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Enhancing Nitrite Reductase Activity of Modified Hemoglobin: Bis-tetramers and Their PEGylated Derivatives[†]

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ABSTRACT: The clinical evaluation of stabilized tetrameric hemoglobin as alternatives to red cells revealed that the materials caused significant increases in blood pressure and related problems and this was attributed to the scavenging of nitric oxide and extravasation. The search for materials with reduced vasoactivity led to the report that conjugates of hemoglobin tetramers and polyethylene glycol (PEG) chains did not elicit these pressor effects. However, this material does not deliver oxygen efficiently due to its lack of cooperativity and high oxygen affinity, making it unsuitable as an oxygen carrier. It has been recently reported that PEG-conjugated hemoglobin converts nitrite to nitric oxide at a faster rate than does the native protein, which may compensate for the scavenging of nitric oxide. It is therefore important to alter hemoglobin in order to enhance nitrite reductase activity while retaining its ability to deliver oxygen. If the beneficial effect of PEG is associated with the increased size reducing extravasation, this can also be achieved by coupling cross-linked tetramers to one another, giving materials with appropriate oxygen affinity and cooperativity for use as circulating oxygen carriers. In the present study it is shown that cross-linked bis-tetramers with good oxygen delivery potential have enhanced nitrite reductase activity with k_{obs} = 0.70 M^{-1} s^{-1} (24 °C), compared to native protein and cross-linked tetramers, k_{obs} = 0.25 M^{-1} s^{-1} and k_{obs} = 0.52 M^{-1} s^{-1}, respectively, but are less active in reduction of nitrite than Hb-PEG5K$_2$ (k_{obs} = 2.5 M^{-1} s^{-1}). However, conjugation of four PEG chains to the bis-tetramer (at each β-Cys-93) produces a material with greatly increased nitrite reductase activity (k_{obs} = 1.8 M^{-1} s^{-1}) while retaining cooperativity (P_{50} = 4.1, n_{50} = 2.4). Thus, PEGylated bis-tetramers combine increased size and enhanced nitrite reductase activity expected for decreased vasoactivity with characteristics of an acceptable HBOC.

Hemoglobin-based oxygen carriers (HBOCs)[1] are being developed as stable and sterile alternatives to red cells in transfusions (*1*−*3*). Reports of clinical trials indicate that specific problems with the tested materials include increases in blood pressure and other cardiovascular complications (*4*, *5*). A recent meta-analysis of the risks, benefits, efficacy, and safety of the materials that have thus far been evaluated concluded that none was worthy of further study (*6*). Evaluations by regulatory agencies of clinical trials of HBOCs led to cessation of the trials and decisions that terminated related operations. These results heightened the need for further research to find improved materials that are safe and effective for the many important needs that stable, sterile, universally acceptable alternatives to red cells could fulfill.

A promising approach arises from the observation of Vandegriff et al. that the pressor effect is not present where hemoglobin is conjugated with polyethylene glycol (PEG). (*7*, *8*). We have previously shown that the addition of PEG−hemoglobin conjugates significantly increases the rate of reduction of nitrite to nitric oxide (NO) compared to the activity seen with native hemoglobin (*9*). As nitric oxide is a signaling species that creates a metabolic cascade that leads to relaxation of the blood vessel, only small amounts would have to escape from association with heme in hypoxic regions (*10*). The increased rate of reduction of nitrite may therefore serve as the basis for the PEG conjugate's desirable lack of a pressor effect as it compensates for scavenged nitric oxide (*5*, *11*−*14*).

However, certain problems limit the potential of PEG−hemoglobin conjugates that have been evaluated: high oxygen affinity combined with low cooperativity (P_{50} = 3.6, n_{50} = 1.8) and the propensity to dissociate into $\alpha\beta$-dimers may make oxygen delivery inefficient (*15*). Vandegriff and Winslow reported the critical observation that suitable HBOC materials must have a high oxygen affinity in order to avoid loss of oxygen at relatively high oxygen tensions where red cells are available, giving protection from a homeostatic vasoconstriction as a response (*7*, *8*). However, high oxygen affinity must be associated with high cooperativity in order to permit oxygen delivery in the desired range. The low n_{50} associated with most PEG−hemoglobins could seriously hinder oxygen off-loading. As a

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[1]Abbreviations: Hb, hemoglobin; HBOC, Hb-based oxygen carrier; metHb, methemoglobin; NOHb, nitrosylhemoglobin; NO, nitric oxide; PEG, polyethylene glycol; Mal-PEG5K, methoxypolyethylene glycol (5000)−maleimide; BT-Hb, bis-tetramers of hemoglobin; BT-Hb-PEG5K$_4$, bis-tetramers of hemoglobin PEGylated at β-Cys-93 with Mal-PEG5K; αα-Hb, αα-diasprin cross-linked hemoglobin; αα-Hb-PEG5K$_2$, αα-diasprin cross-linked β-Cys-93 Mal-PEG5K conjugated hemoglobin; Hb-PEG5K$_2$, β-Cys-93 Mal-PEG5K conjugated hemoglobin; k_{obs}, the bimolecular nitrite reductase rate constant; PEGylation, conjugation of PEG chains to proteins; CD, circular dichroism; NEM, *N*-ethylmaleimide; P_{50}, oxygen pressure at which Hb is half-saturated: n_{50}, Hill's coefficient of cooperativity at half-saturation.

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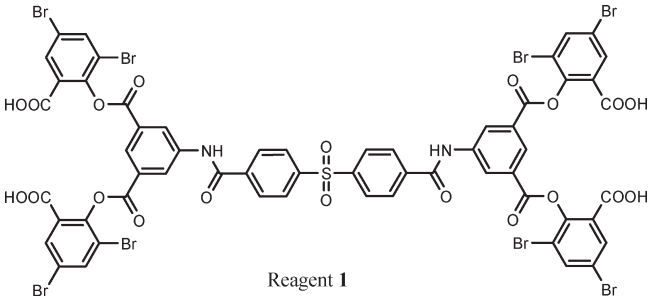
Chart 1: Cross-Linking Reagent (**1**) Used in Reactions with Hemoglobin To Produce Bis-tetramers



Reagent **1**

result, PEG-Hb has been limited to being considered as a volume expander rather than an HBOC (*16*).

PEG conjugation causes a substantial increase in size of the conjugated entity compared to the native protein.(*17*) If the decreased vasoactivity of PEG hemoglobins is associated with the increased size of the conjugate, then alternative ways to produce a larger entity would also be effective. Efficient chemical procedures have recently been reported that produce cross-linked hemoglobin bis-tetramers (BT-Hb), larger materials with promising oxygen binding properties (*18*, *19*). Unlike PEG conjugation, coupling tetramers maintains tetrameric stability and the oxygen transport capabilities of the protein. We have now examined the nitrite reductase properties of BT-Hb derivatives as well as their PEG conjugates. We find that the effect of conjugation of PEG at β-Cys-93 is associated with enhanced nitrite reductase activity in BT-Hb as it is in simple tetramers. Further investigation into features that modulate the protein heme−nitrite reactions indicates that while heme redox potentials clearly play a role in the increased nitrite reductase activity of PEG-Hbs, factors that control ligand affinity and cooperativity also contribute to the enhanced rate of reaction.

EXPERIMENTAL PROCEDURES

Materials and Methods. All chemical reagents were purchased and used as obtained. Anhydrous tetrahydrofuran (THF) was obtained by distillation from metallic sodium. Human hemoglobin A was obtained as a gift from Oxygenix, Inc., or isolated and purified from red cells obtained from the Canadian Blood Services through volunteers with their informed consent. Concentrations of hemoglobin solutions were determined using the cyanomethemoglobin assay (*20*), and the purity of hemoglobin was determined through reverse-phase HPLC analysis as described by Jones (*21*). The tetrafunctional reagent *N*,*N*′-bis[bis-(3,5-dibromosalicyl)isophthalyl]-5,5′-sulfonyl bis[1,4-(phenylenecarbonylimino)]bis(1,3-benzenedicarboxylate) (reagent **1**) was synthesized according to Hu et al. (Chart 1) (*19*).

Cross-Linking. Bis-tetramers of hemoglobin were produced through the reaction of the tetrameric acylating reagent **1** with native unmodified hemoglobin. The concentration of hemoglobin tetramers was maintained at 0.5 mmol/L for all cross-linking reactions. A solution of carboxy-Hb in sodium borate buffer (0.05 M, pH 7.4) was oxygenated under a stream of oxygen at 0 °C with tungsten lamp irradiation and stirring for 2 h before deoxygenation under a stream of humidified nitrogen for 2 h at 37 °C. Since reagent **1** is not readily soluble in aqueous media, DMSO was used to dissolve the reagent. The concentration of DMSO in all reactions did not exceed 2.5%. The dissolved reagent in DMSO is then added to the deoxy-Hb to make the final concentrations of Hb 0.5 mM and reagent **1** 1.0 mM. The reaction was then allowed to proceed for 18 h under a stream of humidified nitrogen at 37 °C to ensure full deoxgenation during the cross-linking reaction. The mixture was cooled on ice and placed under a stream of carbon monoxide for 10 min and then passed through a column of Sephadex G-25 equilibrated with MOPS buffer (0.1 M, pH 7.2) to remove excess reagent. The cross-linked hemoglobin product was concentrated through a membrane with centrifugation (3000 rpm for 30 min) and stored at 4 °C until further purification.

Isolation of Modified Hemoglobins. Purification of bis-tetramers (BT-Hb) was carried out using gel filtration chromatography (Sephadex G-100, 1000 × 35 mm) under slightly dissociating conditions. The eluent used was 25 mM Tris-HCl, pH 7.4, containing 0.5 M magnesium chloride. The high salt concentration causes partially modified hemoglobin tetramers to dissociate, allowing for separation of larger bis-tetramers from the mixture. Fractions were collected, concentrated, and analyzed using Superdex G-200 size exclusion chromatography, C4 reverse-phase analytical HPLC, and SDS−PAGE gel electrophoresis.

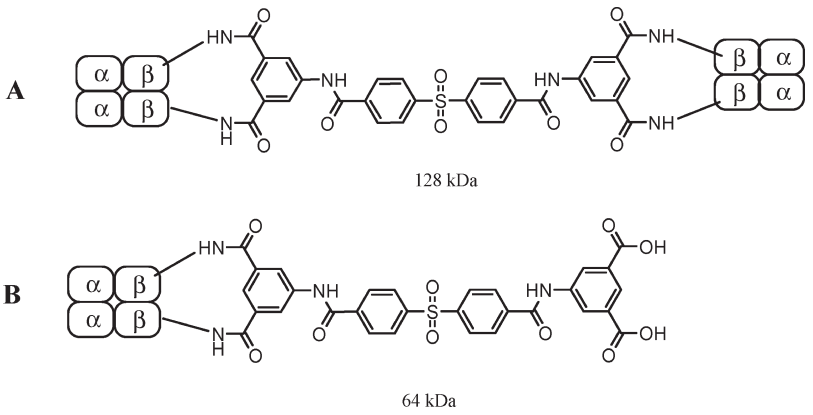
Conjugation with Polyethylene Glycol (*PEG*). Protein solutions of purified bis-tetramers of hemoglobin (0.5 mM) in its carbonmonoxy form were combined with 10 equiv of methoxypolyethylene-glycol (5000)−maleimide (Mal-PEG5K) in sodium phosphate buffer (0.1 M, pH 7.4) and kept at 37 °C overnight to give products with two PEG chains per tetramer: BT-Hb-$(PEG5K)_4$. The resulting mixture was passed through a Sephadex G-25 column equilibrated with sodium phosphate buffer (0.1 M, pH 7.4), concentrated, and stored at 4 °C. Separation from excess PEG reagents was carried out by dialysis in phosphate buffer, (I = 0.1 M, pH 7.4) at 4 °C. The buffer was replaced three times, at 12 h intervals. The PEG-conjugated hemoglobins were then removed, and the solution was treated with carbon monoxide to maintain the carbonmonoxy derivatives. These were stored at 4 °C.

HPLC Analysis of Modified Hemoglobins. Hemoglobin bis-tetramers were analyzed using analytical reverse-phase HPLC with a 330 Å C-4 Vydac column (4.6 × 250 mm) to determine the sites of globin chain modifications (*22*). Modified and unmodified globin chains were separated using an eluting solvent containing 0.1% trifluoroacetic acid and a gradient beginning with 20% and ending with 60% acetonitrile (vol %) in water (*23*). The effluent was monitored at 220 nm. PEG-conjugated bis-tetramers (BT-Hb-$(PEG5K)_4$) were analyzed using a Superdex G-200 HR (10 × 300 mm) preparative size exclusion column. Protein samples (0.5 mM) were eluted under partially dissociating conditions by the addition of 0.5 M magnesium chloride in buffer (25×10^{-3} M Tris-HCl, pH 7.4). The effluent was monitored at 280 nm.

SDS−PAGE Analysis. Protein standards, reaction samples, and native Hb were prepared by combining 2−4 μL with the loading buffer (16−18 μL), consisting of 0.0625 M Tris-HCl (pH 6.8), 1.3 M glycerol, 2% SDS, 0.0125 (w/v) bromophenol blue, and 0.7 M β-mercaptoethanol. The samples were denatured by heating at 95 °C for 10 min. Then, a 7 μL sample was loaded onto a polyacrylamide slab (12% Tris-HCl). The gel was processed in a dual-slab cell apparatus at 200 mV in 0.12 M Tris, 1 M glycine, and 0.014 M SDS running buffer. The gels were stained with Coomassie Brilliant Blue R-250 and then destained with 30% methanol−10% acetic acid solution.

CD Spectroscopy. In order to determine the effects of PEG modification on the overall structural stability of the protein, we

Chart 2: Mixtures of Hemoglobin from the Protein Modification Reaction. (A) Bis-tetramers of Hemoglobin Result from Full Acylation Reactions. (B) Mono-tetrameric Cross-Linked Hemoglobin as a Result of Competing Hydrolysis of Acylation Groups



compared the CD spectra of native hemoglobin with that of the PEG-conjugated materials. Protein samples (5 μM) were prepared in 0.01 M phosphate buffer (pH 7.4), and the CD spectrum from 200 to 260 nm was obtained in triplicates.

Oxygen Binding Analysis. Oxygen binding affinity was measured (28 °C, pH 7.4) with a Hemox analyzer that measures the oxygen pressure for half-saturation (P_{50}) and Hill's coefficient of cooperativity at half-saturation (n_{50}). Hemoglobin samples, BT-Hb and BT-Hb-(PEG5K)$_4$ (~1 g/L), in sodium phosphate buffer (I = 0.01 M, pH 7.4) were oxygenated before analysis. The oxygenated hemoglobin samples were then deoxygenated by bubbling nitrogen through the sample until the P_{O_2} reached a minimum value. The data were then fitted to the Adair equation to obtain P_{50} and n_{50} (*24*).

Kinetic Measurements. Kinetic experiments were conducted in the same manner as described in Lui et al. (*9*) Briefly, 1 mL hemoglobin samples (5−10 μM) in Bis-Tris (0.01 M, pH = 7.2) buffer were thoroughly flushed with nitrogen and the resulting solutions transferred anaerobically using a gastight syringe into a sealed cuvette. Oxygen-free solutions of nitrite were then added to give final concentrations of nitrite of 50−200 μM. The formation of methemoglobin (MetHb) and iron nitrosylhemoglobin (HbNO) was followed by recording spectra from 500 to 650 nm, and spectral data were analyzed by multiple linear regression analysis using separately obtained spectra of the individual components as a basis set (*25*). All kinetic runs were maintained at 24 °C with a circulation bath through the jacketed cell compartment of a UV−vis spectrophotometer. The initial reaction rate is for ferric heme formation as the average rate over the first 100 s after mixing.

RESULTS

Characterization of Bis-tetramers. Two equivalents of the cross-linking reagent **1** was required for complete modification of at least 1 equiv of native hemoglobin. Since the formation of bis-tetramers is entropically unfavorable, acylation of lysine residues in the BPG binding site is comparatively slow, allowing for competing hydrolysis of the esters. This yields a mixture of modified hemoglobins, ranging from cross-linked bis-tetramers (128 kDa) to cross-linked tetrameric hemoglobin (64 kDa) (Chart 2).

Optimal conditions that favor acylation reactions with reactive lysine residues over base-catalyzed hydrolysis of the ester groups were determined by a pH dependence study (Figure 1). At higher pH (pH = 8.5), the amino groups of lysine residues are available

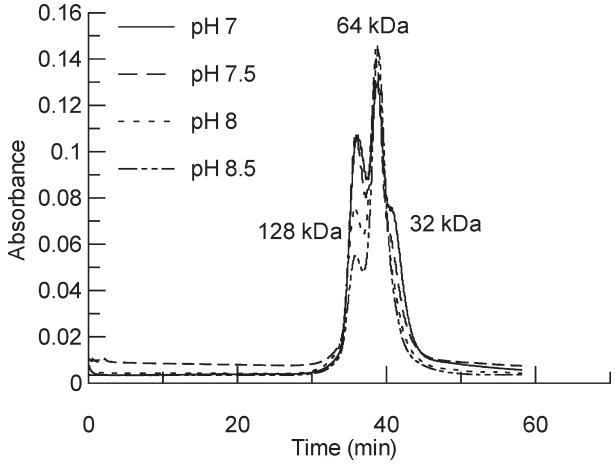


FIGURE 1: Dependence of pH on the formation of hemoglobin bis-tetramers (128 kDa).

for reaction with the DBS-acyl groups of reagent **1**. However, base-catalyzed hydrolysis of the reagent is a significant problem. At pH <7, base-catalyzed hydrolysis is slower but the lysine residues are mostly protonated, decreasing the rate of acylation. Reactions at pH 7.4 are a good compromise and gave the bis-tetramer in 40% yield with little unmodified hemoglobin (which decomposes to 32 kDa $\alpha\beta$ dimers). Interestingly, during further protein characterization, it was found that the oxygen affinity of hemoglobin changes depending on the pH at which cross-linking is carried out. Modifications at higher pH (~9) led to a species with higher oxygen affinity. This is attributed to the difference in nucleophilicity between Val-1 and Lys-82, previously shown to affect heme oxygen binding properties (*26*).

Separation of the bis-tetramers (BT-Hb) through a size exclusion column under partially dissociating conditions gave bis-tetramers in 95% purity as confirmed by G-200 size exclusion HPLC (Figure 2A). Globin chain analysis through C4 reverse-phase HPLC indicated that only the β-subunits are modified (Figure 2B). The cross-linked β-subunits appear as a broad peak. Since there are four β-subunits covalently linked, the elution time increases, and it appears that the molar absorptivity of these species decreases.

Characterization of PEG Conjugated Bis-tetramers. PEG conjugation of BT-Hb at both β-subunits was carried out by reaction at each β-Cys-93 with Mal-PEG5K to give BT-Hb-PEG5K$_4$ (Scheme 1). Although these reactions are typically carried out at 4 °C (*9*, *27*), these reactions had to be carried out

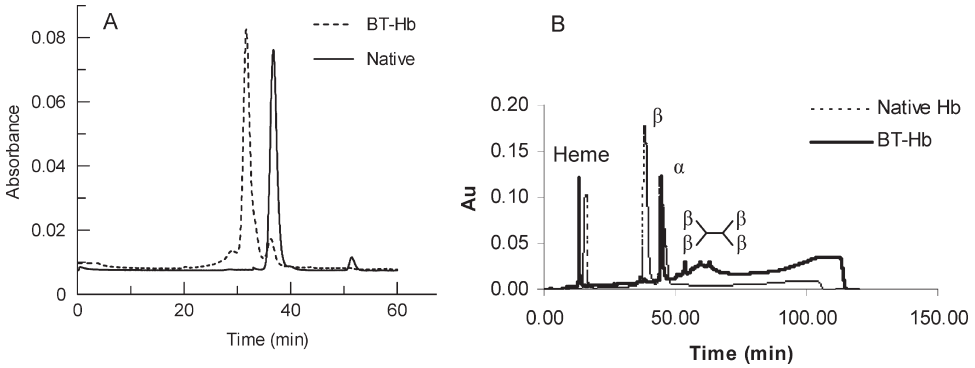


FIGURE 2: Analysis of purified bis-tetramers of hemoglobin (BT-Hb). (A) Size exclusion G-200 HPLC indicates that >95% of the hemoglobin is modified as bis-tetramers. (B) C-4 rpHPLC chromatography indicates that β-subunits are fully modified while α-subunits remain unmodified. New cross-linked β-subunits elute at 52−60 min.

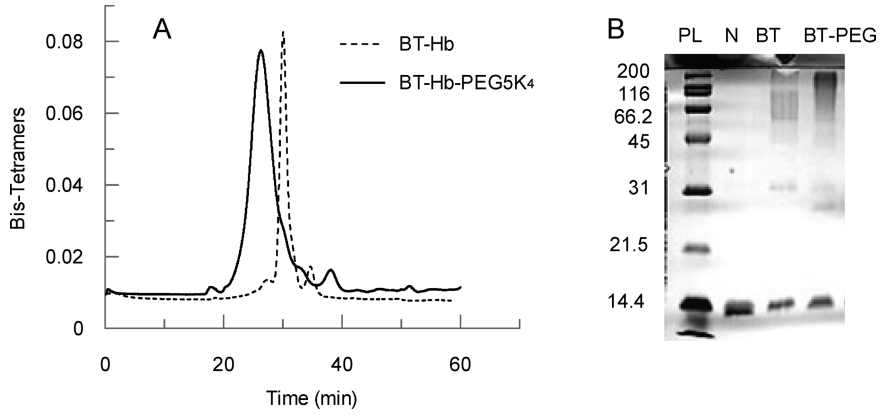
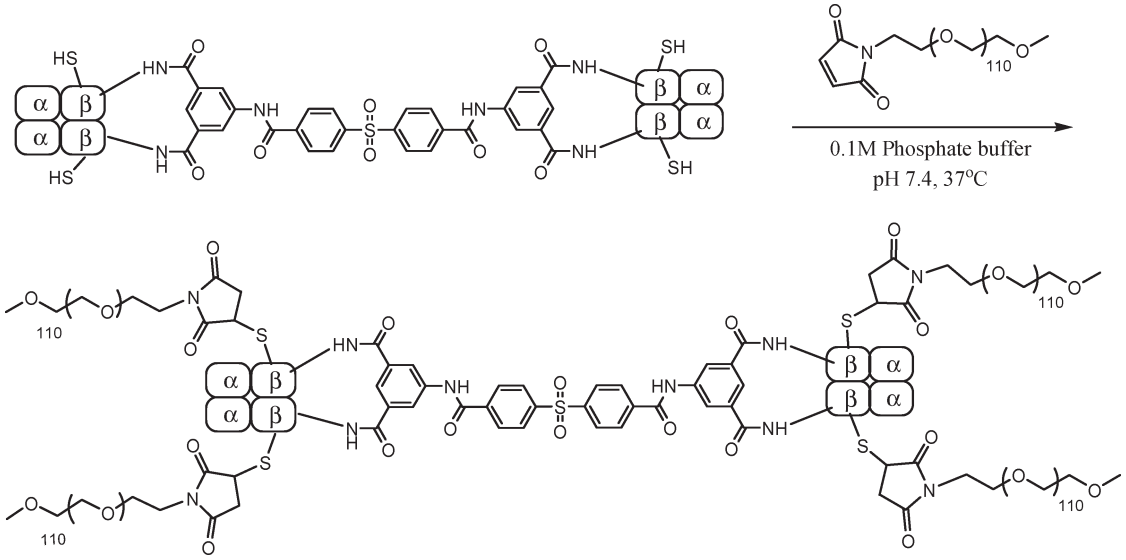


FIGURE 3: Characterization of PEGylated bis-tetramers. (A) Size exclusion G-200 HPLC indicates that the earlier eluting BT-Hb-PEG5K$_4$ is fully modified. (B) SDS gel electrophoresis shows higher molecular mass bands ~110 kDa when BT-Hb is PEGylated.

Scheme 1: Reaction of Purified Bis-tetramers of Hemoglobin with Malemide-Active PEG Results in Complete PEGylation at β-Cys-93



at elevated temperatures (37 °C) to obtain complete modification. This is consistent with the fact that the reactivity of β-Cys-93 decreases upon modification of the β-subunit (*26*). Size exclusion HPLC analysis shows BT-Hb-PEG5K$_4$ (~148 kDa) as a broad peak eluting ahead of the unconjugated bis-tetramers (128 kDa) (Figure 3A).

SDS−PAGE analysis (Figure 3B) gives the molecular masses of the modified subunits. The conditions used show distinct α- and β-globin chains for native hemoglobin at 16 kDa (lane "N"). In the lane marked "BT" there is a band that corresponds to the covalently joined subunits of the $\beta\beta$-cross-linked bis-tetramers (64 kDa) and another corresponding to unmodified α-subunits at 16 kDa. Conjugation of maleimido-PEG at each β-Cys-93 thiol gives a material that produces a band at ~110 kDa, indicating PEG conjugation at all β-Cys-93 groups has occurred (lane "BT-PEG") while unmodified α-subunits remain at 16 kDa. There is no evidence of material with only one subunit conjugated to maleimido-PEG. The large increase in mass after PEGylation is a

result of the polydispersity of PEG, making the protein run ~30 kDa higher than expected.

Oxygen Affinity and Cooperativity. The oxygen binding properties of both BT-Hb and BT-Hb-PEG5K$_4$ were measured and compared with those of unmodified Hb and αα-fumaryl cross-linked hemoglobin (listed in Table 1). The P_{50} of BT-Hb (P_{50} = 9.3) is about twice that of native Hb at pH 7.4, but upon PEGylation to give BT-Hb-(PEG5K)$_4$ (P_{50} = 4.1), the P_{50} is comparable to native hemoglobin (P_{50} = 5.0).

The Hill coefficients at pH 7.4 of both BT-Hb and BT-Hb-(PEG5K)$_4$ are somewhat reduced compared with that of unmodified hemoglobin, with n_{50} values of 2.7 and 2.4, respectively. While the decrease in cooperativity and subsequent increase in oxygen affinity as a result of PEGylation of the bis-tetramer appears to be a direct result of PEG attachment to β-Cys-93, BT-Hb-(PEG5K)$_4$ is still more cooperative than non-cross-linked β-Cys-93 PEGylated hemoglobin (Hb-PEG5K$_2$). It is likely that interprotein interactions of BT-Hb have the dominant effect on the cooperativity of BT-Hb-(PEG5K)$_4$, such that subsequent alterations at β-subunit residues do not further affect the protein conformation significantly. A more direct comparison is with cross-linked PEGylated hemoglobin: β-Cys-93 PEGylated αα-fumaryl-Hb (αα-Hb-PEG5K$_2$). PEGylation of both BT-Hb and αα-Hb results in an ~2-fold decrease in P_{50} (increased oxygen affinity) and subsequent decrease in cooperativity. As a result, it appears that well-defined properties of these proteins can be predicted based on specific structural alterations.

Table 1: Rate Constants for the Reaction of Nitrite and Oxygen Binding Data for Modified Hbs[a]

		oxygen binding (28 °C)	
modified Hbs	nitrite reaction, k_{obs} (M^{-1} s^{-1}, 24 °C)	P_{50}	n_{50}
native	0.25 ± 0.02	5	3
Hb-PEG5K$_2$[b]	2.5 ± 0.03	3.6	1.8
αα-Hb	0.52 ± 0.03	13.9	2.6
αα-Hb-PEG5K$_2$	1.4 ± 0.03	7.9	2.4
BT-Hb	0.7 ± 0.05	9.3	2.7
BT-Hb-PEG5K$_4$	1.8 ± 0.05	4.1	2.4

[a]Oxygen binding data are from reactions in 0.01 M phosphate buffer at pH 7.4. [b]Data taken from ref *38*.

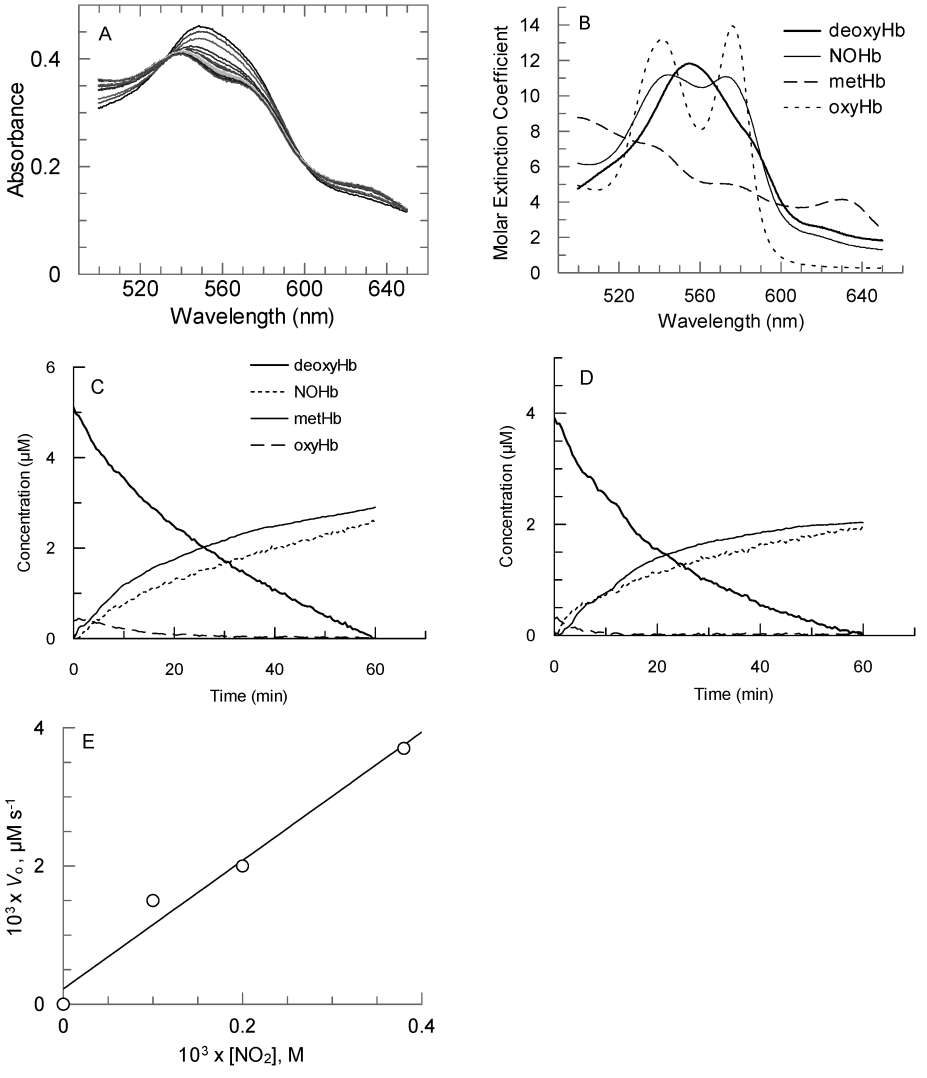


FIGURE 4: Reaction of modified hemoglobins with nitrite. (A) Reaction profile of BT-Hb with nitrite over time. (B) Molar extinction coefficients of pure deoxy-, met-, NO-, and oxyhemoglobin. (C) Reaction progress of 5 μM BT-Hb with 0.4 mM NO_2^- showing concurrent formation of methemoglobin and nitrosylhemoglobin. (D) Reaction progress of 5 μM BT-Hb-PEG$_4$ with 0.2 mM NO_2^-. (E) Initial rate plot as a function of nitrite concentration for BT-Hb-PEG$_4$. The bimolecular rate constant was determined from the formation of methemoglobin at the beginning of the reaction.

CD Spectroscopy. The CD spectra (200−260 nm, which is indicative of secondary structure change) of all cross-linked and PEG-conjugated hemoglobins were identical to that of native hemoglobin (*28*). Although protein side chains had been modified, no changes to the tertiary structure of the protein are evident.

Nitrite−Heme Kinetic Analysis. Nitrite−heme kinetic reactions of native, BT-Hb, and BT-Hb-(PEG5K)$_4$ were measured by acquiring spectra at specific time intervals throughout the course of the reaction (Figure 4A). The changes indicate that deoxy-Hb is converted to equal amounts of metHb and NOHb. Spectra that were recorded over the course of the reaction were analyzed by multiple linear regressions using a basis set of the spectra of pure deoxy-, oxy-, met-, and nitrosylhemoglobin (Figure 4B). The results of multiple regression analysis yielded individual component concentrations (deoxy-, oxy-, met-, NO-) at each reaction time point (Figure 4C,D). The rate constant for formation of metHb was then derived from the observed initial rates (Figure 4E).

The kinetic data for the modified hemoglobins (Table 1) indicate that conjugation with PEG increases the rate of reaction with nitrite: bis-tetramers of hemoglobin (BT-Hb) have nitrite−heme reactivities similar to their cross-linked counterpart, αα-fumaryl-Hb, with reaction rates only slightly faster than that of native hemoglobin. Both have decreased oxygen affinity, with P_{50} = 9.3 and P_{50} = 13.9, respectively. In contrast, PEG conjugation of the bis-tetramer (BT-Hb-PEG5K$_4$) results in a 7-fold increase in the rate of reaction with nitrite compared to that of native Hb, in addition to an increased affinity for oxygen (P_{50} = 4.1). It is therefore apparent that the PEG-conjugated bis-tetramer has nitrite reductase activity similar to that of other PEGylated hemoglobins (Table 1). Although BT-Hb-PEG5K$_4$ has nitrite reductase activity comparable to that of αα-Hb-PEG5K$_2$, they exhibit significantly different oxygen binding properties, indicating that the properties are not directly related. The low oxygen affinity of αα-Hb-PEG5K$_2$ would lead to early off-loading of oxygen in arterioles, which can lead to vasoconstriction as a response (*29*). BT-PEG has a suitably high affinity for oxygen (as has been previously observed for other ββ-cross-linked hemoglobins) (*30*). Unlike its un-cross-linked hexa-PEGylated counterpart, Hb-PEG5K$_6$, oxygenation is highly cooperative. BT-Hb-PEG5K$_4$ (P_{50} = 4.1) also has an oxygen affinity that is comparable to hexa-PEGylated hemoglobin (P_{50} = 3.6). The latter is reported by Vandegriff and Winslow to be non-vasoactive (*7*). PEGylation of bis-tetramers thus results in a product that has increased nitrite reductase activity combined with appropriate oxygenation properties.

DISCUSSION

The nitrite reductase activity of hemoglobin has received much attention as a potential source of bioactive NO during hypoxia/ischemia (*25*, *31*), both in the tissues and in the vasculature (*32*). Nitrite is present in the blood at fairly high levels (0.1−0.5 μM) (*14*, *33*), and its transduction to NO is made possible through an allosterically controlled nitrite reductase reaction with the heme moiety of hemoglobin (*10*). Modifications to the protein that alter ligand binding affinity to the heme or its redox potential could contribute to enhanced nitrite reductase activity. A primary goal in the development of safe and effective HBOCs is thus to predict altered hemoglobin functions based on molecular design.

Bis-tetramers of hemoglobin (BT-Hb) can be produced from one-step conversions of hemoglobin with reagents that produce cross-links within tetramers along with covalent linkages between tetramers. Such interprotein linkages effectively bind two hemoglobin tetramers together, increasing size and cooperativity. Kinetic analysis of BT-Hb heme−nitrite reaction rates indicates that while the bis-tetramer reacts 3 times faster than native hemoglobin, it reacts with rates comparable to αα-fumaryl cross-linked hemoglobin (k_{obs} = 0.70 M^{-1} s^{-1} and k_{obs} = 0.52 M^{-1} s^{-1}, respectively). It appears that tethering two hemoglobin tetramers has little effect on features that govern nitrite−heme reaction rates. Rather, BT-Hb bears a close resemblance to its cross-linked counterpart, in both oxygen binding properties and nitrite reductase activity. In contrast, the combination of interprotein cross-linking and PEGylation yields a PEGylated bis-tetramer (BT-Hb-PEG5K$_4$) that has improved oxygen affinity, good cooperativity, and increased nitrite reductase activity.

The increase in nitrite reductase activity after PEGylation has been reported to be a direct result of β-Cys-93 and R-state stabilization, as in the case of BT-Hb-PEG5K$_4$ (*9*, *25*). It is well-established that the rates of nitrite reaction correlate with the allosteric state of hemoglobin: R-state stabilized hemoglobin reacts ∼50 times faster than T-state stabilized hemoglobin (*34*). However, the actual mechanistic features that contribute to the higher reactivity of R-state hemes remain unclear: this may result from the lower heme redox potential of R-state ferrous hemes or from the high ligand affinity geometry of R-state tetramers that facilitate nitrite binding (*35*) or both. Experiments conducted with sickle hemoglobin (HbS), which has a lower redox potential than HbA but similar oxygen affinity and cooperativity, suggest that enhanced nitrite reductase activity might be the result from decreased heme redox potentials (*35*). However, there may be other features that contribute to increased heme−nitrite reaction rates that do not involve heme reduction potentials. Since ligand binding kinetics and allosteric cooperativity are inseparable components of tetrameric hemoglobin, it is likely that high ligand binding affinity contributes to the high nitrite reactivity of R-state hemes.

Since the ligand binding ability of tetrameric hemoglobin is reflected in its ability to bind oxygen, the degree to which altered hemoglobin binds oxygen is reflected by its conformational state (R or T). R-state stabilized hemoglobin will bind oxygen with increased affinity and likewise facilitate nitrite binding. This will contribute to enhanced formation of nitric oxide from nitrite. It is also known that in the presence of an activator the concentration of substrate binding R-state increases because it is stabilized by the activator (*36*). The activator therefore increases the protein's substrate binding affinity (R-state stabilization), although it decreases the protein's degree of substrate binding cooperativity (*36*). It follows that the increased rate of reduction of nitrite could correlate with oxygen affinity. Hemoglobins that bind oxygen with higher affinity will have enhanced heme−nitrite combination rates. The increased reaction should likewise depend on the presence of an activator that stabilizes the R-state, in this case, PEGylation at β-Cys-93, along with allosteric cooperativity.

Comparison of hemoglobin P_{50} and nitrite reductase activity (NiR) indicates that a good correlation exists for different altered hemoglobins. Altered hemoglobins with high oxygen affinities show an increase in nitrite reduction (Figure 5A). Since native hemoglobin is neither R- nor T-state stabilized, its position on the

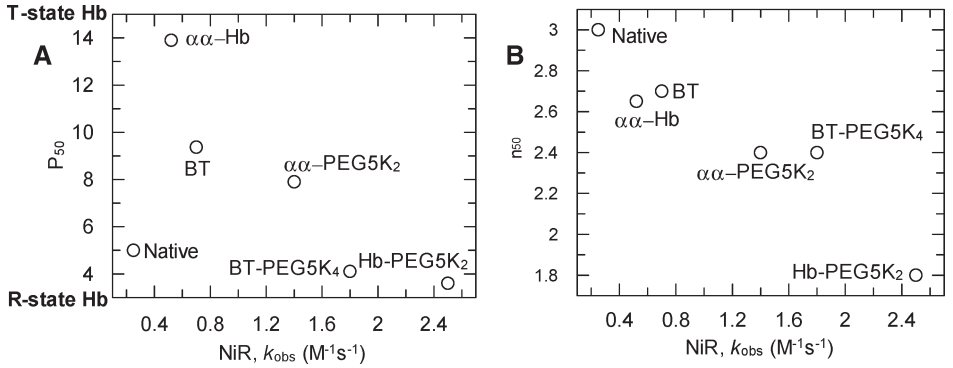


FIGURE 5: Correlation between oxygen binding properties of modified hemoglobins and nitrite reductase activity (NiR). (A) Increased oxygen affinity results in increased rates of reaction with nitrite, while (B) decrease in cooperativity results in an increased rate of reaction with nitrite.

graph may not be an accurate representation, lending more credit toward the trend between P_{50} and NiR. As well, it is clear that β-Cys-93 activated hemoglobin exhibits an overall increase in oxygen affinity, with Hb-PEG5K$_2$ (P_{50} = 3.6) and αα-Hb-PEG5K$_2$ (P_{50} = 7.9) in the lower right area of the graph. These species react with nitrite according to a similar trend, with Hb-PEG5K$_2$ (k_{obs} = 2.5 M^{-1} s^{-1}) having the faster reaction rate and αα-Hb-PEG5K$_2$ (k_{obs} = 1.4 M^{-1} s^{-1}) being slower. The high oxygen affinity PEGylated bis-tetramer (BT-Hb-PEG5K$_4$; P_{50} = 4.1) thus converts nitrite to nitric oxide at rates comparable to the nonvasoactive Hb-PEG5K$_2$. Nonetheless, while an apparent correlation between oxygen affinity and nitrite reductase activity exists (Figure 5A), a more direct correlation between cooperativity and reactivity with nitrite exists, a trend that has not previously been noted (Figure 5B).

Since unliganded subunits in the R-state are already in their oxygen binding conformation, these subunits have an increased oxygen affinity. The oxygen binding curve of Hb-PEG5K$_2$ is thus hyperbolic (*37*), since binding a ligand at one subunit is unaffected by the state of the other subunit in the absence of a quaternary structural change. This loss of quaternary structural change is also clearly reflected in the protein's reduced Hill coefficient for oxygen binding. For hemoglobin derivatives with a low P_{50}, the loss of cooperativity means each monomer is frozen in the R-state, and the loss of quaternary structural changes means that all four protein subunits bind ligands at rapid rates. This further explains the correlation between n_{50} and nitrite reductase activity (Figure 5B). High oxygen affinity hemoglobins with lower cooperativity will have increased ligand binding rates independent of quaternary changes and will thus bind nitrite at faster rates for all four subunits. Therefore, R-state stabilized Hb-PEG5K$_2$ (n_{50} = 1.8) exhibits the highest heme−nitrite reaction rates due not only to the decrease in heme reduction potential but also to increased ligand binding rates reflected by its loss of cooperativity.

Nonetheless, a species with high oxygen affinity and very low cooperativity will not be effective as an oxygen therapeutic, regardless of its increased production of nitric oxide, since oxygen release may be hindered. Instead, a compromise between favorable oxygen binding and releasing properties and features that enhance the nitrite reductase activity may be the first step to constructing a functional HBOC. PEGylated bis-tetramers (BT-Hb-PEG5K$_4$) appear to have suitable characteristics: increased molecular size to prevent extravasation, high oxygen affinity (P_{50} = 4.1) targeting oxygen delivery to capillary beds, significant cooperativity (n_{50} = 2.4) that maintains useful release of oxygen, and an increased rate of production of nitric oxide from heme−nitrite reactions.

CONCLUSIONS

We have produced PEGylated bis-tetramers of hemoglobin (BT-Hb-PEG5K$_4$), high molecular mass material with high oxygen affinity and enhanced nitrite reductase activity, features that are important for the development of functional oxygen therapeutics. We have shown that hemoglobin nitrite reductase activity also correlates with oxygen affinity and allosteric stabilization; derivatives with high oxygen affinity also have faster heme−nitrite reaction rates. As well, the degree of cooperativity reflects rates of nitrite binding, contributing to effects that modulate the protein's nitrite reductase activity. Predicting function based on the nature of structural alterations requires understanding the relationship between P_{50}, n_{50}, and NiR. This will require determining redox potentials of the various hemoglobin species in order to provide additional perspectives into features that contribute to hemoglobin's nitrite reductase activity.

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After this paper was published ASAP November 20, 2009, a correction was made to the abstract; the corrected version was reposted November 23, 2009.

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