hb; lane 6, $(SP\text{-}PEG10K)_2$-Hb; lane 7, $(SP\text{-}PEG20K)_2$-Hb; lane 8, $(Propyl\text{-}PEG5K)_2$-Hb; lane 9, $(SP\text{-}PEG5K)_2$-αα-Hb; Lane 10, αα-fumaryl Hb. Proteins were identified by Coomassie Blue staining. IEF (B) was operated using precast resolve gels from Isolab and a blend of pH 6−8 resolve ampholytes. Lanes 1 and 10, HbA; lane 2, $(SE)_2$-Hb; lane 3, $(SP\text{-}PEG3K)_2$-Hb; lane 4, $(SP\text{-}PEG5K)_2$-Hb; lane 5, $(SP\text{-}PEG10K)_2$-Hb; lane 6, $(SP\text{-}PEG20K)_2$-Hb; lane 7, $(Propyl\text{-}PEG5K)_2$-Hb; lane 8, αα-fumaryl Hb; lane 9, $(SP\text{-}PEG5K)_2$-αα-Hb. The + and − signs indicate the anode and the cathode during electrofocusing, respectively. HPLC analysis (C) was carried out on a Vydac C4 column (0.46 × 25 cm^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.

column volumes at a flow rate of 2.0 mL/min. The desired fractions were pooled and concentrated.

Analytical Methods. 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 precast resolve gels from Isolab and a blend of pH 6−8 resolve ampholytes. Gels were electro-focused for 3 h to resolve the components in the sample completely. HPLC analysis was carried out on a Vydac C4 column (4.6 × 250 mm^2) as described previously (*19*). Molecular radius of the Hb samples (1 mg/mL) was measured by a dynamic light scattering instrument (DynaPro, Protein Solutions, Lakewood, NJ).

Autoxidation Experiments. Autoxidation experiments were carried out by incubating 25 μM Hb samples in PBS buffer (pH 7.4) containing 300 μM EDTA at 37 °C in sealed tubes. Aliquots of the samples were taken out at various time

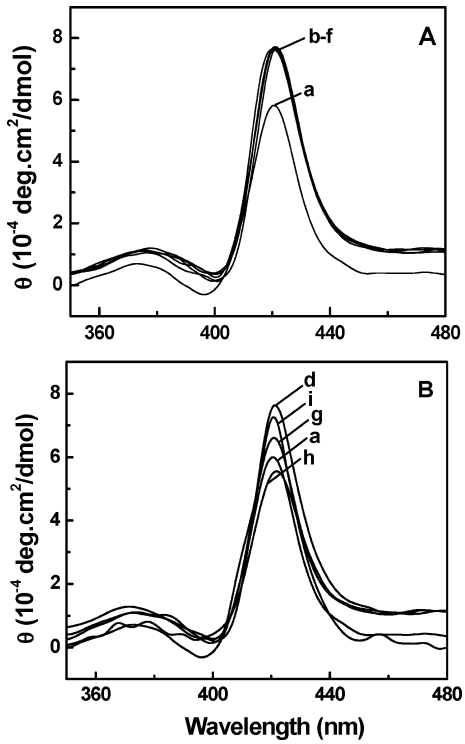

FIGURE 3: Influence of PEGylation on the heme environment of Hb. The CD spectra of (a) HbA, (b) $(SE)_2$-Hb, (c) $(SP\text{-}PEG3K)_2$-Hb, (d) $(SP\text{-}PEG5K)_2$-Hb, (e) $(SP\text{-}PEG10K)_2$-Hb, (f) $(SP\text{-}PEG20K)_2$-Hb, (g) $(Propyl\text{-}PEG5K)_2$-Hb, (h) αα-fumaryl Hb and (i) $(SP\text{-}PEG5K)_2$-αα-Hb were measured using 25 μM Hb in PBS (pH 7.4) at 25 °C.

intervals. Absorbance changes in 500−700 nm due to the autoxidation of Hb were recorded in a spectrophotometer (Shimadzu TCC-240A). The levels of metHb, oxyHb and deoxyHb were measured as described previously (*25*). The first-order autoxidation rate constant (k_{ox}) of the Hb samples were calculated from the fit of a single-exponential equation: $Y = Y_{max}(1 - e^{-kt}) + Y_0$. Y is the percentage of metHb. Y_{max} is the total percentage change in metHb at the end of reaction. The t is the time. Y_0 is the percentage of metHb at $t = 0$.

Autoxidation Experiments in the Plasma. The PEGylated Hbs (40 mg/mL) in PBS (pH 7.4) were mixed with the bovine plasma (Sigma) at volume ratio of 1:1, 1:2, and 1:4. These diluted samples were used to simulate 50%, 33% and 20% exchange transfusion of the blood volume, respectively. In addition, the PEGylated Hbs (40 mg/mL) in PBS (pH 7.4) was mixed with the plasma or PBS buffer at the equal volume, respectively. The autoxidation of the diluted samples are measured at 37 °C with or without catalase (CAT, Sigma) and superoxide dismutase (SOD, Sigma). The percentage of plasma metHb was measured as previously described (*19*).

Azide Mediated Oxidation of the PEGylated Hbs. The Hb samples (25 μM) were incubated with 0.1 M sodium azide (Sigma) in PBS (pH 7.4) containing 300 μM EDTA at 37 °C in sealed tubes. Aliquots of the sample were taken out at various time intervals. Absorbance changes in the range of 500−700 nm during oxidation were recorded. The metHb content was calculated as described elsewhere (*26*).

The Activation Energy. The autoxidation of the PEGylated Hbs was investigated and quantitated in terms of the activation energy (E_a). The E_a has been calculated from an Arrhenius plot, a plot of $\ln(k_{ox})$ versus $1/T$ from 35 °C to 41°C (*27*).

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra of the Hb samples were recorded in PBS buffer (pH 7.4) on a JASCO-720 spectropolarimeter (JASCO, Tokyo, Japan) using a 0.2 cm light path cuvette (310 μL) at 25 °C. Soret CD spectra (350−480 nm) were obtained using 25.0 μM Hb samples (in tetramer). Far UV region CD spectra (200−250 nm) were obtained using 1.25 μM Hb samples (in tetramer). The molar ellipticity (θ) is expressed in deg·cm^2/dmol on a heme basis. The α-helix contents of Hb samples were estimated from the far UV spectra at 222 nm as described previously (*28*).

Heme Exchange Experiment. The rate of heme exchange between metHb and human serum albumin (HSA, Sigma) was measured as described elsewhere (*29, 30*). The metHb was formed by reaction of oxyHb with potassium ferricyanide (*31*). The ferro- and ferricyanide were removed by passing the modified protein through a Sephadex G-25 column in 50 mM BisTris-Ac buffer (pH 7.5) containing 0.1 M NaCl. The heme exchange reaction was initiated by adding 50 μL of 1 mM HSA to a cuvette containing 0.45 mL of 0.5 M Tris-Ac buffer (pH 9.05) and 0.5 mL metHb (∼2 mg). The mixture is at a final volume of 1 mL and at pH 9.0. The absorbance at 578, 620 and 700 nm was recorded every 2 min. MetHb and metHSA (MHA) were calculated as described elsewhere (*26*).

Hydrogen Peroxide Mediated Oxidation of the Hb Samples. The Hb samples (3 mL) consisting of 300 μM xanthine (Sigma), 20 μM desferrioxamine (Sigma) and 50 μM Hb in PBS (pH 7.4) were incubated at 37 °C for 30 min (*32*). The H_2O_2 mediated oxidation of the Hb samples was initiated by the addition of 54 milliunits of xanthine oxidase (EC 1.1.3.22, Sigma). An aliquot (0.2 mL) was taken every two minutes and dissolved in 0.8 mL of PBS (pH 7.4) for measuring the metHb content. In addition, the Hb samples (25 μM) in PBS (pH 7.4) were incubated with H_2O_2 solution (Sigma) for measuring the structural loss of Hb. The incubation is at the molar ratio of H_2O_2 to heme equal to 0, 0.5, 1, 2 and 4 in final volume of 1 mL at room temperature for 1 h. The reaction was terminated by adding excess CAT. The resultant oxidized product was subjected to CD spectral measurement.

Oxygen Affinity Measurements. Oxygen affinity of the Hb samples was measured on a Hemox analyzer (TCS Scientific, PA) at 37 °C, using the Hemox buffer (pH 7.4). Values for P_{50} (the O_2 pressure at which Hb is half-saturated) are obtained from the oxygen equilibrium curves (*33*−*35*).

RESULTS

Characterization of the Site-Specifically PEGylated Hbs. HbA and $(SE)_2$-Hb show a closely placed doublet corresponding to α- and β-chains (lanes 2−3, Figure 2A). $(SP\text{-}PEG3K)_2$-Hb, $(SP\text{-}PEG5K)_2$-Hb, $(SP\text{-}PEG10K)_2$-Hb and $(SP\text{-}PEG20K)_2$-Hb show two well-separated bands. One is at the position of the original band. Another one shows progressively decreased mobility (lanes 4−7) as the size of the PEG chain increased from 3K to 20K. $(Propyl\text{-}PEG5K)_2$-Hb (lane 8) shows two bands with the same mobility as $(SP\text{-}PEG5K)_2$-Hb (lane 5). The α-chain of $(SP\text{-}PEG5K)_2$-αα-Hb (lane 9) and αα-fumaryl Hb (lane 10) showed a slower mobility, as a result of the intramolecular cross-link. All the Hb samples focused as a single compact band on isoelctric focusing that

Table 1: Molecular Volume, Oxygen Affinity and the Autoxidation Rate of the PEGylated Hbs[a]

sample	radius (nm)	volume (nm^3)	P_{50}[b] (mmHg)	n[c]	k_{ox}[d] (h^{-1})	E_a[e] (kJ/mol)
HbA	3.11	126.0	13.7	2.9	0.011	128
$(SE)_2$-Hb	3.07	121.2	8.1	2.3	0.019	150
$(SP\text{-}PEG3K)_2$-Hb	3.93	254.2	6.7	2.1	0.024	146
$(SP\text{-}PEG5K)_2$-Hb	4.45	369.1	6.7	1.9	0.027	144
$(SP\text{-}PEG10K)_2$-Hb	5.31	627.1	6.9	2.0	0.030	137
$(SP\text{-}PEG20K)_2$-Hb	6.79	1311.2	6.9	2.0	0.035	119
$(Propyl\text{-}PEG5K)_2$-Hb	4.09	286.6	6.3	2.0	0.016	140
αα-fumaryl Hb	3.16	132.2	29.2	2.1	0.016	144
$(SP\text{-}PEG5K)_2$-αα-Hb	4.46	371.6	12.7	2.0	0.031	172

[a] Molecular radius of the Hb samples was measured by dynamic light scattering at a protein concentration of 1 mg/mL. [b] Partial oxygen pressure at half-saturation. [c] Hill coefficient [d] First-order autoxidation rate constant. [e] Activation energy of autoxidation.

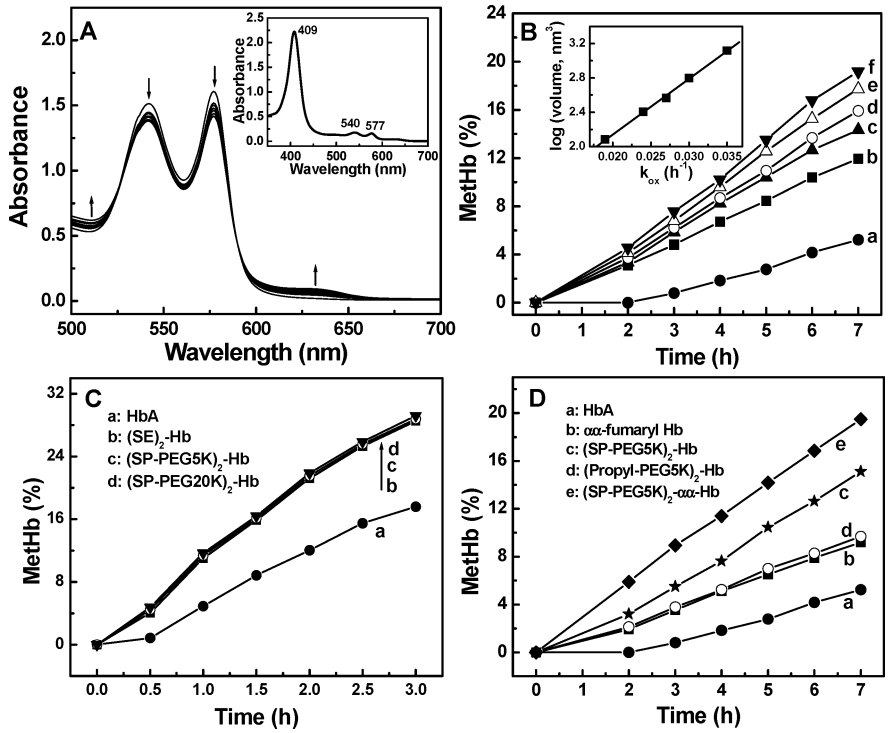

FIGURE 4: Influence of PEGylation on the autoxidation of Hb. Panel A shows the spectral change in 500−700 nm when $(SP\text{-}PEG3K)_2$-Hb was autoxidized in PBS (pH 7.4) at 37 °C for 0, 2, 3, 4, 5, 6, and 7 h. The inset shows the spectral changes in 350−700 nm for the fully oxidized $(SP\text{-}PEG3K)_2$-Hb. Panel B exhibits the kinetics of the metHb formation of HbA (a), $(SE)_2$-Hb (b), $(SP\text{-}PEG3K)_2$-Hb (c), $(SP\text{-}PEG5K)_2$-Hb (d), $(SP\text{-}PEG10K)_2$-Hb (e) and $(SP\text{-}PEG20K)_2$-Hb (f) in PBS (pH 7.4) at 37 °C. The inset shows the k_{ox} of the Hb samples as a function of the log of their molecular volume. Panel C shows the azide mediated oxidation of the PEGylated Hbs. The oxidation of the PEGylated Hbs was measured in PBS (pH 7.4) containing 0.1 M azide at 37 °C. Panel D shows the kinetics of metHb formation of the Hb samples in PBS (pH 7.4) at 37 °C.

clearly reflects their molecular homogeneity, regardless of the different band mobility (Figure 2B). Reverse-phase HPLC shows that αα-fumaryl Hb is composed of intact β-chain and the cross-linked α-chain (Figure 2C). There is no indication of any $\beta\beta$-fumaryl cross-link in the αα-fumaryl Hb. Thus, these results established the purity of these site-specifically PEGylated Hbs.

Structure and Oxygen Affinity of the Site-Specifically PEGylated Hbs. (i) The heme environment of the PEGylated Hbs. The CD spectrum of Hb in the Soret region is primarily due to the relative position of the heme to the Phe and Tyr residues near the heme pocket (*36*). Accordingly, it was used to investigate the heme environment of the PEGylated Hbs. As shown in Figure 3A, $(SE)_2$-Hb displayed higher ellipticity than HbA. The results suggest that NEM modification at Cys-93(β) of Hb facilitates the opening of the heme pocket toward the solvent. On the other hand, the CD profiles for the four PEGylated Hbs are practically superimposable on that of $(SE)_2$-Hb (Figure 3A). This demonstrates that the PEG chains do not increase the exposure of the heme pocket to the solvent. $(SP\text{-}PEG5K)_2$-αα-Hb showed slightly lower ellipticity than $(SP\text{-}PEG5K)_2$-Hb (Figure 3B). This suggests that αα-fumaryl cross-link can slightly reduce the perturbation of the heme environment. The ellipticity of $(Propyl\text{-}PEG5K)_2$-Hb (Figure 3B) is lower than that of $(SP\text{-}PEG5K)_2$-Hb. Thus, PEGylation at Cys-93(β) has a higher propensity to perturb the heme environment than that at Val-1(β).

(ii) Oxygen Affinity of the PEGylated Hbs. As noted in Table 1, NEM modification at Cys-93(β) significantly increases the oxygen affinity of Hb (i.e., lowered the P_{50}). Further conjugation of the PEG chains has minimal influence on the oxygen affinity. The P_{50} of $(SP\text{-}PEG5K)_2$-αα-Hb is lower than that of αα-fumaryl Hb and higher than that of $(SP\text{-}PEG5K)_2$-Hb. $(Propyl\text{-}PEG5K)_2$-Hb exhibits a P_{50} comparable to that of $(SP\text{-}PEG5K)_2$-Hb, in spite of their different perturbation of the heme environment.

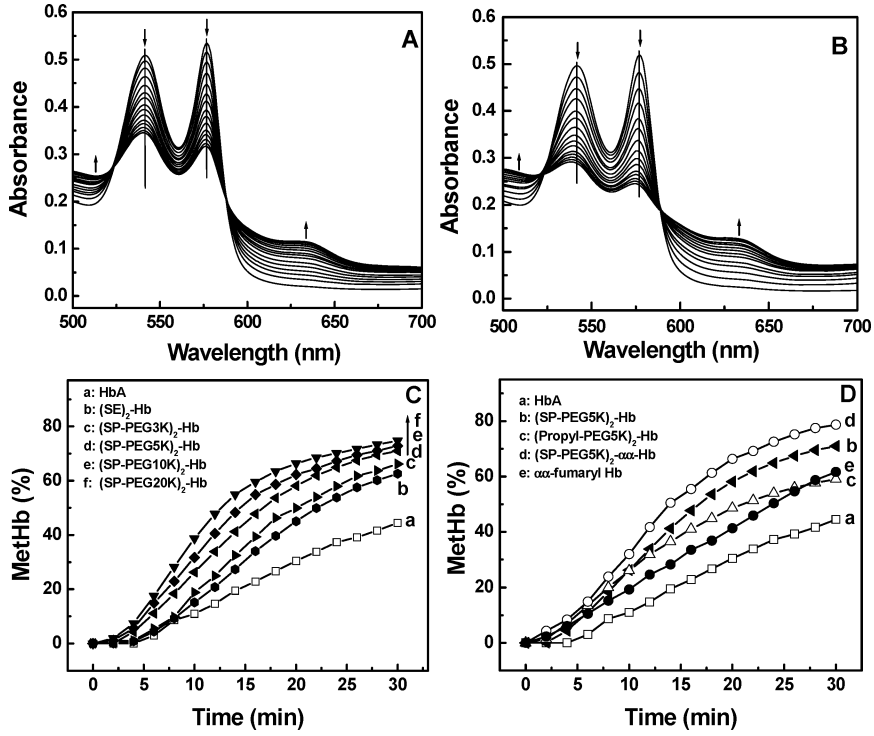

FIGURE 5: H_2O_2 mediated oxidation of the PEGylated Hb. The spectral changes of HbA (A) and (SP-PEG5K)$_2$-Hb (B) were observed when exposed to H_2O_2 generated from xanthine/xanthine oxidase system. The spectra were obtained every 2 min. Panels C and D showed the metHb formation of the PEGylated Hbs under H_2O_2 oxidation at 37 °C as function of time.

Effects of Various Factors on the Autoxidation of the PEGylated Hbs. (SP-PEG3K)$_2$-Hb, (SP-PEG5K)$_2$-Hb, (SP-PEG10K)$_2$-Hb and (SP-PEG20K)$_2$-Hb can be considered as the homologues of (SE)$_2$-Hb linked by phenyl PEG-3K, 5K, 10K and 20K chains, respectively (Figure 1).

(i) Spectral Change on the Autoxidation of (SP-PEG3K)$_2$-Hb. Figure 4A showed the spectral change when (SP-PEG3K)$_2$-Hb is autoxidized at 37 °C as a function of time. Two isobestic points were observed at 524 and 589 nm, along with a decrease in the maxima at 577 and 541 nm and an increase in the maxima at 630 nm. The spectra indicate that oxyHb is converted to metHb within several hours. Moreover, the fully autoxidized (SP-PEG3K)$_2$-Hb exhibited a typical spectrum of a six coordinated low-spin hydroxyl met form with the maxima at 409, 540 and 577 nm (inset, Figure 4A).

(ii) Effect of the PEG chains. Figure 4B shows the kinetics of the metHb formation of the PEGylated Hbs. (SE)$_2$-Hb exhibited a higher k_{ox} than HbA (Table 1), even though (SE)$_2$-Hb showed higher oxygen affinity that can attenuate the autoxidation rate of Hb (*1*). The k_{ox} of (SE)$_2$-Hb increased further as a function of the length of the PEG chains (Table 1). Moreover, the k_{ox} of these PEGylated Hbs is a direct correlate of the log of their molecular volumes (inset, Figure 4B). Therefore, the PEG chains were assumed to act as a reservoir of the water molecules (a weak nucleophile) to promote the autoxidation of Hb. However, the k_{ox} of (SE)$_2$-Hb was marginally changed in the presence of free PEG chain (data not shown). Presumably, the free PEG is excluded from the protein domain.

(iii) Effect of Azide. Azide, a strong nucleophile, can promote Hb oxidation by stabilizing the heme in ferric state (*26*). (SE)$_2$-Hb shows a higher metHb formation rate than HbA in the presence of 0.1 M azide (Figure 4C). On the other hand, the PEG chains have little influence on the azide-mediated oxidation. Since the PEG chains did not alter the heme environment of Hb, the water-mediated oxidation of Hb was presumably overwhelmed by the azide-mediated oxidation.

(iv) Influence of Oxygen Affinity and the Sites of PEGylation on the Autoxidation. (Propyl-PEG5K)$_2$-Hb exhibited a significantly lower k_{ox} than (SP-PEG5K)$_2$-Hb, regardless of their comparable P_{50} (Figure 4D). This indicates that the sites of PEGylation may influence the autoxidation of Hb (Table 1). Presumably, this influence is due to the alteration in the heme environment. The k_{ox} of (SP-PEG5K)$_2$-αα-Hb is higher than that of (SP-PEG5K)$_2$-Hb (Table 1), even though the heme environment of (SP-PEG5K)$_2$-αα-Hb is less perturbed. This suggested that the autoxidation of the PEGylated Hb was decreased by high oxygen affinity (low P_{50}).

(v) Effect of Temperature. As shown in Table 1, the activation energy (E_a) for the autoxidation of Hb increased upon modification at Cys-93(β) and decreased by conjugation of the PEG chains. Apparently, the PEG chains can stabilize Hb structure and decrease the temperature-dependent autoxidation. The E_a of (SP-PEG5K)$_2$-Hb is slightly higher than that of (Propyl-PEG5K)$_2$-Hb. This reflects a temperature-dependent conformational change of Hb that facilitates the autoxidation of the PEGylated Hb. (SP-PEG5K)$_2$-Hb shows a lower E_a than (SP-PEG5K)$_2$-αα-Hb, indicating that conserving the deoxy conformation facilitates the autoxidation of the PEGylated Hb.

Inhibition of the Autoxidation of the PEGylated Hbs by Antioxidant Enzymes. The autoxidation of the PEGylated Hbs was measured in the presence of the antioxidant enzymes, SOD and CAT. The inclusion of SOD and CAT efficiently inhibited but cannot completely suppress the autoxidation

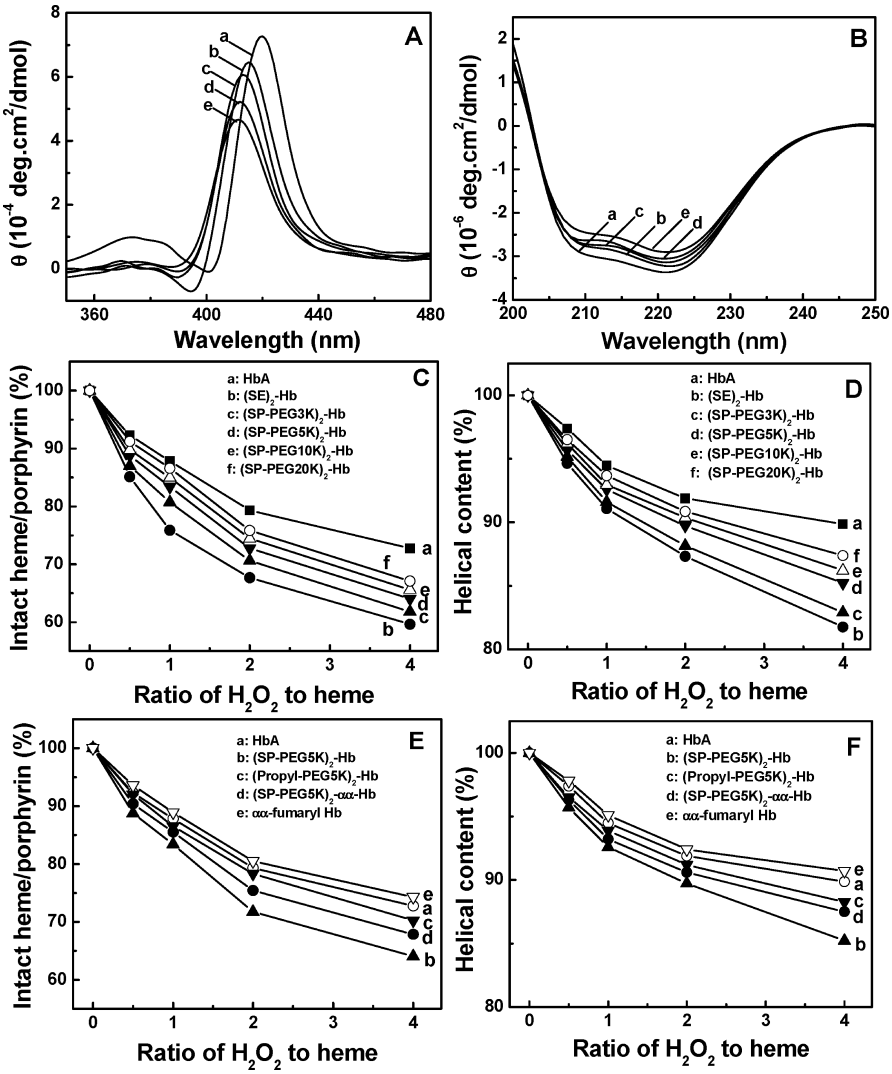

FIGURE 6: The H_2O_2 mediated structural loss of the PEGylated Hbs. CD spectra of $(SP\text{-}PEG5K)_2$-Hb in the Soret region (A) and in the far UV region (B) are shown at the molar ratio of H_2O_2 to heme equal to 0 (a), 0.5 (b), 1 (c), 2 (d) and 4 (e), respectively. The loss of intact heme/porphyrin (C and E) and α-helical structure (D and F) of the PEGylated Hbs were also shown. The Hb samples (25 μM) in PBS (pH 7.4) were incubated with H_2O_2 solution (Sigma). The incubation was at the molar ratio of H_2O_2 to heme equal to 0, 0.5, 1, 2 and 4 in final volume of 1 mL at room temperature for 1 h. The incubation was terminated by adding excessive CAT.

of the Hb samples. This indicates the production and the buildup of ROS during the autoxidation of Hb. Minor inhibition of the autoxidation was achieved when SOD is added. Nearly 50% inhibition of the autoxidation of the Hb samples was observed in the presence of CAT. Presumably, the superoxide generated from the autoxidation is rapidly dismutated to H_2O_2 that can be efficiently removed by the presence of CAT.

Peroxide Mediated Oxidation of the PEGylated Hbs. Figures 5A and 5B show the spectral alterations of HbA and $(SP\text{-}PEG5K)_2$-Hb during H_2O_2 mediated oxidation, respectively. The increase in the absorbance at 630 and 700 nm is indicative of the metHb formation as well as the degree of denaturation and precipitation. An observable ferryl complex was absent for the lack of the peaks at 545 and 580 nm, along with two isobestic points at 523 and 589 nm. Moreover, the original peaks at 541 and 576 nm for $(SP\text{-}PEG5K)_2$-Hb is left shifted as a function of time. This suggested that oxidation of ferric iron to ferryl iron was inhibited and hemichrome was formed during the oxidation process.

The metHb formation rate of $(SE)_2$-Hb is faster than that of Hb (Figure 5C), which is further increased by the PEG chains. In addition, $(SP\text{-}PEG5K)_2$-Hb shows a higher metHb formation rate than $(Propyl\text{-}PEG5K)_2$-Hb (Figure 5D). This indicated that the H_2O_2 mediated oxidation of the PEGylated Hbs is promoted by the PEG chains and is dependent on the sites of PEGylation. $(SP\text{-}PEG5K)_2$-αα-Hb shows a higher metHb formation rate than $(SP\text{-}PEG5K)_2$-Hb. This indicated that the H_2O_2 mediated oxidation of the PEGylated Hb is dependent on the oxygen affinity.

The CD measurement was used to evaluate the H_2O_2 mediated structural loss of Hb. The ellipticity of $(SP\text{-}PEG5K)_2$-Hb at 421 nm decreased in the presence of H_2O_2. The peak with a maximum at 421 nm slowly shifted to approximately 412 nm (Figure 6A), reflecting the loss of heme/porphyrin (*12*). The absolute value of the ellipticity of $(SP\text{-}PEG5K)_2$-Hb at 222 nm also decreased as the H_2O_2 concentration was increased (Figure 6B). This decrease reflects the loss of α-helical content of Hb. The loss rates of heme/porphyrin (Figure 6C) and α-helix content (Figure 6D) of Hb are lowered by the presence of the PEG chains. The

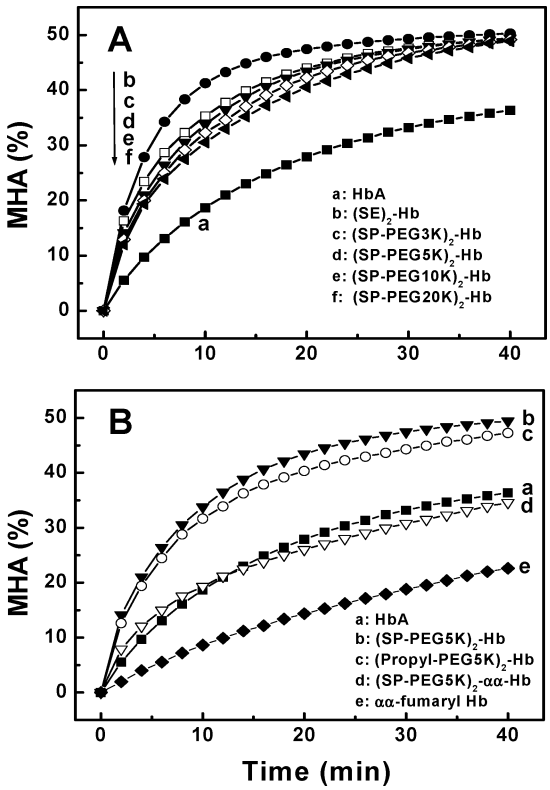

FIGURE 7: Kinetics of the heme transfer from the oxidized Hb samples to human serum albumin. Percent metheme-albumin (MHA) = [MHA]/([metHb] + [MHA]) × 100%.

Table 2: The Heme Loss of the PEGylated Hbs[a]

sample	k_{fast}[b] (min^{-1})
HbA	0.044
$(SE)_2$-Hb	0.161
$(SP\text{-}PEG3K)_2$-Hb	0.108
$(SP\text{-}PEG5K)_2$-Hb	0.100
$(SP\text{-}PEG10K)_2$-Hb	0.092
$(SP\text{-}PEG20K)_2$-Hb	0.082
$(Propyl\text{-}PEG5K)_2$-Hb	0.089
αα-fumaryl Hb	0.019
$(SP\text{-}PEG5K)_2$-αα-Hb	0.036

[a] The k_{fast} is calculated from the fit of a single-exponential equation: $Y = Y_{max}(1 - e^{-kt}) + Y_0$. Y is the percentage of MHA. Y_{max} is the total percentage change in metHb at the end of reaction. The t is the time. Y_0 is the percentage of MHA at $t = 0$. [b] The fast-phase heme loss rate constant, $P < 0.0001$ and $R > 0.995$.

loss rates of intact heme/porphyrin (Figure 6E) and α-helical content (Figure 6F) of $(SP\text{-}PEG5K)_2$-Hb are higher than those of $(Propyl\text{-}PEG5K)_2$-Hb and $(SP\text{-}PEG5K)_2$-αα-Hb. Thus, the structural loss of the PEGylated Hb is dependent on the site of PEGylation and attenuated by the PEG chains and αα-fumaryl cross-link.

Heme Loss of the PEGylated Hb. Oxidation of ferrous heme iron to the ferric state weakens its fifth coordinate bond to proximal His residue and thereby increases the probability of the heme loss (*37*). The kinetics of the heme loss from metHb to HSA (the heme acceptor with strong binding site for heme) has been measured (Figure 7). The fast-phase heme loss rate constant (k_{fast}) was used to evaluate the heme loss of the PEGylated Hbs (Table 2). The k_{fast} of $(SE)_2$-Hb is higher than that of HbA and further decreased by the PEG chains. The k_{fast} of $(SP\text{-}PEG5K)_2$-Hb was higher than that of $(Propyl\text{-}PEG5K)_2$-Hb. Thus, the heme loss of the PEGylated Hb was decreased by conjugation of the PEG chains and was dependent on the sites of PEGylation. The αα-fumaryl cross-link decreases the heme loss of $(SP\text{-}PEG5K)_2$-Hb. This indicated that the tetramer stability of Hb might enhance the heme stability of Hb.

The Autoxidation of the PEGylated Hbs in the Plasma. Typically, the *in vivo* oxidation of Hb depends on the capacity of the plasma and the tissue to maintain Hb in a reduced state (*38*). Accordingly, the autoxidation of the PEGylated Hb was investigated in the plasma at 37 °C to simulate the *in vivo* oxidation.

(i) Effect of PEGylation on the Autoxidation of Hb in the Plasma. The autoxidation of all the Hb samples in the plasma followed pseudo-first-order kinetics (data not shown). The k_{ox} of the PEGylated Hbs is attenuated in the plasma (Figure 8A−C). PEGylation at Cys-93(β) led to a much higher increase in the k_{ox} of Hb in the plasma than that in PBS buffer. However, PEGylation at Val-1(β) led to a marginal increase in the k_{ox} in the plasma. Presumably, the components in the plasma may stabilize the heme environment of the PEGylated Hbs and thus attenuate the autoxidation of Hb. On the other hand, αα-fumaryl cross-link led to an increase in the k_{ox} in the plasma comparable to that in PBS buffer. Thus, the effect of the oxygen affinity on the autoxidation of the PEGylated Hb was not altered in the plasma.

(ii) Effect of the Protein Concentration. As Hb concentration increased from 8 to 20 mg/mL (Figure 8A−C), the k_{ox} of HbA increased slightly. The k_{ox} of Hb was more dependent on the protein concentration upon modification at Cys-93(β) and conjugation of the PEG chains both in the plasma and in PBS. However, this dependence is less pronounced in the plasma than that in PBS.

(iii) Effect of SOD and CAT. The inclusion of excessive CAT and SOD lowered the k_{ox} of these Hb samples. The k_{ox} of the PEGylated Hbs was about 50% inhibited by the enzymes in PBS buffer and about 30% inhibited in the plasma (Figure 8D). This suggests that the presence of the antioxidant species in the plasma partially suppressed the oxidation of the PEGylated Hbs.

DISCUSSION

In the present work, we investigated the influence of PEGylation on the autoxidation of Hb. The most significant findings are that (i) PEGylation perturbed the heme environment, increased the solvent accessibility to the heme pocket and increased the *in vitro* autoxidation of Hb; (ii) conjugation of the PEG chains did not perturb the heme environment; (iii) the PEG chains are assumed to act as a reservoir of waters to promote the autoxidation of Hb; (iv) the H_2O_2 mediated structural loss of Hb is decreased by the PEG chains and αα-fumaryl cross-link; (v) the autoxidation of the PEGylated Hb is significantly attenuated in the presence of the plasma.

The embedding of the heme group within the globin chains provides a significant protection against the autoxidation of Hb (*39*). The heme is covalently bound to the globin chains via the proximal His-92(β) and is wedged into its pocket by a Phe residue (Phe CD1) (*40*). Cys-93(β) is located at ~1.4 nm from the iron and is adjacent to the proximal His-92(β) (*41*). The proton NMR spectra showed that chemical modification of Cys-93(β) or its mutation significantly influenced the proximal heme pocket of the β chain (*42*).

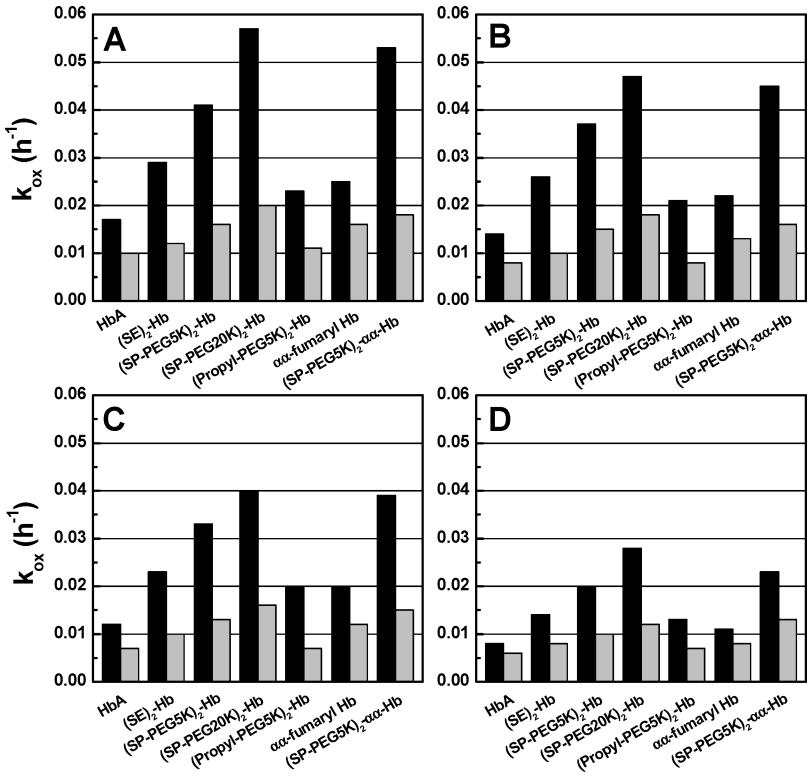

FIGURE 8: The autoxidation of the PEGylated Hbs in the plasma. The k_{ox} of the Hb samples in plasma (gray bar) and in PBS (black bar) were measured at 20 mg/mL (A), 13.3 mg/mL (B) and 8 mg/mL (C) and 20 mg/mL with SOD and catalase (D) at 37 °C, respectively.

Because Val-1(β) is far from the proximal His-92(β), PEGylation at Val-1(β) induces less perturbation of the heme environment.

The PEG chains are expected to bind 2−3 water molecules per repeating unit and can create a water dense domain around the protein (*21*). Roche et al. (*43*) proposed three hydration layers around Hb, i.e., (i) the surface waters that are in very close contact with the surface residues of Hb, (ii) the waters that comprise the hydration layer of Hb and (iii) the bulk water phase. The PEG chains exhibit both hydrophobic and hydrophilic properties. Accordingly, the PEG chains could interact with nonpolar protein surface residues in competition with exclusion forces (*44*). Along with the increased solvent accessibility to heme pocket, the water-rich PEG domain is assumed to facilitate the entrance of water to the heme pocket and accelerate the water-mediated autoxidation of Hb. On the other hand, the free PEG is preferentially excluded from protein domain by steric exclusion and shows a marginal influence on the autoxidation of Hb.

The autoxidation of Hb and its derivatives has been suggested to correlate inversely to the oxygen affinity of Hb (*1, 36*). Vandegriff et al. (*19*) suggested that there is a better correlation between k_{ox} and k_{off} (oxygen dissociation). Our present studies reveal that the autoxidation of the PEGylated Hb is assumed to involve two other factors, i.e., nucleophilic displacement of dioxygen from oxyHb by the water molecule (through the PEG chains) and the increased solvent accessibility to the heme pocket. The effects of these two factors may counteract the contribution of the oxygen affinity on the autoxidation of the PEGylated Hb.

Vandegriff et al. (*19*) have reported that MP4 displayed faster heme loss than Hb. However, the contribution of PEGylation on the heme loss of Hb is not clear. Our studies revealed that the heme loss of the PEGylated Hb is promoted by modification at the specific sites and attenuated by conjugation of the PEG chains. The heme loss from the metHb is related to the geometry of the heme pocket and dynamics of Hb molecule (*37*). This also reflects an increasing instability of globin as the heme loss continues. Because of the alteration in the local environment and the dynamics of the Hb domain, the chemical modification increases the heme loss of Hb. However, the PEG chains maintain the native conformation of the protein (*45*) and decrease the heme loss of Hb.

Our data demonstrates that the k_{ox} of the PEGylated Hb was proportional to oxyHb concentration. The k_{ox} of the PEGylated Hb is assumed to be additive with the oxidation promoting ligand induced reaction: $k_{ox} = k_1[\text{oxyHb}][H_2O]$, where k_1 is strongly sensitive to the nature of the ligand (e.g., H_2O) and the heme environment. On the other hand, Hb at low concentration has a high autoxidation rate, due to the dissociation of the tetrameric Hb to dimers (*7, 8*). Thus, the correlation between Hb with dimer formation and the autoxidation rate (*7, 8*) is distinct from the correlation shown here for the PEGylated Hb. It should be mentioned that the autoxidation condition here is different from the earlier report (*1*). The Hb samples (50 μM) in 50 mM potassium phosphate buffer (pH 7.4) containing 100 μM EDTA were used in that study (*1*). However, 25 μM Hb samples in PBS (pH 7.4) containing 300 μM EDTA were used here. The k_{ox} values of αα-Hb at 25 μM and 50 μM are 0.016 h^{-1} and 0.019 h^{-1}, respectively. Besides the protein concentration, EDTA and the buffer may influence the k_{ox}.

H_2O_2 is a powerful oxidant to produce cellular damage. Alayash et al. (*12*) reported that intact heme/porphyrin and α-helical structure were lost upon H_2O_2 oxidation of Hb. This structural loss is partly related to oxidative modification

of the amino acids in the β globin susceptible to H_2O_2 oxidation. Modification at the specific sites, e.g., Cys-93(β), may lead to the heme instability, alter the local environment of some key amino acids and enhance their susceptibility to H_2O_2 oxidation. The $\alpha\alpha$-fumaryl cross-link and the PEG chains could increase the heme stability by stabilizing Hb structure and shield some key amino acids from oxidative modification.

Formation of metHb *in vivo* from MP4 following its transfusion in rats and humans was slower than that predicted based on the *in vitro* autoxidation rates (*19*). Vandegriff et al. suggested that the ability of blood to keep cell-free Hb Fe^{2+} in the reduced state probably depends on the presence of free radical-scavenging antioxidant (*19*). Our present data supports this suggestion and demonstrates that the autoxidation of the PEGylated Hbs (i) may be partially attenuated by the antioxidant species in the plasma; (ii) was dependent on the Hb concentration in the plasma; and (iii) was less influenced by PEGylation in the plasma than in PBS buffer.

As noted earlier, two PEG chains of MP4 are on Cys-93(β) and the rest four are on the Lys residues of Hb (*18*). HexaPEGylation of Hb resulted in a 3-fold increase in the k_{ox} of Hb (*19*). Our present study shows that PEGylation at Cys-93(β) increased the k_{ox} of Hb by nearly 2.5-fold. Thus, PEGylation at the Lys residues has very limited influence on the autoxidation of Hb. Similarly, PEGylation at Val-1(β) has a limited influence on the autoxidation of Hb. Thus, avoiding PEGylation at Cys-93(β) is expected to generate a PEGylated Hb with low autoxidation rate.

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