# Polyethylene Glycol Conjugation Enhances the Nitrite Reductase Activity of Native and Cross-Linked Hemoglobin[†]

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ABSTRACT: Although stabilized hemoglobins have been evaluated as oxygen-carrying replacements for red cells in transfusions, in vivo evaluations have noted that these materials are associated with vasoactivity, a serious complication. Scavenging of endogenous nitric oxide by the deoxyheme sites of the stabilized proteins is one likely source of vasoactivity. Recent reports indicate that modification of cell-free hemoglobin derivatives with multiple chains of polyethylene glycol (PEG) suppresses vasoactivity. Gladwin and co-workers observed that the nitrite reductase activity of hemoglobin serves as a major endogenous source of nitric oxide. If PEG conjugation leads to enhanced nitrite reductase activity, this could compensate for scavenged endogenous nitric oxide. To test this possibility, the rates of conversion of nitrite ion to nitric oxide by altered hemoglobins with and without PEG were measured at 25 °C. Fumaryl (α99−α99) cross-linked hemoglobin reacts with nitrite with a bimolecular rate constant of 0.52 $M^{-1}$ $s^{-1}$, which is comparable to that associated with native hemoglobin (0.25 $M^{-1}$ $s^{-1}$). Addition of PEG chains to the cross-linked hemoglobin at $\beta$-Cys93 (αα-Hb-PEG5$K_2$) results in a material that produces nitric oxide much more rapidly ($k$ = 1.41 $M^{-1}$ $s^{-1}$). R-State-stabilized hemoglobins with multiple PEG chains (Hb-PEG5$K_2$ and Hb-PEG5$K_6$) react 10 times faster with nitrite to produce nitric oxide than does native hemoglobin ($k$ = 2.5 and 2.4 $M^{-1}$ $s^{-1}$, respectively). These results, showing enhanced production of nitric oxide resulting from an increased proportion of the protein residing in the R-state, are consistent with the decrease in vasoactivity associated with PEG conjugation.

Stabilized hemoglobins (Hb)[1] have been produced by both chemical methods and protein engineering, motivated by a need for acellular oxygen carriers that can provide alternatives to red cells in transfusions (*1*). Ideally, these hemoglobin-based oxygen carriers (HBOCs) would be readily sterilized, nonimmunogenic, stable in storage, and clinically benign. While many of the objectives have been realized, clinical observations of the effects of currently tested HBOCs have led to questions regarding their efficacy and safety, with problems resulting at least partially from their apparent vasoactivity (*2*). While the specific cause of the complications has not been identified, one possibility has been that the heme iron of the protein combines rapidly and irreversibly with the endogenous vasodilator, nitric oxide (NO), causing constriction of blood vessels (*3*). Since the need for such a product remains critical, it is important to understand the potential reactivity patterns that can contribute to the observed complications. In one approach, Winslow and co-workers made the promising discovery that vasoactivity is minimized in a chemically modified hemoglobin containing multiple chains of polyethylene glycol (PEG) (*4*). Since nitric oxide binds rapidly to such species, explanations of vasoactivity that invoke scavenging of nitric oxide appear to be inadequate (*5*). Winslow and co-workers suggest instead that vasoactivity is a homeostatic response to locally increased oxygen concentrations: the addition of PEG overcomes this response by slowing the release of oxygen.

Gladwin and co-workers observed that nitrite ion (or nitrous acid) in vivo serves as a stable, circulating precursor of nitric oxide (NO) (*6*). The nitrite reductase activity of hemoglobin (*7*) reduces ferrous heme-bound nitrite to nitric oxide while ferrous ion is oxidized to the ferric state, producing a subunit of methemoglobin. The resulting NO is released and may combine with a ferrous heme. The presence of ferric ion within the tetramer also facilitates nitrite reduction at the remaining ferrous hemes within the tetrameric protein (*8*). They conclude that faster reduction of nitrite occurs where the protein is in the relaxed or "R" conformation. As a result, the reduction of nitrite by hemoglobin is subject to allosteric effects similar to those accompanying the binding of oxygen.

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[1] Abbreviations: Hb, hemoglobin; PEG, polyethylene glycol; metHb, methemoglobin; NOHb, nitrosylhemoglobin; NO, nitric oxide; Mal-PEG5K, methoxypolyethylene glycol 5000 maleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoate); αα-Hb, αα-diasprin cross-linked hemoglobin; αα-Hb-PEG5$K_2$, αα-diasprin cross-linked, $\beta$-Cys93 Mal-PEG5K-conjugated hemoglobin; Hb-PEG5$K_2$, $\beta$-Cys93 Mal-PEG5K-conjugated hemoglobin; Hb-PEG5$K_6$, thiol-mediated, Mal-PEG5K-conjugated hemoglobin; Hb-$NEM_6$, $\beta$-Cys93 NEM-modified hemoglobin; $k_{obs}$, bimolecular nitrite reductase rate constant; HBOC, Hb-based oxygen carrier; PEGylation, conjugation of PEG chains to proteins; CD, circular dichroism; NEM, *N*-ethylmaleimide; $p_{50}$, oxygen pressure at which Hb is half-saturated.

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The nitrite reductase reactions for individual subunits (*5*) are shown in eqs 1 and 2:

$$Hb(Fe^{II})^{2+} + ONOH \rightarrow Hb(Fe^{III})^{3+} + NO + OH^- \quad (1)$$

$$Hb(Fe^{II})^{2+} + NO \rightarrow Hb(Fe^{II}NO)^{2+} \quad (2)$$

If addition of PEG to hemoglobin enhances its nitrite reductase activity, the modification would counteract consumption of endogenous nitric oxide by hemoglobin. Thus, we examined the effects of the addition of PEG on the nitrite reductase activity of both native and cross-linked hemoglobins. We find that the added PEG chains produce a significant increase in the rate of reduction of nitrite. In addition, simple modification of the thiol of $\beta$-Cys93 with *N*-ethylmaleimide (NEM) also yields R-state-stabilized hemoglobin with increased nitrite reductase activity. On the other hand, modifications that stabilize the T-state of hemoglobin, such as that resulting from formation of the cross-linked α99−α99-bis-fumaryl amide, produce lower nitrite reductase activity.

## EXPERIMENTAL PROCEDURES

*Materials and Methods.* Human hemoglobin A was isolated and purified from red cells obtained from the Canadian Blood Services through volunteers with their informed consent or as a gift from Oxygenix, Inc. All reagents were purchased and used as obtained. Methods for purifying, analyzing, and storing hemoglobin were as described by Jones (*6*).

*Cross-Linking.* Hemoglobin was cross-linked between α-subunits as a bis-fumaryl amide of the ε-amino groups derived from the side chains of Lys99 (*7*). This cross-linked hemoglobin is often designated as "diaspirin cross-linked hemoglobin" (DCLHB), despite the fact that it neither contains nor is made from ASA. Our procedure followed that reported by Walder and co-workers (*8*). A solution of deoxyHb was converted to oxyHb under a stream of oxygen at 0 °C with tungsten lamp irradiation and stirring for 2 h. Five equivalents of inositol hexaphosphate ($3.75 \times 10^{-4}$ mol) was added to the solution and the mixture placed under a stream of humidified nitrogen for 2 h at 37 °C. Four equivalents of solid 3,5-dibromosalicyl fumarate ($3 \times 10^{-5}$ mol) (*8*) was added and the solution stirred for 18 h. Glycine was then added to the mixture to destroy excess reagent. The mixture was cooled in ice, placed under a stream of carbon monoxide for 10 min, and then passed through a column of Sephadex G-25 that had been equilibrated with MOPS buffer (0.1 M, pH 7.2). The cross-linked hemoglobin product was concentrated through a membrane with centrifugation (3000 rpm for 30 min) and stored at 4 °C.

*Conjugation with Polyethylene Glycol (PEG).* Native hemoglobin and αα-cross-linked hemoglobin (0.5 mM) were separately converted to analogous PEG derivatives. The protein solution was combined with 10 equiv of methoxypolyethylene glycol 5000 maleimide (Mal-PEG5K) in sodium phosphate buffer (0.1 M, pH 7.4) and kept at 4 °C overnight to give products with two PEG chains per tetramer: Hb-PEG5K$_2$ and αα-Hb-PEG5K$_2$. A species with six PEG chains conjugated to hemoglobin (Hb-PEG5K$_6$) was prepared as described by Vandegriff (*9*). 2-Iminothiolane (10 equiv) and Mal-PEG5K (20 equiv) were added to the hemoglobin mixture in a single step. The reaction mixture was stirred for 16−20 h at 4 °C. The resulting mixtures were passed through a column containing Sephadex G-25 equilibrated with sodium phosphate buffer (0.1 M, pH 7.4), concentrated, and stored at 4 °C. Separation from excess reagents was achieved 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 produce the carbonmonoxy derivatives. These were stored at 4 °C.

*HPLC Analysis of Modified Hemoglobins.* Cross-linked hemoglobins were analyzed using analytical reverse-phase HPLC with a 330 Å C-4 Vydac column (4.6 mm × 250 mm) in determining the sites of globin chain modifications (*14*). Modified and unmodified globin chains were separated using an eluting solvent containing 0.1% trifluoroacetic acid and a gradient from 20 to 60% acetonitrile (volume percent) in water. The effluent was monitored at 220 nm. PEG-conjugated hemoglobins were individually analyzed using a preparative size-exclusion column (Superdex G-200 HR, 10 mm × 300 mm). Protein samples (0.5 mM) were eluted under partially dissociating conditions by the addition of 0.5 M magnesium chloride in buffer [$25 \times 10^{-3}$ M Tris-HCl (pH 7.4)]. The effluent was monitored at 280 nm.

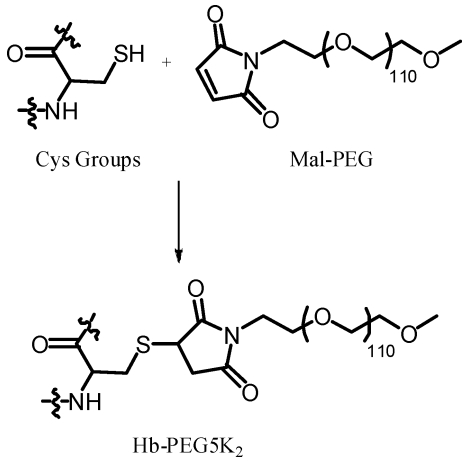
*SDS−PAGE Analysis.* Protein standards, reaction samples, and native Hb were prepared by combining 2−4 μL with the loading buffer (16−18 μL), consisting of 0.0625 M Tris-HCl (pH 6.8), 1.3 M glycerol, 2% SDS, 0.0125 (w/v) bromophenol blue, and 0.7 M $\beta$-mercaptoethanol. The samples were denatured by being heated 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 a 30% methanol/10% acetic acid solution.

*CD Spectroscopy.* To determine the effects of PEG modification on the overall structural stability of the protein, we 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 200−260 nm was obtained in triplicate.

*Thiol Determination with 5,5′-Dithiobis(2-nitrobenzoate) (DTNB).* Accessible sulfhydryl groups were quantified by observing the results of the disulfide exchange reaction of the $\beta$-Cys93 thiols and DTNB (*10*) at 412 nm. The conditions for the DTNB titration were as follows: 50 mM Bis-Tris (pH 7.4), 1 mM EDTA, 0.1−0.4 mg/mL protein, and 20 μM DTNB. We produced a standard curve using known concentrations of $\beta$-mercaptoethanol. Thus, spectroscopic measurements of the DTNB reaction mixture with modified hemoglobins provided the residual thiol concentration after subtraction of background absorbance.

*Kinetic Measurements.* Solutions containing αα-Hb, αα-Hb-PEG5K$_2$, Hb-PEG5K$_2$, or Hb-PEG5K$_6$ in buffers other than Bis-Tris were exchanged for Bis-Tris (0.01 M, pH 7.2). Samples (1 mL, 0.05 mM) were thoroughly flushed with nitrogen and the resulting solutions transferred anaerobically into a sealed cuvette. Oxygen-free solutions of nitrite were then added to give final concentrations of nitrite of 0.05−1.5 mM. The formation of methemoglobin (MetHb) and iron nitrosyl hemoglobin (HbNO) was followed by recording spectra from 500 to 650 nm. Spectral data were analyzed

Scheme 1: Conjugation of Mal-PEG5K to a Thiol at $\beta$-Cys93 of Hemoglobin (Hb-PEG5K$_2$)




by multiple-linear regression analysis of data at 1 nm intervals using separately obtained spectra of the individual components as a basis set (*7*). The initial reaction rate is the rate of ferric heme formation at the beginning of the reaction calculated as the average rate over the first 100 s.

## RESULTS

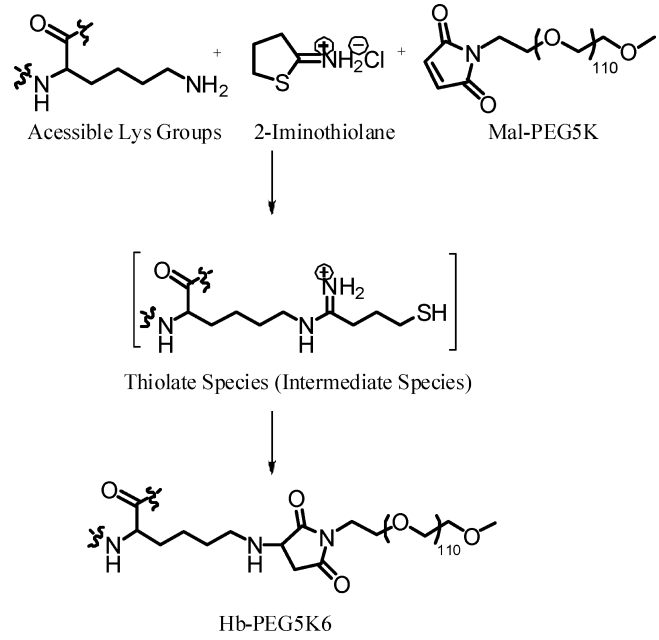
*Characterization of Cross-Linked and PEG-Conjugated Hbs.* PEG was conjugated to αα-cross-linked hemoglobin at both $\beta$-subunits by reaction of the thiol of each $\beta$-Cys93 with malemide-activated PEG5K to give αα-Hb-PEG5K$_2$ (Scheme 1). We produced a similar derivative of native hemoglobin (Hb-PEG5K$_2$) in the same manner. A conjugate of six PEG chains and hemoglobin (Hb-PEG5K$_6$) was prepared via the initial reaction of exposed lysine $\varepsilon$-amino groups with 2-iminothiolane (Scheme 2) (*16*) to produce thiols at each amino group that reacts.

*(i) HPLC Analysis.* We separated and analyzed the products of cross-linking and conjugation using HPLC (Figures 1 and 2) and analyzed the fractions by SDS−PAGE (Figure 3). As described in Experimental Procedures, cross-linked hemoglobins were separated by HPLC and analyzed by being passed through a C4 reversed-phase HPLC column. Analysis of the α99−α99-fumaryl cross-linked hemoglobin (DBSF-Hb) indicated that this produces >98% site-specific modification with less than 5% unmodified α-subunits remaining.

Conjugation of PEG to DBSF-modified Hb (64 kDa) gives αα-Hb-PEG5K$_2$ (74 kDa). This appears as an earlier-eluting peak on size-exclusion HPLC, while the material with six PEG chains (Hb-PEG5K$_6$, 94 kDa) elutes ahead of the material with two PEG chains (Hb-PEG5K$_2$, 74 kDa), as previously reported by Acharya (*17*). The chromatograms indicate that addition of two PEG chains at $\beta$-Cys93 gives a homogeneous material. We observe that the material with six PEG chains conjugated to hemoglobin is the predominant species in its fraction, along with some lower-molecular mass materials (fewer PEG chains) as indicated by smaller, broader bands eluting at 30−40 min. This variation in the extent of PEG modification is typical of protein modification with PEG reagents.

*(ii) SDS−PAGE Analysis.* The gel shown in Figure 3 gives the molecular masses of the modified subunits. The conditions used separate native hemoglobin into 16 kDa α- and $\beta$-globin chains (lane N). In the lane marked αα, there is a band that corresponds to the covalently joined subunits of αα-fumaryl cross-linked subunits at 32 kDa and another corresponding to unmodified $\beta$-subunits at 16 kDa. Conjugation of maleimido PEG at each $\beta$-Cys93 thiol gives a material that produces a band at 26 kDa, indicating PEG conjugation at both $\beta$-Cys93 groups (lane ααPEG), while cross-linked α-subunits remain at 32 kDa. A similar band at 26 kDa indicative of PEG conjugation at $\beta$-Cys93 is observed in native PEG-conjugated hemoglobin (2PEG = Hb-PEG5K$_2$). There is no evidence of material with only one subunit conjugated to maleimido-PEG. The sites of conjugation of maleidmido-PEG to the product of reaction of lysyl amino groups with 2-iminothiolane were previously identified by Winslow and co-workers (*16*). They observed modification of $\beta$-Cys93 and partial modification of five lysine residues: Lys40(α), Lys120($\beta$), Lys61(α), Lys7(α), and Lys8($\beta$). The large diffuse band from that species is presented in lane

Scheme 2: Cyclic 2-Iminothiolane Reacts with $\varepsilon$-Amino Groups of Lysines To Produce an Extended Thiol That Reacts with Free Mal-PEG5K[a]




[a] Conjugation of PEG to $\beta$-Cys93 of the same hemoglobin also occurs, generating a hexa-PEG conjugate (Hb-PEG5K$_6$).

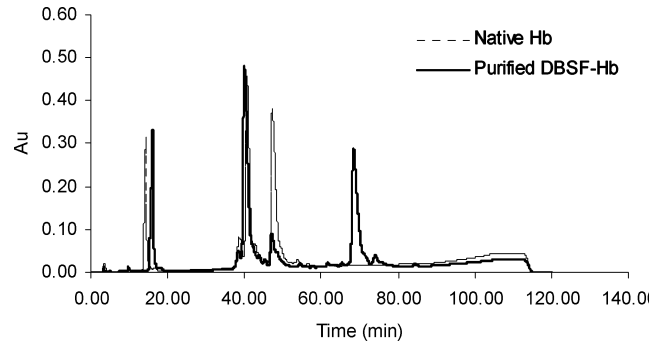



FIGURE 1: C4 reversed-phase HPLC of purified αα-fumaryl cross-linked hemoglobin (αα-Hb). The peak at 19 mins = heme, 40 mins = $\beta$ subunit, 44 mins = α subunit, 70 mins α-α′ cross-linked subunits.

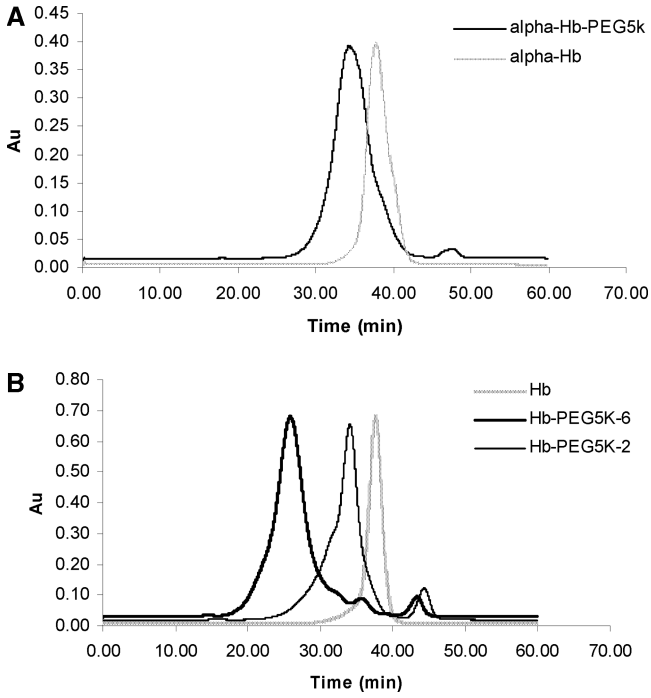



FIGURE 2: Superdex G-200 size-exclusion HPLC of (A) αα-Hb and αα-Hb-PEG5$K_2$ or (B) Hb, Hb-PEG5$K_2$, and Hb-PEG5$K_6$. Separation is dependent on increasing molecular size.

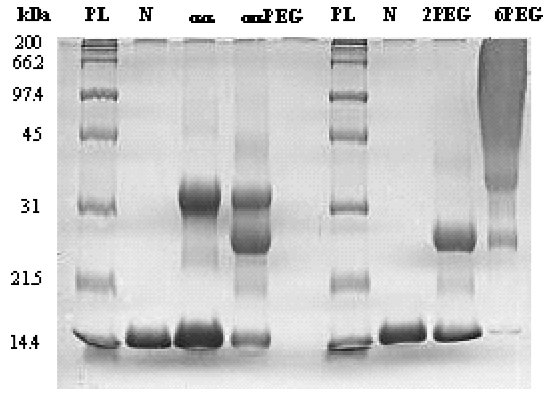



FIGURE 3: SDS−PAGE analysis (12%) of native Hb (N), fumaryl-cross-linked Hb (αα-Hb), and PEG-conjugated hemoglobins (ααPEG = αα-Hb-PEG5$K_2$, 2PEG = Hb-PEG5$K_2$, and 6PEG = Hb-PEG5$K_6$).

6PEG and is likely a composite of various PEG-conjugated subunits and a result of the polydispersity of PEG.

*(iii) Thiol Concentration.* Thiol determination using a DTNB (Ellman's reagent) analysis was used to determine the extent of PEG conjugation of accessible thiols. This ranges from 97% in αα-Hb-PEG to 100% in both Hb-PEG5$K_2$ and Hb-PEG5$K_6$.

*Kinetic Analysis.* We followed the reactions of modified hemoglobins with nitrite by acquiring spectra at specific time intervals (Figure 4A). The changes are very similar to those observed by Doyle (*5*) for reactions of the native protein. They indicate that deoxyHb is converted to equal amounts of metHb and NOHb. Spectra that were recorded over the course of the reaction were analyzed by multiple linear regression 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, and NO) at each reaction time point (Figure 4C−F). A similar method of analysis was employed for all derivatives (cross-linked and PEG-conjugated) of hemoglobin. Although there appear to be small amounts of oxygen present, exclusion of oxyHb from the fitting routines does not increase the magnitudes of the residuals (Figure 5B), and the residuals remain randomly scattered. In any event, the presence of oxyHb is not significant and does not contribute to the nitrite reductase activity of hemoglobin. The rate constant for formation of metHb was then derived from the observed initial rates (Figure 6).

The CD spectra (200−260 nm, indicative of secondary structure change) of all cross-linked and PEG-conjugated hemoglobins were identical to that of native hemoglobin. Although protein side chains had been modified, no disturbances to the tertiary structure of the protein are likely to have occurred.

According to the reaction scheme in eqs 1 and 2, MetHb and NOHb are produced in equal amounts in the absence of oxygen (*7*). We observe this to be the case for all species of modified hemoglobin, regardless of the method of modification (cross-linking or PEG conjugation). Also, the progress curve for conversion of the initial spectrum of αα-fumarylHb shows sigmoidal character similar to that reported for native hemoglobin, indicating autocatalysis or cooperativity (Figure 4C). This is consistent with the allosteric effect observed by Gladwin and co-workers, where R-state Hb reduces nitrite faster than T-state Hb (*19*). The T-state-stabilized αα-fumarylHb retains a high degree of cooperativity ($n_{50} = 2.6$) similar to that of native hemoglobin ($n_{50} = 3$), which is reflected in its nitrite−heme reaction. In contrast, the reactions of nitrite with PEG-conjugated hemoglobins (αα-Hb-PEG5$K_2$, Hb-PEG5$K_2$, and Hb-PEG5$K_6$) did not exhibit allosterism (Figure 4D−F). This is consistent with the observation that PEG-conjugated hemoglobins have reduced cooperativity in binding oxygen, suggesting that addition of PEG interferes with features that permit allosterism to occur.

The initial rate of metHb formation for all different species was derived from multiple linear regression fits at constant hemoglobin concentrations and plotted as a function of increasing nitrite concentration (Figure 6). We followed the formation of metHb, rather than the consumption of deoxyHb, since formation of metHb is due exclusively to the reaction of deoxyHb with nitrite. The depletion of deoxyHb is dependent on both its reaction with nitrite (to form metHb) and its combination with nitric oxide generated in the reduction of nitrite (to form nitrosyl Hb). The initial rate of formation of metHb exhibits a linear dependence on nitrite concentration under these conditions for all of the modified hemoglobins. Assuming that this rate depends only on the reaction between nitrite and Hb, the apparent rate constant ($k_{int}$) for each of the different hemoglobin species can be determined (Table 1) in the same manner.

The kinetic data for the modified hemoglobins (Table 1) indicate that conjugation with PEG increases the rate of reaction with nitrite: αα-fumarylHb is T-state-stabilized, with a consequently low oxygen affinity ($p_{50} = 13.9$). The rate of reaction of αα-fumarylHb with nitrite is comparable to that of hemoglobin. PEG conjugation (αα-fumarrylHb-PEG5$K_2$) results in a 6-fold increase in the rate of reaction with nitrite compared to that of native Hb, as well as an increased affinity for oxygen. The R-state stabilizing effect of $\beta$-Cys93 PEG overwhelms the T-state stabilizing effect

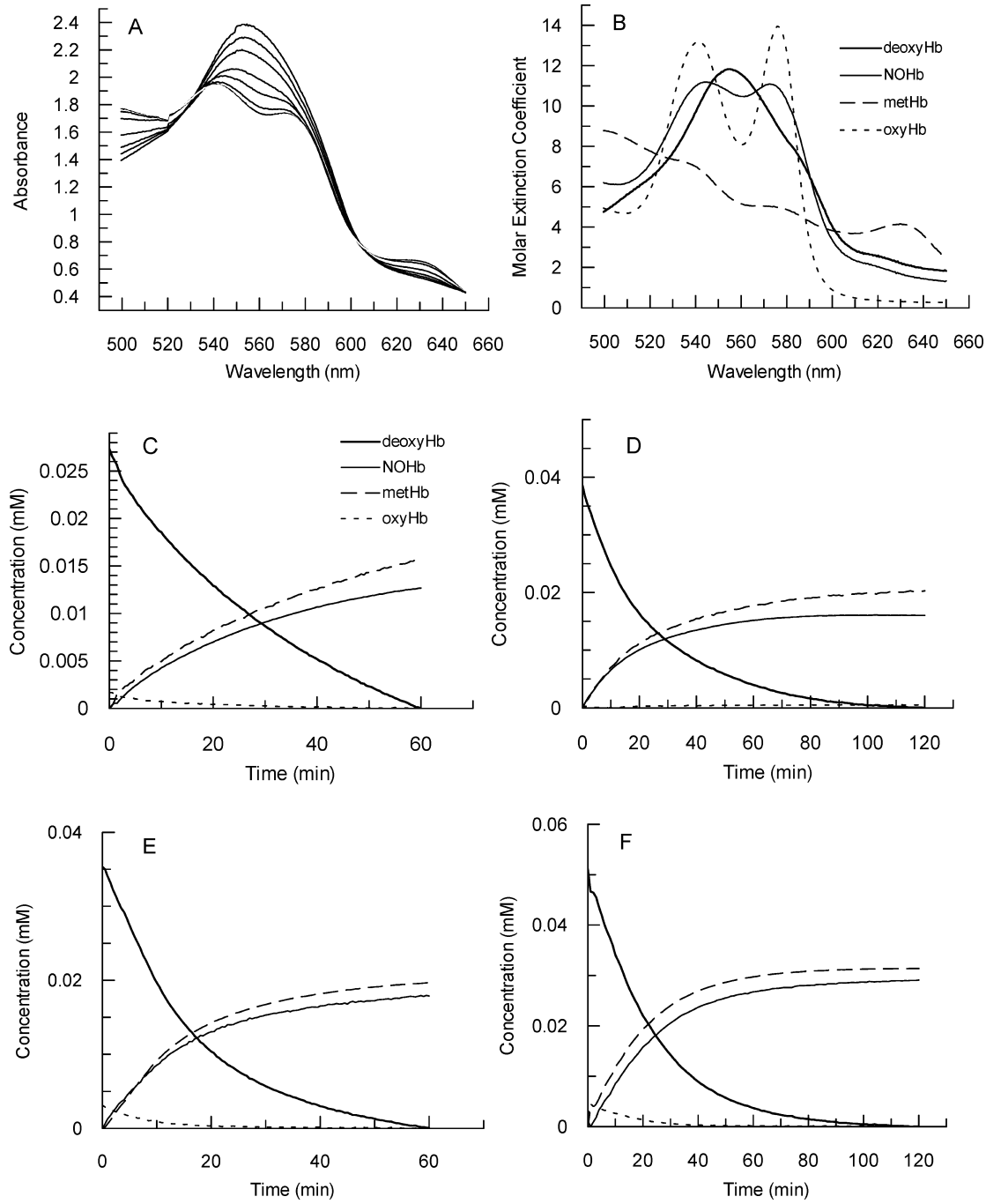



FIGURE 4: Excess nitrite is reacted separately with modified deoxyhemoglobins (0.03 mM) in a 0.4 cm path length cuvette under nitrogen flow, and spectral data were collected at 30 s intervals. (A) Time scans for the reaction of Hb-PEG5K$_2$ from 0, 0.5, 1, 2, 4, 6, 8, and 9 min. (B) Molar extinction coefficients obtained from spectra of deoxy-, NO-, Met-, and oxyHb used in the regression analysis. Progress of the reactions between (C) αα-Hb and 1.5 mM nitrite, (D) αα-Hb-PEG5K$_2$ and 0.75 mM nitrite, (E) Hb-PEG5K$_2$ and 0.5 mM nitrite, and (F) Hb-PEG5K$_6$ and 0.5 mM nitrite.

of αα cross-linking, increasing the rate of reaction with nitrite as well as the oxygen affinity ($p_{50} = 7.9$).

Comparison of the reaction of native Hb with its PEG-conjugated analogue, Hb-PEG5K$_2$, indicates that the modified protein reacts 10 times more rapidly with nitrite to give nitric oxide. Both Hb-PEG5K$_2$ and Hb-PEG5K$_6$ produce nitric oxide from the reaction with nitrite at a higher rate than do native or αα-cross-linked hemoglobins, but there is little difference between the reaction rates of the two species with nitrite. This similarity is also reflected in the oxygen affinity and cooperativity of these two species; both have high oxygen affinities ($p_{50} = 3.6$) and reduced cooperativity ($n_{50} = 1.8$). It thus appears that the R-state stabilizing effect of PEG conjugation at $\beta$-Cys93 alone is responsible for the increased nitrite reductase activity in both Hb-PEG5K$_2$ and Hb-PEG5K$_6$. Further modification at lysine residues to produce Hb-PEG5K$_6$ does not alter the heme−nitrite reaction rates.

In a control study, we observed the nitrite−heme reaction for hemoglobin that had been modified with *N*-ethylmaleimide (NEM) in the presence of 2-iminothiolane, giving Hb-NEM$_6$. This simple modification yields R-state-stabilized hemoglobin whose initial rate of reaction with nitrite is 0.9 $M^{-1}$ $s^{-1}$, only 4 times faster than that of native hemoglobin,

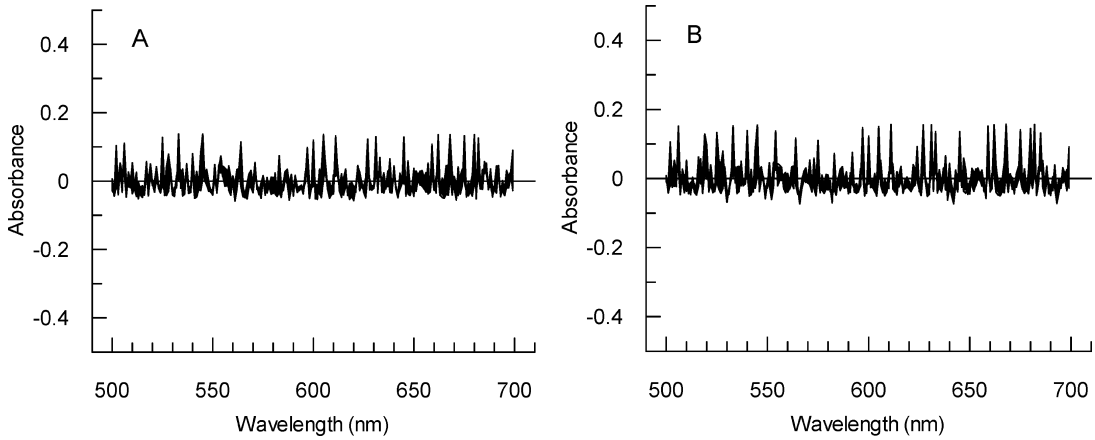



FIGURE 5: Residuals between multiple-linear regression analysis and spectra for the reaction between αα-Hb and 1.5 mM nitrite (Figure 4C) for (A) all components (deoxy-, NO-, met-, and oxyHb) and (B) without oxyHb.

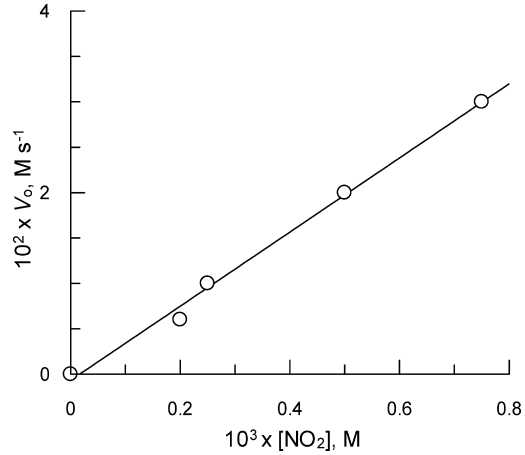



FIGURE 6: Initial rate plot as a function of nitrite concentration for PEG-conjugated αα-fumarylHb (αα-Hb-PEG5$K_2$). The bimolecular rate constant ($k_{int}$) was determined from the formation of met-hemoglobin at the beginning of the reaction.

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

| Hb | nitrite reaction $k_{int}$ ($M^{-1}$ $s^{-1}$) | oxygen binding[a] | |
|---|---|---|---|
| | | $p_{50}$ | $n_{50}$ |
| native | 0.25 ± 0.02 | 5 | 3 |
| αα-Hb | 0.52 ± 0.03 | 13.9 | 2.6 |
| αα-Hb-PEG5$K_2$ | 1.4 ± 0.03 | 7.9 | 2.4 |
| Hb-PEG5$K_2$[b] | 2.5 ± 0.03 | 3.6 | 1.8 |
| Hb-PEG5$K_6$[b] | 2.4 ± 0.05 | 3.7 | 1.8 |

[a] Oxygen binding data were recorded in 0.01 M phosphate buffer at pH 7.4. [b] Data from ref *16*.

and 3 times slower than that of PEG-conjugated hemoglobin. This establishes that the addition of PEG is essential for the large increase in the rate of formation of nitric oxide.

## DISCUSSION

Gladwin has noted that nitrite may provide a large vascular storage pool as a precursor of NO (*20*), and we observe that modification of hemoglobin has a strategic effect on this process. Our results show that there is a distinct variation in the reaction of nitrite among the various cross-linked and PEG-conjugated hemoglobins. There is little difference in the rate of association with nitrite in native hemoglobin and αα-fumarylHb. However, there is a significant increase in the rate of reaction with nitrite with the two R-state-stabilized hemoglobins, Hb-PEG5$K_2$ and Hb-PEG5$K_6$. Both of these hemoglobins have high oxygen affinities and stabilized R-states. Another source of the increased rate of reaction is the decrease in cooperativity (indicated by the lower magnitudes of $n_{50}$) in reactions of PEG-conjugated hemoglobins. The lower cooperativity maintains the R-conformation. On the other hand, αα-cross-linked hemoglobin has a much lower oxygen affinity and is a T-state-stabilized hemoglobin. This is reflected in the observation that αα-Hb reacts with nitrite at a rate similar to that of native hemoglobin.

Gladwin and co-workers (*8*) have noted that the nitrite reductase activity of hemoglobin correlates with both its allosteric state and the number of deoxygenated hemes. In oxygenated hemoglobin (R-state), the hemes of the tetramer exhibit a decreased reduction potential, resulting in an increase in the rate of reduction of nitrite. Thus, R-state hemoglobin gives a bimolecular rate constant of 6 $M^{-1}$ $s^{-1}$ for reduction of nitrite as compared to a rate of 0.12 $M^{-1}$ $s^{-1}$ for T-state hemoglobin (*6*). While the R-state of hemoglobin possesses the higher nitrite reductase activity, the T-state will have more heme sites available for binding and reaction. A balance of available deoxyheme sites versus oxyheme sites with a higher bimolecular rate constant occurs at ~50% oxygen saturation ($p_{50}$) (*7*).

The increased rates of production of nitric oxide from the reaction of nitrite with PEG-modified hemoglobin occur in those derivatives that produce the least vasoactivity. The resulting level of nitric oxide depends on the rate of the reaction between deoxyHb and nitrite and the rate of consumption of nitric oxide from its combination with deoxyHb (eq 3). An increase in the rate of reaction with nitrite ($k_1$) results in an increased production of nitric oxide (Figure 7). Since the reaction of nitric oxide with free deoxyHb ($k_2$) is much faster (~$10^7$-fold), modifications of hemoglobin do not significantly alter the rate of this reaction: the increased production of nitric oxide depends exclusively on the magnitude of $k_1$.

$$\frac{d[NO]}{dt} = k_1[\text{deoxyHb}][NO_2] - k_2[\text{deoxyHb}][NO] \quad (3)$$

It appears that there is no difference in the heme−nitrite reaction of conjugates with two PEG chains or those with six PEG chains. This is important because although there are significant differences in the physical properties of the

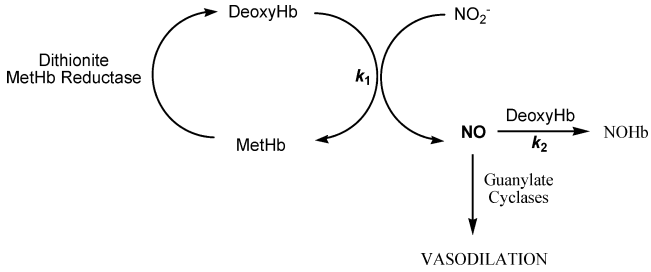



FIGURE 7: Increase in $k_1$ as a result of covalent modifications to deoxyHb will increase the level of formation of NO.

modified proteins (*16*), there is little difference in the heme−nitrite reactivity. It is thus likely that the rate of the heme−nitrite reaction is a direct result of the R-state stabilizing effect of PEG conjugation at $\beta$-Cys93. Both Hb-PEG5$K_2$ and Hb-PEG5$K_6$ are PEG-conjugated at $\beta$-Cys93, and further modifications at accessible lysine residues to produce Hb-PEG5$K_6$ do not alter the heme−nitrite reaction rates. Since both Hb-PEG5$K_2$ and Hb-PEG5$K_6$ react with nitrite to produce NO at identical rates, they should also exhibit identical vasoactivity in vivo if this property is reflected. Consistent with this is the report that both Hb-PEG5$K_2$ and Hb-PEG5$K_6$ are not vasoactive in animal tests and that there is little difference in their ability to deliver oxygen (*21*).

While further PEG conjugation at lysine residues (e.g., Hb-PEG5$K_6$) does not contribute to increased heme−nitrite reaction rates, PEG conjugation at $\beta$-Cys93 to hemoglobin is essential to the major increase in nitrite reactivity. Modification of $\beta$-Cys93 without addition of PEG (Hb-$NEM_6$) gives a material that reacts more slowly with nitrite. Thus, the R-state stabilizing effect alone that results from modification of the protein side chains is not the sole cause of the increased reactivity. In addition, different modifications at $\beta$-Cys93 by NEM or PEG can result in different redox potentials that affect their heme−nitrite rates (*8*).

Studies of PEG in solution indicate that two to three water molecules are tightly associated with each ethylene glycol subunit, effectively increasing its size in solution (*11*). While this effect was originally considered to result in a decrease in the binding affinity of hemoglobin for NO, there is little difference in NO binding affinity between native and PEG-conjugated hemoglobins (*5*). An alternative may be that the polar aprotic properties of PEG decrease the net polarity of the solvating water on the surface of the protein (*12*), ultimately affecting the heme−nitrite reaction rates.

Structural analysis indicates that longer PEG chains adopt two extreme structural conformations (*13*). In one, there may be no PEG−protein interaction: the conjugate adopts a helical structure stabilized by hydrogen bonds and fluctuates freely in solution. In the other, water solvates the hydrophilic regions around the protein while hydrophobic PEG clusters interact with hydrophobic surfaces of the protein, creating a shell-like structure (*13*). Such a conformation has a significant effect on the polarity of the solvent on the surface of the protein, which will enhance the rate of heme−nitrite reactions.

The role of nitrite−Hb reactions in the control of vasoactivity through production of nitric oxide has been considered in terms of the kinetics of reactions of nitric oxide with vicinal deoxy- and oxyHbs, which are efficient scavengers of nitric oxide. It has been proposed that dinitrogen trioxide ($N_2O_3$) is a likely intermediate in the nitrite−hemoglobin reaction (*25*), leading to a mechanism in which the highly electron deficient HbFe(III)−$NO_2^-$ intermediate, with HbFe(II)−$NO_2$ character, undergoes a radical combination reaction of the coordinated nitrite with NO, resulting in the formation of $N_2O_3$. This also would compete with NO−heme interactions that otherwise limit NO bioavailability. Although PEG-conjugated hemoglobin still reacts with NO, it is less likely to extravasate and scavenge more NO in the interstitial space, and we have shown that this NO scavenging effect is compensated by its nitrite-mediated NO production.

## CONCLUSIONS

We observe that all PEG-conjugated hemoglobins produce nitric oxide in a heme−nitrite reaction at a faster rate than native hemoglobin or T-state cross-linked hemoglobin without PEG. This suggests that the reduced vasoactivity reported for PEG-conjugated hemoglobins could be, in part, the result of increased nitrite reductase activity.

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