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**PEGylation Promotes Hemoglobin Tetramer Dissociation**

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Hemoglobin conjugated with poly(ethylene glycol) (PEG) acts as an oxygen carrier free in plasma, substituting red blood cells in supplementing oxygen in hypo-oxygenation pathologies. Given the complexity of oxygen delivery controls, subtle structural and functional differences in PEGylated hemoglobins might be associated with distinct physiological responses and, potentially, adverse effects. We have compared hemoglobin PEGylated under anaerobic conditions, called PEG-Hb<sub>deoxy</sub>, with hemoglobin PEGylated under aerobic conditions, called PEG-Hb<sub>oxy</sub>, a product that mimics Hemospan, produced by Sangart, Inc. SDS PAGE and MALDI-TOF analyses demonstrated that PEG conjugation yields products characterized by a broad distribution of PEG/hemoglobin ratios. The elution profiles in size-exclusion chromatography indicate that both products exhibit a more homogeneous distribution of molecular weight/hydrodynamic volume under deoxy conditions and at higher concentrations. PEG-Hb<sub>oxy</sub> shows high oxygen affinity, low modulation of allosteric effectors, almost no cooperativity, a fast and monophasic CO binding, and a limited dependence of functional properties on concentration, whereas PEG-Hb<sub>deoxy</sub> exhibits oxygen binding curves that significantly depend on protein concentration, and a slow CO binding, similar to native hemoglobin. PEGylated CO-hemoglobins, probed by flash photolysis, exhibited a lower amplitude for the geminate rebinding phase with respect to native hemoglobin and a negligible T state bimolecular CO rebinding phase. These findings are explained by an increased dissociation of PEGylated hemoglobins into dimers and perturbed T and R states with decreased quaternary transition rates. These features are more pronounced for PEG-Hb<sub>oxy</sub> than PEG-Hb<sub>deoxy</sub>. The detected heterogeneity might be a source of adverse effects when PEGylated Hbs are used as blood substitutes.
of byproduct and heterogeneity generated by the chemical procedures or slight deviation of the functional properties from the physiological needs might be responsible for some of the observed adverse effects. However, so far, no investigation has been carried out to link structural and functional properties of products with the observed adverse effects. One of these products, MP4, trade name Hemospan, is obtained via the conjugation of Hb with an average of 6–7 PEG 5000 (M1) chains under oxygenated conditions in a two-step procedure: first, oxyHb is thiolated by treatment with iodoacetamide (IMT), and then, thiols react with the PEG-maleimido derivative MAL-PEG (16, 26). Hemospan has completed phase III clinical trials to test its capacity for prevention and treatment of hypotension during orthopedic surgery (22, 25, 27). Hemospan exhibits high oxygen affinity and low cooperativity, which makes it similar to Hb dimers than to the physiological tetrameric oxygen carrier. These features might have originated from the interference of PEG molecules with Hb amino groups of the organic phosphate binding pocket and the sulfhydryl tetrameric oxygen carrier. These features might have originated.

**Experimental Procedures**

**Materials.** IMT, IHP, NEM, molecular weight markers, BSA, and MALDI calibration kit were purchased from Sigma Aldrich. Reagents and equipment for SDS-PAGE were from Biorad Laboratories Inc. Ultrafiltration membranes and kits were from Sartorius Stedim and Millipore. MAL-PEG (molecular mass 5.6 kDa) was from Nektar Molecule Engineering (Nektar Therapeutics).

**Protein Modification.** Conjugated Hbs were prepared by treating HbA in 50 mM Na-phosphate, 100 mM NaCl, 0.5 mM EDTA, pH 7 at 20 °C, with an excess of IMT and MAL-PEG, or NEM, under anaerobic conditions in the presence of IHP, as previously described (34) to produce PEG-Hb\textsuperscript{oxy}. The amount of IMT and the reaction time were adjusted to achieve the desired degree of thiolation and, thus, the number of conjugated PEG molecules. Concentrated and deoxygynated lysine and cysteine solutions were added to stop IMT and MAL-PEG reactions, respectively. PEG-Hb\textsuperscript{oxy} was then equilibrated with 100 mM NaCl by seven ultrafiltration cycles, under aerobic conditions, at 5 °C, using a 30 kDa cutoff Amicon membrane. The solution was concentrated to one-third of the initial volume before redilution with NaCl solution. The number of conjugated PEG chains was estimated by treating deoxy Hb with IMT under the same conditions without the addition of MAL-PEG. The thiol titer determined by a biochemical method (39), corrected for the titer determined after treatment with MAL-PEG, or NEM, yielded the number of conjugated PEG/tetramer, or NEM/tetramer (34). PEG-Hb\textsuperscript{oxy} was prepared as described by Vandegriff et al. (16). Hb, PEG-Hb\textsuperscript{oxy}, and PEG-Hb\textsuperscript{deoxy} were stored in liquid nitrogen. The ferric Hb content was less than 2%. Hb concentration is expressed on a hemoglobin basis. BSA (68 kDa, 59 lysines) was PEGylated under aerobic conditions by the same procedure described for PEG-Hb\textsuperscript{oxy} to obtain a protein conjugated with an average number of 6 PEG/molecule. Since the BSA sample was contaminated by 5% IgG immunoglobulin, the product was also contaminated by PEGylated IgG, which showed up in size exclusion chromatography as an inflection of the leading edge of the elution profile of PEG-BSA. The characteristics of the various preparations are summarized in Table 1.

**SDS-PAGE and Protein Extraction.** SDS-PAGE was carried out according to Laemli (40) by acrylamide and piperazine diacrylamide as the cross-linker. Gels were stained with BioSafe G-250 Coomassie Brilliant Blue compatible with MS analysis and scanned using Quantity One software. Protein bands were excised from the gels and destained with a 40/10 (v/v) mixture of methanol/acetic acid. The proteins were recovered by passive elution at 37 °C using the SDS-PAGE running buffer and concentrated by Vivasin 2 (Sartorius) centrifugal concentrators, which facilitates SDS removal.

**Table 1. Biochemical Characterization of Hb Derivatives\textsuperscript{a}**

<table>
<thead>
<tr>
<th>species</th>
<th>reaction cycles</th>
<th>total SH</th>
<th>free SH</th>
<th>conjugation (NEM or MAL-PEG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA</td>
<td>2 cycles IMT+NEM</td>
<td>7.7</td>
<td>2.3</td>
<td>5.3 (NEM)</td>
</tr>
<tr>
<td>NEM-Hb</td>
<td>1 cycle IMT+MAL-PEG</td>
<td>9.0</td>
<td>2.3</td>
<td>6.7 (MAL-PEG)</td>
</tr>
<tr>
<td>PEG-Hb\textsuperscript{oxy}</td>
<td>1 cycle IMT+MAL-PEG</td>
<td>6.9</td>
<td>1.0</td>
<td>5.9 (MAL-PEG)</td>
</tr>
<tr>
<td>PEG-BSA</td>
<td>1 cycle IMT+MAL-PEG</td>
<td>5.9</td>
<td>0.2</td>
<td>5.7 (MAL-PEG)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} IMT-modified amino groups before (total SH) and after reaction with NEM or MAL-PEG (free SH) as determined on a tetramer basis by thiol titration with the method of Ampulsky et al. (39) The number of residues conjugated with NEM or MAL-PEG was equal to the difference between total SH and free SH. The titre of Cys/393 of native Hb yielded by the method was 2.3.

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Mass Spectrometry. A layer of sinapinic acid in acetonitrile was spotted onto the sample well before adding 1 µL protein aliquots diluted with matrix solution containing 30 mg/mL sinapinic acid in 50/50 (v/v) ACN/0.1% TFA. MALDI TOF mass spectra were obtained using a MALDI-LR mass spectrometer (Waters Italia) operated in negative linear mode. External calibration was carried out in the 7–70 kDa mass range using protein standards from the ProteoMass Peptide and Protein MALDI-MS Calibration kit: aprotinin (equine) (M+H+): 16 952.27), aldolase (M+H+): 39 212.28), albumin (M+H+): 66 430.09) and High Mass Post Acceleration Linear detector to increase the sensitivity for the PEGylated molecules.

Static and Dynamic Light Scattering. Laser light scattering was carried out with a goniometer (BI-200SM Brookhaven Instrument Corporation) equipped with a BI-9K Digital correlator and a solid-state 676 nm light source. Independent SLS and DLS measurements were carried out at 20 °C, under aerobic conditions, on Hb, PEG-Hbdeoxygenated, and PEG-Hbdeoxygenated in a solution containing 50 mM Na-phosphate, 100 mM NaCl, 0.5 mM EDTA, pH 7, at 40–280 µM concentration range. The hydrodynamic radii were determined by Cumulant and Fourier Transform analysis of the intensity correlation function measured by DLS (41). Independent SLS measurements provided information about the average molecular weight and concentration of macromolecules after absolute intensity calibration with a 0.02 mg/mL C₁₂E₈ solution (42).

Size Exclusion Chromatography. Chromatography was carried out in 50 mM Na-phosphate, 100 mM NaCl, 0.5 mM EDTA, pH 7, with an AKTA Prime instrument and a HiLoad Superdex 200 column (≈120 mL bed volume, 600 kDa mass exclusion limit), thermostatted at 20 °C, at flow rate of 1.5 mL/min (Amersham Pharmacia Biotech). The retention time was 40–70 min using 0.5 mL samples of PEGylated Hb. To carry out anaerobic chromatography, the buffer reservoir was kept under nitrogen and 1 mM Na-dithionite was added to the buffer. Samples were loaded after deoxygenation by nitrogen tonometry and addition of Na-dithionite. The eluate from Hb chromatographic analysis was monitored at 280 nm to avoid dithionite interference, whereas the flowthrough of BSA chromatographic analysis was monitored at 280 nm. Chromatograms were normalized to absorbance values versus molecular mass derived from standard proteins calibration.

To determine the tetramer–dimer equilibrium constant of oxy Hb and oxy NES-Hb, a HiLoad Sephacryl S-200 column was used (bed volume ≈79 mL, matrix volume ≈3 mL, according to the manufacturer specifications, and void volume ≈38 mL, as determined by Blue Dextran, 250 kDa mass exclusion limit). Solute saturation was achieved by loading 15 mL samples. The column was run at 0.5 mL/min flow rate at 20 °C. A solute in rapid tetramer–dimer equilibrium, as Hb, loaded on such a column under solute saturating conditions, shows an elution profile characterized by a plateau region between a leading and a trailing edge. The solute elution volume at the inflection point of the leading edge, \( V \), is related to the fraction of dissociated tetramers, \( \alpha \), through eq 1 (43):

\[
V = V_0 + [\sigma_T + \alpha(\sigma_D - \sigma_T)]V_i
\]  

where \( V_0 \) is the void volume, \( V_i \) the internal matrix volume, provided by the manufacturer, and \( \sigma_T \) and \( \sigma_D \) are parameters that characterize the matrix interactions with tetramers and dimers, respectively. These parameters were obtained using enough high and low Hb concentrations to justify the assumption that the protein was in tetrameric and dimeric form, respectively. The gradient of the elution profile was analyzed to precisely determine the elution volume at the inflection point of the leading edge. The dissociation constant is related to \( \alpha \) through eq 2:

\[
K_d = \alpha^2 C/(1 - \alpha)
\]  

where \( C \) is the solute plateau concentration. Under deoxy conditions, the method could not be used since both normal and NES-Hb require dilution much beyond the limit of concentration detection of the technique and the tetramer–dimer equilibration is not rapid relative to the time scale of the chromatographic run.

Oxygen Affinity Measurements. Oxygen dissociation curves (ODCs) as a function of protein concentration were carried out at 15 °C in a solution containing the enzymatic metHb reducing system (44), at pH 7.0, using a novel homemade apparatus (45). The gas mixture generated by Environics 200 and 4000 (Environics, Inc.) flows in a thermostatted humidifier and then in the sample chamber consisting of a 2 mm optical path cuvette fused to a 25 mL open-top threaded reservoir with fittings for inlet and outlet gas lines. The chamber is thermostatted in a shaking bath. The sample compartment of a CARY 400 spectrophotometer (Varian, Inc.) is also thermostatted. Fractional oxygen saturations and oxidized hemehs were determined by fitting the observed spectra to a linear combination of reference spectra (46, 47). ODCs of PEG-Hbdeoxygenated, in the absence of allosteric effectors, at different protein concentrations, were globally fitted using eq 3, assuming two independent species \( a \) and \( b \), characterized by \( p_{50a} \) and Hill coefficient \( n_a \), and \( p_{50b} \) and Hill coefficient \( n_b \), respectively, varying only the relative fraction of species \( a, f_a \), as a function of concentration:

\[
Y = f_a \frac{pO_2^a}{pO_2^a + pSO_2^a} + (1 - f_a) \frac{pO_2^b}{pO_2^b + pSO_2^b}
\]

ODCs in the presence of the allosteric effectors Cl⁻ and CO were fitted with eq 1, constraining \( p_{50a} \) and \( n_a \) to be equal to those calculated in the absence of allosteric effectors. It was assumed that functional properties of species \( a \) are unaffected by effectors because this species corresponds to Hb dimers, whereas functional properties of species \( b \) are affected because this species corresponds to Hb tetramers (see Results). ODCs of PEG-Hbdeoxygenated and Hb were fitted to a single species characterized by \( p_{50} \) and Hill coefficient \( n \).

CO Binding Kinetics. CO binding to HbA, PEG-Hbdeoxygenated, and PEG-Hbreoxygenated was followed by stopped-flow apparatus (SX 18 MV, Applied Photophysics) at a single wavelength (435 nm). Two millimolar Na-dithionite was added to deoxygenated protein and ligand solutions to completely remove oxygen. Depending on Hb concentration, a path length of 0.2 or 1 cm was used.

Flash photolysis experiments were performed with a setup described elsewhere (48, 49), following the absorbance change at 436 nm. Photolysis yields for all species were evaluated and found to be similar. The rebinding kinetic traces were first converted to fraction of deoxy-protein as a function of time. The lifetime distribution associated with the observed CO rebinding kinetics could not be used since both normal and NES-Hb require dilution much beyond the limit of concentration detection of the technique and the tetramer–dimer equilibration is not rapid relative to the time scale of the chromatographic run.

RESULTS

Analysis of PEGylated Hbs by SDS-PAGE and Mass Spectrometry. A previous mass spectrometry (MS) study of Hb PEGylated under anaerobic conditions showed that 5 lysines
per subunit were modified by IMT at a significantly higher rate than three additional lysines, both on the α and β chains (34). The reactivity differences between these 5 reactive lysines do not allow the preparation of a homogeneous product. Only 6—7 residues per tetramer are PEGylated, showing a significant number of conjugated PEG chains. However, a shift in the apparent molecular weight from 540 to 650 kDa was eluted as a single, nearly symmetrical peak, which shifted to lower values upon dilution due to the increase in the molecular mass, suggesting a Hb core surrounded by a 15—20 Å thick shell of highly hydrated flexible PEG monomers.

**Analysis of PEGylated Hbs by Static and Dynamic Light Scattering.** Solutions containing HbA, PEG-Hbdeoxygenated, and PEG-Hbdeoxygenated, at a concentration of 40—280 μM, were characterized by static light scattering (SLS) and dynamic light scattering (DLS) measurements. The observed average weight molecular masses were 68 ± 5, 105 ± 2, and 99 ± 1 kDa, respectively. The average number of PEG chains per tetramer, 6.6 (PEG-Hbdeoxygenated) and 5.6 (PEG-Hbdeoxygenated), calculated from these mass values agreed with the values obtained by the biochemical characterization and mass spectroscopy measurements (Table 1). The average hydrodynamic diameters of HbA, PEG-Hbdeoxygenated, and PEG-Hbdeoxygenated were 59, 105, and 104 Å, respectively, within 5% error. Using the static and dynamic measurements, it was calculated that Hb hydrodynamic radius agreed with the dimension of a globular particle with 66 kDa molecular mass. In the case of PEG-Hbdeoxygenated and PEG-Hbdeoxygenated, PEGylation brought about a significant increase in hydrodynamic radius with respect to the increase in the molecular mass, suggesting a Hb core surrounded by a 15—20 Å thick shell of highly hydrated flexible PEG monomers.

**Analysis of PEGylated Hbs by Size Exclusion Chromatography.** Column chromatography of oxy Hb and oxy NES-Hb, at 20 °C in 50 mM phosphate, 100 mM NaCl, 0.5 mM EDTA, 0.1 mM NaCN, pH 7, under aerobic conditions and protein column saturation, yielded single plateau regions at all protein concentrations in the 4—4000 μM range. The averaged dimer/tetramer Kd(deoxygenated) values were 0.26 ± 0.08 and 0.42 ± 0.07 μM, respectively, comparable to published values of 0.48—1.9 μM for liganded human Hbs under similar conditions (36, 37). Under anaerobic conditions, the dimer/tetramer Kd(deoxygenated) values of deoxy NES-Hb could not be obtained by this approach due to the extremely low value of the equilibrium dissociation constant, as already known for Hb (54).

The chromatography of PEG-Hbdeoxygenated and PEG-Hbdeoxygenated, under aerobic conditions, failed to yield a single plateau because of either the heterogeneity of the PEGylated proteins or the slow equilibration of the species during chromatography. To clarify this issue, we carried out size exclusion chromatography using nonsaturating column conditions. Oxy Hb at all concentrations was eluted as a single, nearly symmetrical peak, which shifted from 65 ± 3 kDa at 800 μM to lower values upon dilution due to the increased fraction of dimers in rapid equilibrium with tetramers (data not shown). Deoxy Hb was eluted as a single, 65 kDa symmetrical peak at all concentrations (data not shown). Chromatography of PEG-BSA (Figure 2A, inset) showed a single main peak with an inflection in the leading edge due to the presence of PEGylated IgG immunoglobulin. This clearly indicates that under the conditions of the chromatography the molecular sieve could not resolve species differing in the number of conjugated PEG chains. However, a shift in the apparent molecular weight from 540 ± 25 to 640 ± 25 kDa occurred
upon dilution (Figure 2A, inset), due to non-ideality of the solute–matrix interactions. The non-ideality effect was also observed using Hb at high concentrations where dissociation into dimers is not significant (data not shown). At 800 μM concentration, oxy PEG-Hb<sub>deoxy</sub> was eluted as a main species with an apparent molecular weight of 460 ± 25 kDa, more than four times the value calculated on the basis of the average PEG/tetramer number (Figure 2A). A trailing edge, however, indicated the presence of small amounts of other components. 

At 2 μM concentration, four additional unidentified components, partially or completely resolved, were observed (Figure 2A). The formation of low relative molecular mass (M<sub>r</sub>) components upon dilution was reversible, since a 8 μM sample of oxy PEG-Hb<sub>deoxy</sub> reconstituted oxygenated to 800 μM was eluted as the original undiluted 800 μM sample (data not shown). PEG-Hb<sub>deoxy</sub> chromatography under anaerobic conditions, at all concentrations, yielded a single peak with an apparent molecular weight of 500 kDa. However, the slight trailing observed with diluted samples depended on the modality of sample dilution. When a 800 μM oxygenated sample was deoxygenated before dilution to 8 μM, trailing was minimal (Figure 2B). If dilution to 8 μM was carried out under aerobic conditions followed by rapid deoxygenation with Na-dithionite, trailing was more pronounced (Figure 2B). Under aerobic conditions and at 8 μM concentration (Figure 2C), PEG-Hb<sub>oxy</sub> showed at least a five-component elution pattern with the main component, I (Figure 2C), shifted to an apparently lower molecular weight (380 kDa) than component I in the PEG-Hb<sub>deoxy</sub> elution pattern, Figure 2A, and a significant increase in the relative proportions of partially or completely resolved species as compared with PEG-Hb<sub>deoxy</sub> under the same conditions (Figure 2C). Under anaerobic conditions and when rapid deoxygenation of 8 μM oxy PEG-Hb<sub>oxy</sub> was carried out by Na-dithionite addition (Figure 2C), the apparent M<sub>r</sub> of the main peak was 430 kDa and the presence of incompletely reass ociated species was more evident than in the case of PEG-Hb<sub>deoxy</sub> (Figure 2B).

Oxygen Binding Curves of PEG-Hb<sub>deoxy</sub> and PEG-Hb<sub>oxy</sub> as a Function of Protein Concentration, in the Absence and Presence of Allosteric Effectors. ODCs were carried for PEG-Hb<sub>deoxy</sub> at protein concentrations between 11 and 550 μM, for PEG-Hb<sub>oxy</sub> at 219 μM and for HbA at 13 and 193 μM in a solution containing 100 mM Hepes, 1 mM EDTA, pH 7, at 15 °C (Figure 3). The apparent oxygen affinity for PEG-Hb<sub>deoxy</sub> was found to increase as protein concentration decreased. ODCs were globally fitted to two components, a with p50<sub>a</sub> = 0.80 ± 0.12 Torr and n<sub>a</sub> = 1.33 ± 0.09 and b with p50<sub>b</sub> = 3.18 ± 0.08 Torr and n<sub>b</sub> = 2.54 ± 0.19. The fraction of species a, f<sub>a</sub>, decreases with increasing Hb concentration, remaining constant above 200 μM (Figure 3, inset). The dependence of f<sub>a</sub> on Hb concentration was fitted to a binding isotherm with a dissociation constant K<sub>a</sub> of 21.5 ± 2.3 μM.

ODCs for HbA (Figure 3) do not exhibit variation as a function of protein concentration and were fitted to an equation for a single oxygen binding species with a p50 of 2.65 ± 0.02 Torr and Hill n = 2.39 ± 0.05, values very close to those exhibited by species b. ODC for PEG-Hb<sub>oxy</sub> (Figure 3) was well fitted to a single species with p50 of 0.66 ± 0.03 Torr and Hill n of 1.36 ± 0.03, values very close to those exhibited by species a. However, when ODCs were measured for PEG-Hb<sub>oxy</sub> fractions separated by native PAGE (data not shown), p50 values were found to even 2-fold different between them.

PEGylated Hbs obtained under anaerobic conditions exhibited nearly normal oxygen affinity modulation by 2,3-BPG, due to the organic phosphate binding pocket protection by IHP during the chemical modification and a Bohr effect reduced by 20–25% (34). However, more relevant in view of the use of PEG-Hbs as HBOC are the effects of CO₂ and Cl⁻, which are allosteric

Figure 2. Size exclusion chromatography. A HiLoad Superdex 200 column (about 120 mL bed volume) was equilibrated at 20 °C with 50 mM Na-phosphate, 100 mM NaCl, 0.5 mM Na-EDTA, 0.1 mM NaCN, pH 7. Samples (0.5 mL) were eluted at a 1.5 mL/min flow rate. The elution volumes in the abscissa were transformed into molecular weight units (kDa) by column calibration using known standards. Absorbance values on the ordinate were normalized to the highest value. (A) (dash line) 800 and (solid line) 8 μM oxy PEG-Hb<sub>deoxy</sub>. The species eluted in peaks 1–5 were not identified. The apparent molecular weight of peak 1 is 460 kDa. Inset: 800 M (solid line) and 8 μM (dash line) PEG-BSA. The apparent molecular weights are 540 and 640 kDa, respectively. The inflection of the leading edge indicates the presence of PEGylated IgG, which contaminated the native BSA sample (5%). (B) 8 μM deoxy PEG-Hb<sub>deoxy</sub> in the presence of 1 mM Na-dithionite. (dash line), sample prepared by dilution of a deoxygenated 800 μM solution. (solid line), sample prepared by Na-dithionite addition to a 8 μM oxygenated solution. The apparent molecular weight of the peak is 500 kDa. (C) 8 μM PEG-Hb<sub>deoxy</sub> under aerobic (solid line) and anaerobic (dash line) conditions. The species in peaks 1–4 were not identified. The apparent molecular weight of peak 1 is 380 kDa. The 8 μM deoxy PEG-Hb<sub>deoxy</sub> sample was obtained by the addition of Na-dithionite to a 8 μM oxygenated sample before loading. The apparent molecular weight of the peak is 430 kDa.
Figure 3. Concentration dependence of oxygen binding to PEG-Hb\(deoxy\), PEG-Hb\(oxy\), and Hb. ODCs at 11 (closed up-triangles), 25 (open squares), 165 (open down-triangles), and 550 \(\mu\)M (closed circles) concentrations of PEG-Hb\(deoxy\) were determined in 100 mM Hepes, 1 mM EDTA, pH 7, at 15°C. ODCs were determined under the same conditions for 193 \(\mu\)M HbA (dash line) and 219 \(\mu\)M PEG-Hb\(oxy\) (dash-dot-dot line). ODCs for PEG-Hb\(deoxy\) were globally fitted (solid lines) assuming different fractions of component a (p50\(a\) = 0.80 Torr and \(n_a = 1.33\)) and b (p50\(b\) = 3.18 Torr, \(n_b = 2.58\)). HbA and PEG-Hb\(deoxy\) were fitted to a single species with p50 of 2.61 ± 0.05 Torr and Hill \(n\) of 2.36 ± 0.06 and p50 of 0.85 ± 0.02 Torr and Hill \(n\) of 1.34 ± 0.04, respectively. Inset: dependence of the fraction of component a on concentration. The dependence was fitted to a binding isotherm with an apparent tetramer–dimer equilibrium constant \(K_d = 21.5 ± 2.3\) \(\mu\)M.

CO re-binding to PEG-Hb\(deoxy\) and PEG-Hb\(oxy\), in the absence of IHP, exhibits a lower geminate re-binding amplitude than HbA and an almost negligible T state bimolecular re-binding (Figure 5A). The associated lifetime distributions (Figure 5B) show two major bands centered at \(\approx 90\) ns and \(\approx 200\) \(\mu\)s, corresponding to geminate and bimolecular re-binding, respectively. A barely detectable band appears at 10 ms. The amplitude of the band associated with geminate recombination is lower for PEGylated Hbs than in HbA and becomes broader in the case of PEG-Hb\(oxy\). The band centered around 200 \(\mu\)s displays only minimal differences among samples, suggesting that the rate for CO re-binding to R is similar for all samples.

The presence of the allosteric effector IHP leads to a slight decrease in the amplitude of the geminate phase for both PEG-Hb\(deoxy\) and PEG-Hb\(oxy\) and a concomitant onset of millisecond T state bimolecular re-binding. The comparison of the re-binding kinetics in the absence and presence of IHP for PEG-Hb\(oxy\) (Figure 6A) and the corresponding lifetime distributions (Figure 6B) indicates that, in the presence of IHP, the nanosecond and microsecond bands appear to be slightly shifted toward longer times, with the geminate phase becoming a bit smaller, and a clearly distinguishable additional band at \(\approx 9\) ms. These effects demonstrate for PEG-Hb\(oxy\) and, similarly, for PEG-Hb\(deoxy\) (data not shown) that the allosteric effectors bias the distribution of molecules toward states with T-like reactivity.

CO re-binding kinetics were measured for 13 and 98 \(\mu\)M PEG-Hb\(deoxy\) (Figure 7A). When PEG-Hb\(oxy\) concentration is increased, an additional kinetic phase appears in the millisecond range. Accordingly, three distinct peaks are present in the MEM lifetime distribution (Figure 7B), corresponding to geminate re-binding (100 and 130 ns, at 13 and 98 \(\mu\)M, respectively) and to bimolecular re-binding to R-like structures (230 and 250 \(\mu\)s, at 13 and 98 \(\mu\)M, respectively) and to T-like structures (12 ms at 98 \(\mu\)M; at 13 \(\mu\)M this band is barely detectable and has a maximum at 7 ms). It is worthwhile to notice that the bands become broader when the protein concentration is increased. Analogous results were obtained for PEG-Hb\(oxy\) (data not shown).

Variation of the amplitude of the geminate phase and the concomitant increased amplitude of the slow second-order re-binding in the presence of allosteric effectors (Figure 6), increasing protein concentration (Figure 7), and increasing photolysis level (data not shown) indicate that an R to T quaternary conformational change is occurring upon photolysis also for PEG-Hb\(deoxy\) and PEG-Hb\(oxy\). However, when compared to re-binding to HbA, the bimolecular re-binding to T-like structures measured for PEG-Hb\(deoxy\) and PEG-Hb\(oxy\) exhibits a lower amplitude, suggesting that the bias toward a T-like structure is much lower for these products than for HbA due to a higher concentration of the liganded dimers and a slow kinetics of dimer to tetramer association in the unliganded state.

DISCUSSION

PEG-Hb Heterogeneity. The PEGylation procedure yields topologically heterogeneous products because the similar accessibility of the lysine residues (5–7 per Hb chain) reacting with IMT does not allow an absolute selectivity (34). In addition, these modifiable sites are not fully saturated by PEG conjugation because the usual HBOC preparation adds only 6–8 PEG per Hb tetramer, which leaves a small fraction of unmodified chains. The MALDI MS analyses carried out on the chains isolated by SDS-PAGE allowed a reliable quantitative determination of unmodified and differently PEGylated chains distribution. The PEG moiety distribution for PEG-Hb\(oxy\) indicates that the bulk of this product (65–70%) consists of tetramers conjugated with a number of PEG chains centered at the value of 6.6 ± 1. Procedures have been developed for the direct coupling to Hb

CO re-binding to PEG-Hb\(deoxy\) and PEG-Hb\(oxy\), in the absence of IHP, exhibits a lower geminate re-binding amplitude than HbA and an almost negligible T state bimolecular re-binding (Figure 5A). The associated lifetime distributions (Figure 5B) show two major bands centered at \(\approx 90\) ns and \(\approx 200\) \(\mu\)s, corresponding to geminate and bimolecular re-binding, respectively. A barely detectable band appears at 10 ms. The amplitude of the band associated with geminate recombination is lower for PEGylated Hbs than in HbA and becomes broader in the case of PEG-Hb\(oxy\). The band centered around 200 \(\mu\)s displays only minimal differences among samples, suggesting that the rate for CO re-binding to R is similar for all samples.

The presence of the allosteric effector IHP leads to a slight decrease in the amplitude of the geminate phase for both PEG-Hb\(deoxy\) and PEG-Hb\(oxy\) and a concomitant onset of millisecond T state bimolecular re-binding. The comparison of the re-binding kinetics in the absence and presence of IHP for PEG-Hb\(oxy\) (Figure 6A) and the corresponding lifetime distributions (Figure 6B) indicates that, in the presence of IHP, the nanosecond and microsecond bands appear to be slightly shifted toward longer times, with the geminate phase becoming a bit smaller, and a clearly distinguishable additional band at \(\approx 9\) ms. These effects demonstrate for PEG-Hb\(oxy\) and, similarly, for PEG-Hb\(deoxy\) (data not shown) that the allosteric effectors bias the distribution of molecules toward states with T-like reactivity.

CO re-binding kinetics were measured for 13 and 98 \(\mu\)M PEG-Hb\(deoxy\) (Figure 7A). When PEG-Hb\(oxy\) concentration is increased, an additional kinetic phase appears in the millisecond range. Accordingly, three distinct peaks are present in the MEM lifetime distribution (Figure 7B), corresponding to geminate re-binding (100 and 130 ns, at 13 and 98 \(\mu\)M, respectively) and to bimolecular re-binding to R-like structures (230 and 250 \(\mu\)s, at 13 and 98 \(\mu\)M, respectively) and to T-like structures (12 ms at 98 \(\mu\)M; at 13 \(\mu\)M this band is barely detectable and has a maximum at 7 ms). It is worthwhile to notice that the bands become broader when the protein concentration is increased. Analogous results were obtained for PEG-Hb\(oxy\) (data not shown).

Variation of the amplitude of the geminate phase and the concomitant increased amplitude of the slow second-order re-binding in the presence of allosteric effectors (Figure 6), increasing protein concentration (Figure 7), and increasing photolysis level (data not shown) indicate that an R to T quaternary conformational change is occurring upon photolysis also for PEG-Hb\(deoxy\) and PEG-Hb\(oxy\). However, when compared to re-binding to HbA, the bimolecular re-binding to T-like structures measured for PEG-Hb\(deoxy\) and PEG-Hb\(oxy\) exhibits a lower amplitude, suggesting that the bias toward a T-like structure is much lower for these products than for HbA due to a higher concentration of the liganded dimers and a slow kinetics of dimer to tetramer association in the unliganded state.

DISCUSSION

PEG-Hb Heterogeneity. The PEGylation procedure yields topologically heterogeneous products because the similar accessibility of the lysine residues (5–7 per Hb chain) reacting with IMT does not allow an absolute selectivity (34). In addition, these modifiable sites are not fully saturated by PEG conjugation because the usual HBOC preparation adds only 6–8 PEG per Hb tetramer, which leaves a small fraction of unmodified chains. The MALDI MS analyses carried out on the chains isolated by SDS-PAGE allowed a reliable quantitative determination of unmodified and differently PEGylated chains distribution. The PEG moiety distribution for PEG-Hb\(oxy\) indicates that the bulk of this product (65–70%) consists of tetramers conjugated with a number of PEG chains centered at the value of 6.6 ± 1. Procedures have been developed for the direct coupling to Hb
435 nm followed upon rapid mixing of deoxy the absence of allosteric effectors. Normalized absorbance changes at Figure 4.

For HbA and PEG-Hb

Table 2. Effects of CO₂ and Cl⁻ on Oxygen Affinity

<table>
<thead>
<tr>
<th>species</th>
<th>stripped p50ₜ (Torr)</th>
<th>nₜ</th>
<th>CO₂ p50ₜ (Torr)</th>
<th>nₜ</th>
<th>Cl⁻ p50ₜ (Torr)</th>
<th>nₜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA⁵⁻</td>
<td>2.61 ± 0.05</td>
<td>6</td>
<td>4.09 ± 0.02</td>
<td>6</td>
<td>5.15 ± 0.01</td>
<td>6</td>
</tr>
<tr>
<td>NEM-Hb</td>
<td>2.23 ± 0.03</td>
<td>8</td>
<td>3.16 ± 0.05</td>
<td>8</td>
<td>4.14 ± 0.05</td>
<td>8</td>
</tr>
<tr>
<td>PEG-Hb⁴⁻</td>
<td>3.18 ± 0.08</td>
<td>12</td>
<td>3.71 ± 0.03</td>
<td>12</td>
<td>3.66 ± 0.05</td>
<td>12</td>
</tr>
<tr>
<td>PEG-Hb⁶⁻</td>
<td>0.85 ± 0.02</td>
<td>42</td>
<td>1.23 ± 0.04</td>
<td>42</td>
<td>1.38 ± 0.04</td>
<td>42</td>
</tr>
</tbody>
</table>

⁵ p50ₜ of HbA, NEM-Hb, PEG-Hb⁴⁻, and PEG-Hb⁶⁻, in the absence and presence of physiological concentrations of CO₂ and saturating concentration of Cl⁻ (200 mM), in a solution containing 100 mM Hepes, 1 mM EDTA, at 15°C, pH 7.0. The sample concentration is 110–200 µM. ⁶ For HbA and PEG-Hb⁶⁻, p50 and n were obtained by fitting ODCs to a single species.

of functionalized PEG (61, 62). It cannot be excluded that bound bulky PEG molecules steer the attachment of other PEG molecules leading to products less heterogeneous than those obtained in the two-step PEGylation procedure. However, the issue of molecular weight heterogeneity may also concern these products because of the large number of potential attachment sites, 42 lysines and 4 α-amino groups, with respect to the much lower number, 6–8, of bound PEGs. Thus, it seems unavoidable that PEGylated Hb is a heterogeneous product. This relatively wide species heterogeneity leads to different physicochemical and physiological properties that should be taken into account when in vivo experiments are analyzed and might be responsible for some of the observed adverse effects (23).

**PEGylated Hb Tetramer Stability.** Native Hb undergoes a reversible tetramer–dimer equilibrium as depicted by eq 4:

\[
\frac{(\alpha\beta)(\alpha\beta)}{k_{\text{diss}}} = 2(\alpha\beta)
\]

where \((\alpha\beta)(\alpha\beta)\) represents the tetrameric form and \((\alpha\beta)\) the dimeric form of the protein.

The rate of dissociation, \(k_{\text{diss}}\), is ligand-dependent with \(k_{\text{diss(deoxy)}} \ll k_{\text{diss(oxy)}}\), whereas the rate of association, \(k_{\text{ass}}\), is ligation-independent (37). The reactions in eq 4 have several important implications for evaluation of the in vitro and in vivo properties of PEGylated Hbs: (i) the exchange of dimers among species differing in the number and distribution of conjugated PEG-Hb yields hybrid species. This phenomenon could contribute in vitro to smoothing out the molecular weight heterogeneity of the species and their properties in solution, such as oxygen affinity, allosteric effects, redox potential; (ii) in the circulation, at the interface with tissues, selective permeation of some species may occur leading to a change in the bulk properties of the PEGylated species confined in the plasma; (iii) the order of magnitude of the equilibrium constants for the dissociation of deoxy Hb and oxy Hb are \(10^{-11}\) and \(10^{-6}\) M, respectively. Most Hb mutants with increased oxygen affinity and decreased cooperativity show an increase in \(k_{\text{diss(deoxy)}}\), although a few cases are known in which an increase in \(k_{\text{diss(oxy)}}\) has also been observed (36, 37). Since the tetramer–dimer equilibrium is an oxygen-linked property, it could be involved in the cooperativity reduction of PEGylated Hbs, mimicking mutant species. A direct study of the reactions in eq 4 by various methods, such as haptoglobin binding (63) and hybridization techniques (64, 65) was not feasible due to the interference of conjugated PEG.

The equilibrium gel permeation study of NES-Hb showed that the product exhibits the same \(K_{\text{diss(oxy)}}\) as normal Hb. The \(K_{\text{diss(oxy)}}\) value could not be measured because the product was not significantly dissociated under the most dilute conditions, as known for normal Hb. The various PEGylated species in the polydisperse solution of PEG-BSA were not resolved by size
exclusion chromatography both at high and low product concentration, at which trailing was completely absent. On the contrary, PEG-Hb\textsuperscript{deoxy} under aerobic conditions was resolved into a multiplicity of components upon similar dilution, in agreement with the electrophoretic pattern under native conditions (data not shown). Some of these components had apparent molecular weights consistent with monomeric PEGylated species. Since the phenomenon was completely reversible, the most plausible interpretation is that oxy PEG-Hb\textsuperscript{deoxy} dissociates rapidly into dimers as normal oxy HbA does, but the PEGylated subunits reassociate at a low rate compared with the time scale of the chromatography, in contrast with oxy HbA behavior. This interpretation is consistent with the observation that the slight residual trailing under deoxy conditions was minimal when the sample was obtained by dilution of a deoxygenated solution in which the low molecular weight species had not been given enough time to form (Figure 2B, dashed line). When deoxygenation was carried out rapidly by the addition of dithionite to the diluted oxygenated solution, trailing was more significant because some of the low molecular weight species had not been given enough time to reassociate (Figure 2B, solid line). Two possible explanations for this behavior are the slower translational/rotational coefficient of the PEGylated dimeric species, as suggested by the DLS measurements in dilute solution, also reported by other authors (32, 66), and steric interference by PEG in the mechanism of subunit reassociation. The PEG-Hb\textsuperscript{deoxy} chromatography under anaerobic conditions did not show a multiplicity of components. The slight trailing observed at high concentration was not significantly increased upon dilution.

This can be attributed to the very slow rate of dissociation of the various PEGylated tetramers as compared with the elution rate.

The size exclusion chromatography studies indicate that under aerobic conditions PEG-Hb\textsuperscript{oxy} was more markedly dissociated into dimers than PEG-Hb\textsuperscript{deoxy} and, under anaerobic conditions, a more pronounced trailing was observed if deoxygenation was achieved by dithionite addition to dilute oxy PEG-Hb\textsuperscript{oxy} (Figure 2C). Fully in keeping with this finding, functional properties of PEG-Hb\textsuperscript{oxy} were found to be less affected by dilution than PEG-Hb\textsuperscript{deoxy} (see below). Therefore, PEGylation carried out under aerobic conditions favors the modification of sites crucial for function and tetramer assembly, likely leading to a very large population of dimeric species. These sites are less severely modified in the anaerobic procedure for PEG-Hb\textsuperscript{deoxy} preparation.

**Oxygen Binding Affinity and CO Binding Rate, in the Absence and Presence of Allosteric Effectors.** The concentration dependence of the oxygen affinity of PEG-Hb\textsuperscript{deoxy} (Figure 3) and the corresponding analysis (Figure 3, inset) indicates that at least two species are in equilibrium in solution. On the basis of the p50s, Hill coefficients, and responses to allosteric effectors, the more straightforward interpretation is that one species corresponds to PEG-Hb\textsuperscript{deoxy} dimers, characterized by functional properties very similar to native HbA and the other corresponds to PEG-Hb dimers because its amount increases on dilution. It is interesting to note that the latter species exhibits functional properties close to native HbA and the other corresponds to PEG-Hb dimers because its amount increases on dilution. The oxygen affinity is that the high oxygen affinity species is associated to a perturbed R state tetramer in which the
quaternary transition is either prevented or dramatically slowed down by PEGylation. Such an interpretation is consistent with the hypothesis suggested by Svergun et al. (33) that PEG chains may interfere with the T/R transition by binding at the dimer interface, thereby reducing cooperativity. However, the ODc dependence on concentration favors the former interpretation.

PEG-Hbdeoxy more than PEG-HbO2 responds to the physiological concentrations of CO2 and Cl− by reducing the oxygen affinity, although not to the same extent as native HbA (Table 2). This can be explained since one of the binding sites common to both ligands involves the α-amino groups of the α chains. PEGylation of LysoC7 likely interferes with the salt bridge formed by Val1α and removes part of the oxygen-linked CO2, Cl−, and Bohr effects involving this site (34, 67). However, the protection of the 2.3-BPG binding pocket during PEGylation for PEG-Hbdeoxy, but not PEG-HbO2, allows CO2 modulation through binding in the deoxy structure to the amino groups of Val1β, the major site of CO2 binding (68), and Cl− modulation through binding to Lysβ82 (69). These data support the notion that PEGylation of deoxy Hb exhibits a less dramatic impact on protein functional properties.

The comparison of CO binding and rebinding kinetics of PEG-Hbdeoxy, PEG-HbO2, and HbA, at high and low concentration, in the absence and presence of IHP, allows further identification of functional differences, associated with distinct species. First, it is interesting to note that CO binding to deoxy PEG-Hbdeoxy and PEG-HbO2 is monophasic, lacks the autocatalytic process typical of native Hb, and is concentration-independent over about a 10-fold range of concentrations (10−100 µM). In particular, the observed rate constant for PEG-Hbdeoxy exhibits the same value of the rate associated with the slow phase of HbA, whereas the rate constant for CO binding to PEG-HbO2 is higher but still lower than the rate of the fast phase observed for HbA. These findings suggest that (i) deoxy PEG-Hbdeoxy is predominantly present as T state tetraters converting to the R state slower than CO binding, and (ii) deoxy PEG-HbO2 is present as a mixture of perturbed dimers and T state tetraters, in keeping with the chromatographic measurements, that slowly are converted to the R state. A more detailed analysis is hampered by the fast rate of reaction, leading to the omission of a significant portion of the kinetic traces. Nevertheless, these data provide complementary information with respect to the more robust results generated from flash photolysis experiments. It was found that CO rebinding to both PEGylated samples, in the absence of IHP, did not show significant bimolecular rebinding to T state. This can be explained by either PEGylation reducing the rate of the R to T quaternary transition or the liganded PEGylated Hbs being predominantly present as dimers that, upon deligation, form T state tetraters on a time scale slower than CO rebinding. The reduced amplitude of the geminate rebinding indicates a faster escape to solvent, as observed in myoglobin. The appearance of milliseconds bimolecular rebinding in the presence of IHP, at higher concentrations or higher pulse energy, clearly signals an increased accumulation of T state tetraters. However, their amount is much lower than in native Hb due to a slow quaternary R to T transition and/or dimer association upon deligation.

In a recent paper, CO binding kinetics was measured for Hemospan (70), observing a CO rebinding lacking the geminate phase. This discrepancy with respect to our laser flash photolysis data is most probably due to the instrumental setup used in the previous work that did not allow detection of submicrosecond processes.

Overall, results indicate that PEG-HbO2 is predominantly in a highly reactive state, characterized by high oxygen affinity and a high rate of CO binding. Ligand binding triggers the formation of dimers faster than the quaternary T to R transition.

The same picture holds for PEG-Hbdeoxy, assuming that the PEG-Hbdeoxy tetrameric T state is more stable than PEG-HbO2 and the T to R quaternary transition efficiently competes with tetramer dissociation. Thus, under physiological conditions, PEG-HbO2 and PEG-Hbdeoxy in spite of containing the same average number of PEG molecules, are expected to deliver different amounts of oxygen, react with NO and nitrite with different rates, and trigger different regulatory signals because they exhibit distinct functional and structural properties.

CONCLUSIONS

PEGylation affects Hb in distinct ways: (i) perturbation of the T and R states (31), (ii) perturbation of the quaternary transitions (33), and (iii) influence on the tetramer−dimer equilibrium (38). Although animal studies carried out using PEG-Hbdeoxy and other similar products showed only moderately adverse pressure and renal filtration effects in the short period (2−3 h), the long-term effects of the stability upon dilution of the various PEGylated tetramers and dimers should be considered with regard to both the physicochemical and physiological properties of the bulk product in the circulation. The observed anomalous equilibrium of the PEGylated tetraters and product heterogeneity should also be considered in the evaluation of the extravasation and renal filtration behavior of these HBOCs. In view of the reported adverse effects of most HBOCs (23), it is mandatory to tightly link functional and structural HBOC properties with biochemical and physiological parameters, including proteomic analyses, associated to organ damages, oxidative stress, and inflammatory signals. This is our long-term ongoing challenge.

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LITERATURE CITED


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