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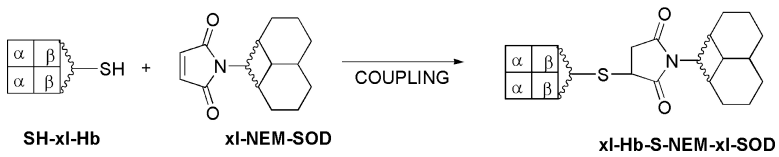

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Hemoglobin−Superoxide Dismutase−Chemical Linkages That Create a Dual-Function Protein

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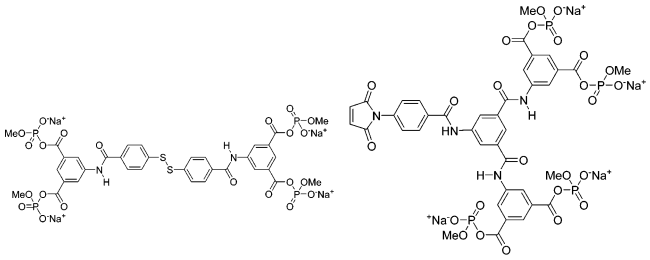
Abstract: Chemical reagents were designed to cross-link and connect hemoglobin and superoxide dismutase, combining the oxygen transport and superoxide-removal capabilities of the red cell in a dual-function protein. Reaction of **1** with thiol-protected hemoglobin followed by reduction produces cross-linked hemoglobin with a free thiol on the cross-link. Reaction of SOD with **5** produces a cross-linked protein with a maleimide on the cross-link. Addition of the hemoglobin-thiol to the SOD-maleimide produces a protein with the desired dual properties. Hemoglobin's oxygenation cooperativity is lowered as a result of being in the conjugate, while SOD's activity is equal to that of the native protein.

Introduction

Oxygen coordinated to ferrous hemes of hemoglobin within red blood cells is subject to reduction to superoxide, a reactive species that is removed by reactions promoted by intracellular superoxide dismutase (SOD), converting superoxide to hydrogen peroxide.[1−4] Hemoglobin-based oxygen carriers (HBOCs) are being evaluated as substitutes for red cells in transfusion medicine. These materials also produce superoxide (and methemoglobin) from coordinated oxygen.[5] This phenomenon is associated with reperfusion injury, tissue damage resulting from transfusion of an oxygen donor after an organism has been subject to an oxygen deficiency.[6]

The potential utility of a combination of hemoglobin and SOD in a circulating oxygen carrier has been demonstrated by Chang and co-workers, who combined an 8-fold excess of glutaraldehyde with hemoglobin and small amounts of SOD (and catalase).[6] The resulting material, which is a heterogeneous mixture from its method of formation, was shown to decrease the concentration of reactive oxygen species in circulation through its enzymic activities. The material has been used successfully in further studies of its physiological effects.[7] This work shows that only a small amount of SOD activity is necessary to protect the system. The accessibility of intracellular catalase may be sufficient to remove peroxide or it may be necessary to form a specific conjugate.

Our interest in the structurally defined chemical stabilization of hemoglobin[8,9] led us to seek a method that would produce a defined conjugate of hemoglobin and SOD in order to assess the effects of a link on the properties of the constituent proteins. We report the preparation of a cross-linked hemoglobin with a free thiol on its cross-link from reagent **1**, formation of a cross-linked SOD with a thiol-accepting maleimide group on its cross-link from reagent **5**, and formation of the conjugate from reaction of the thiol and maleimide groups of the two modified proteins.



1 **5**

Results

Synthesis of Cross-Linkers. Reagents for reaction with each protein component were produced by activation of carboxylic acids, followed by reaction with amines to form amides. Scheme 1 summarizes the route we used to produce **1**, which we designed to stabilize hemoglobin and to introduce an accessible thiol. This material incorporates a disulfide that is converted to a thiol by reduction. This feature simplifies synthetic steps as well as protein modification. Scheme 2 represents the route used

(1) Sutton, H. C.; Roberts, P. B.; Winterbourn, C. C. *Biochem. J.* **1976**, *155*, 503−10.
(2) Winterbourn, C. C.; McGrath, B. M.; Carrell, R. W. *Biochem. J.* **1976**, *155*, 493−502.
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(6) D'Agnillo, F.; Chang, T. M. S. *Nat. Biotechnol.* **1998**, *16*, 667−671.
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(9) Kluger, R.; De Stefano, V. *J. Org. Chem.* **2000**, *65*, 214−219.

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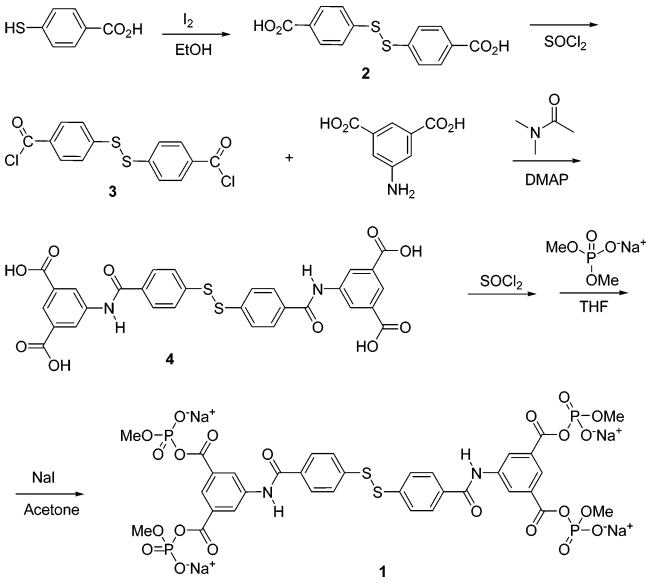
Scheme 1. Synthesis of the Cross-Linking Reagent (**1**) for Hemoglobin



to produce **5**, a cross-linker with a maleimide for stabilizing SOD and introducing a coupling site. We note that there are two types of electrophilic sites on **5** with orthogonal reaction selectivity: acyl phosphate esters to react rapidly with amines and maleimide to react with thiols. Scheme 3 summarizes the overall reaction strategy for forming the protein−protein conjugate.

In order that only the chemically introduced thiol reacts with the introduced maleimide, the two (β-Cys93) surface cysteinyl thiols of hemoglobin were blocked with *N*-ethyl maleimide (NEM). MALDI-TOF mass measurements indicated that each β globin chain was modified with a single molecule of NEM. Preliminary experiments showed that cross-linked hemoglobin without a free thiol group on its cross-link and with β93 Cys residues in their native state (not blocked by NEM), did not produce conjugates when it was mixed with maleimide-containing SOD. The β-82 lysyl ϵ-amino groups in the 2,3-diphosphoglycerate binding site of hemoglobin react with a pair of adjacent acyl phosphate esters of **1**. The reagent introduces cross-links into each of two hemoglobin tetramers, connecting them by a chain containing a disulfide. The reaction was readily monitored by C4 reversed phase HPLC (disappearance of the NEM β globin chains). We tracked the formation of reaction products by size-exclusion chromatography, isolating the cross-linked species. This was reduced with TCEP to liberate the thiol (Scheme 4).

Native hemoglobin dissociates into four 16 kDa monomers (2α and 2β) under the denaturing conditions of SDS-PAGE. Analysis of the purified cross-linked species in the presence of 2-mercaptoethanol produced bands at 16 and 32 kDa, corresponding to the masses of the expected unmodified α chains and cross-linked β chains. In the absence of 2-mercaptoethanol, the same system gives a band at 64 kDa, the combined mass of four β globin chains, consistent with a disulfide-linked bis-tetramer (shown schematically in Scheme 4; the rigidity of the reagent keeps the pairs of reaction sites far apart and in a linear relationship: the two ends must react with different protein molecules.)

We produced a peptide map from HPLC peptide elution patterns resulting from tryptic digestion of modified globin chains to determine the sites that had reacted with **1**. The products were separated by analytical C18 reversed phase HPLC. The modification decreases the relative amounts of peptides βT-1, βT-9, and βT-10. On the basis of the known sequence of these peptides, the result establishes that the β globin chains are cross-linked as amides from the α-amino group of β-Val1 and from the ϵ-amino group of the β-Lys82.

Modification of Superoxide Dismutase. SOD was reacted with **5** to incorporate a thiol-reactive maleimide functional group while cross-linking its subunits. The product mixture was resolved by analytical C4 reversed phase HPLC (Figure 1).

SDS-PAGE analysis of the products gave bands at 32 and 16 kDa. A control lane containing native SOD shows only a 16 kDa band, which is the mass of a single subunit of the enzyme. Native and modified SOD were digested with trypsin to identify the residues that had reacted with **5**. Individual peptides formed during the digest were separated by C18 reversed phase HPLC, whose output was analyzed by electrospray ionization mass spectroscopy (ESI-MS). Comparison of the peptide pattern of digested native and modified superoxide dismutase showed that peaks T1-T2, T4, T6-T7, T5-T7, T11, and T12-T13 were reduced in the modified protein (Figure 2). This suggests that the ϵ-amino groups of Lys3 (in T1-T2) and/or Lys151 (in T12-T13) are cross-linked to the ϵ-amino group of Lys134 (in T11) or Lys67 (in T4) of the other subunit (Supporting Information).

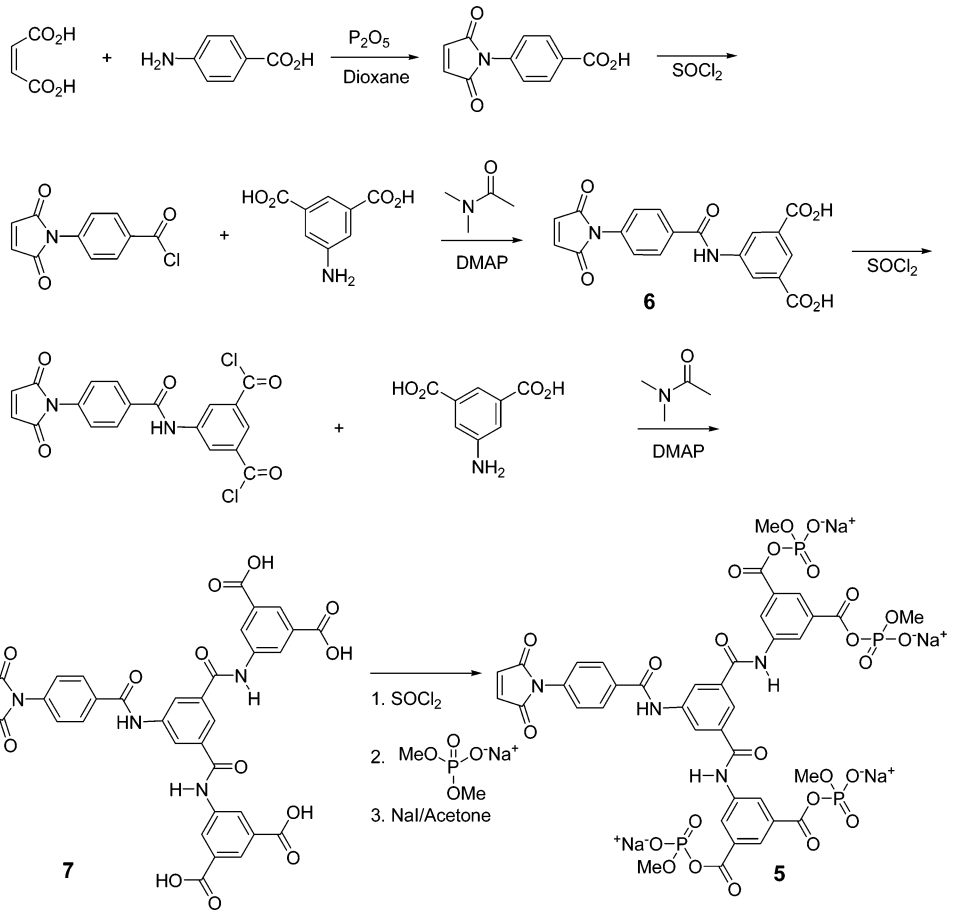
Conjugation of Cross-Linked Hb and Cross-Linked SOD. Cross-linked hemoglobin that been reacted with **1** and reduced was combined with the maleimide-containing cross-linked SOD in the presence of TCEP (Scheme 3). The isolated reaction mixture was resolved by SDS-PAGE into bands at 64, 32, and 16 kDa (Figure 3, lane 2). Thus, the mass of 64 kDa corresponds to the combined masses of cross-linked SOD (32 kDa) and hemoglobin that is cross-linked at the two β subunits of hemoglobin (32 kDa), separated from the α subunits.

These results show that the reaction between the Cys group of SOD with a maleimide group of compound **5** is not prevalent. Formation of a thioether bond between the Cys group and the maleimide on compound **5** would block SOD for conjugation to thiol-containing hemoglobin. Formation of hemoglobin−SOD conjugates demonstrates that the potential side-reaction between a Cys group of SOD and compound **5** is not interfering with the conjugation reaction.

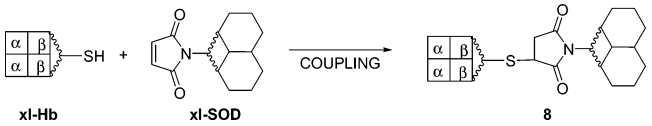
SDS-PAGE analysis of the coupling reaction between cross-linked hemoglobin and cross-linked SOD shows the presence of the protein−protein conjugate as well as excess cross-linked SOD. Size exclusion chromatography was used to separate the intact proteins under conditions that do not separate subunits. The 96 kDa conjugate was separated from the 32 and 16 kDa species (in the presence of TCEP). Fractions were pooled, concentrated, and reloaded onto the column until analysis by gel filtration column showed that the sample was homogeneous (Figure 4). The sample was then dialyzed using a membrane (cutoff $\sim$ 100 kDa).

The material collected by size exclusion chromatography binds and releases oxygen and also has SOD activity. The oxygen binding curve of the conjugate (Figure 5) indicates that the conjugate has a lower oxygen affinity ($P_{50} = 6.1$ Torr) than

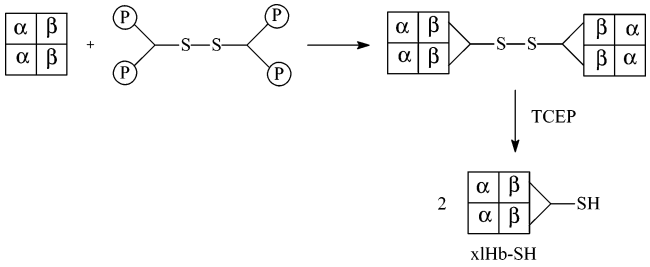
Scheme 2. Synthesis of the Cross-Linking Reagent (**5**) for SOD



Scheme 3. Formation of Hb−SOD



Scheme 4. Formation of Cross-Linked Hemoglobin with a Free Thiol[a]



[a] β-Cys-93 is blocked with NEM.

hemoglobin that is modified only with NEM ($P_{50} = 3.8$ Torr). This suggests that the cross-link may restrict movement necessary to go from the T to the R state upon addition of oxygen, leading to a lower energy requirement to release oxygen. Native hemoglobin binds oxygen with $P_{50} = 5.0$ Torr under the same conditions. The Hill coefficient of the conjugate, n_{50}, is 1.55 (Figure 5), which is lower than the Hill coefficient of NEM-modified hemoglobin ($n_{50} = 2.60$), native hemoglobin ($n_{50} = 2.7$), or cross-linked hemoglobin tetramers that are not conjugated to another protein ($n_{50} = 2.8$).[8,10]

The SOD activity of the conjugate was determined by evaluating the ability of the conjugate to inhibit superoxide-dependent autoxidation of pyrogallol. The activity of the conjugate is equal to that of the native protein (Figure 6).

The ability of the Hb−SOD conjugate to resist superoxide-induced heme oxidation (forming methemoglobin, metHb) was equal to that provided by native SOD in solution with hemoglobin.

The CD spectrum of the Hb−SOD conjugate decreases near 260 nm upon deoxygenation. This change in the CD spectrum is normally associated with the change in the state of ligation of the hemes in hemoglobin. The development of a negative peak at 287 nm is indicative of the conformational change of the aromatic residues trytophan C2(37)β and tyrosine C7(42)β, which are located at the $\alpha 1\beta 2$ interface.[11] These results indicate that hemoglobin in the conjugate undergoes the R to T transition upon deoxygenation.

Discussion

The ability of the thiol and maleimide to join to create the double protein is clearly established by our results. By blocking the accessible native thiols, we can be assured that the connection has proceeded between the desired groups. Chemoselective ligation of two proteins occurs in an addition of the thiol to the maleimide group (Scheme 5).[12]

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(11) Perutz, M. F.; Ladner, J. E.; Simon, S. R.; Ho, C. *Biochemistry* **1974**, *13*, 2163-2173.

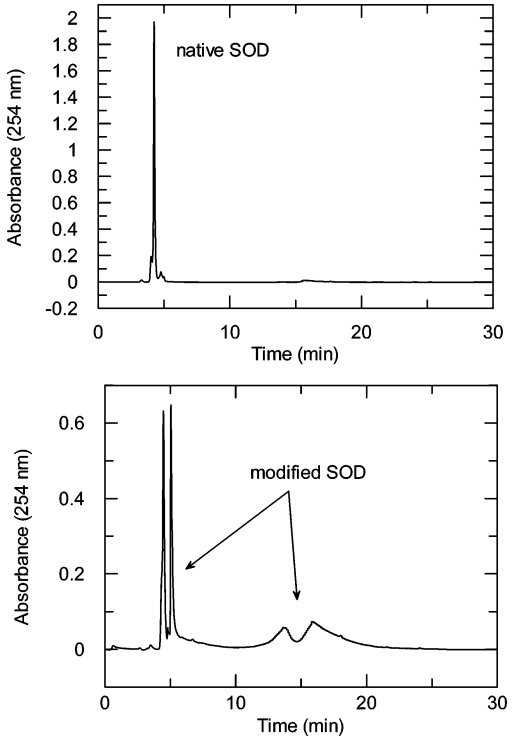


Figure 1. C4 reverse-phase HPLC of the native SOD (a) and of the products of the cross-linking reaction of SOD and **5** (b), monitored at 254 nm.

Conjugation of hemoglobin to SOD results in the formation of a thioether−succinimide bond that permanently links the two proteins. The succinimide amide linkage can undergo hydrolysis after conjugation. However, this reaction does not lead to the cleavage of the bond joining the two proteins.

Evaluation of functional properties of the conjugate indicated that upon conjugation each protein retained its normal function. SOD that is modified with **5** in the vicinity of its active site and conjugated to hemoglobin retains its activity. Thus, conjugation of the modified SOD to hemoglobin does not block the active site. The complex structure of the products and the analysis procedure do not give us a confident quantitative estimation of the yield.

Since superoxide promotes conversion of hemoglobin to the ferric heme form (metHb), the presence of SOD reduces the rate of this destructive process.[13] The autoxidation of hemoglobin occurs through the following reactions:

$$\text{Hb}-\text{Fe(II)} + \text{O}_2 \rightarrow \text{Hb}-\text{Fe(III)} + \text{O}_2^{\bullet -} \quad (1)$$

$$\text{Hb}-\text{Fe(II)} + \text{O}_2^{\bullet -} + 2\text{H}^+ \rightarrow \text{Hb}-\text{Fe(III)} + \text{H}_2\text{O}_2 \quad (2)$$

$$\text{Hb}-\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Hb}-\text{Fe(III)} + \text{HO}^- + \text{HO}^{\bullet} \quad (3)$$

$$\text{Hb}-\text{Fe(II)} + \text{HO}^{\bullet} \text{ f } \text{Hb}-\text{Fe(III)} + \text{HO}^- \quad (4)$$

Our results show that conjugating SOD to hemoglobin does improve the chemical stability of hemoglobin, as we observe a 45% decrease in the rate of metHb formation. This level of protection is likely due to the inhibition of the progress of reaction 2 above and also prevents dissociation. However, both spontaneous and enzyme-catalyzed dismutation of superoxide generates hydrogen peroxide, which contributes to reaction 3 above. This would explain why we observe about 50% metHb formation in the presence of superoxide dismutase alone. When hemoglobin is incubated with catalase (with no SOD), the rate of metHb formation is inhibited by over 80%. This suggests

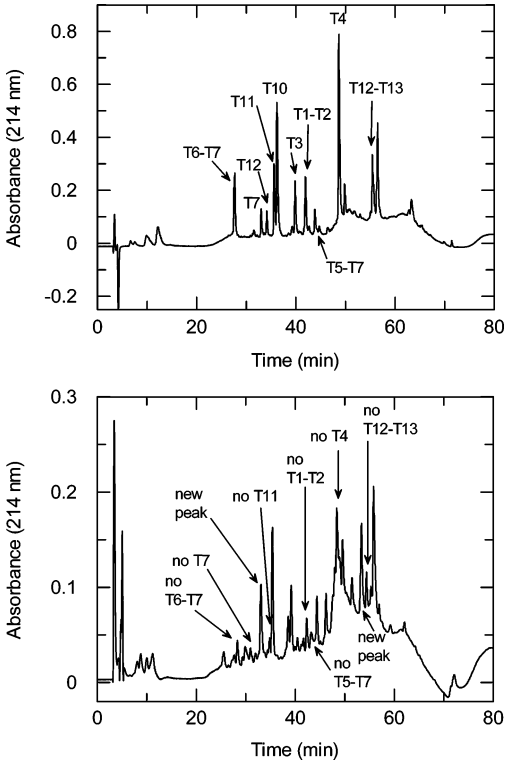


Figure 2. C18 reverse-phase HPLC chromatogram of the tryptic digest of (a) native SOD and (b) SOD that was treated with **5**.

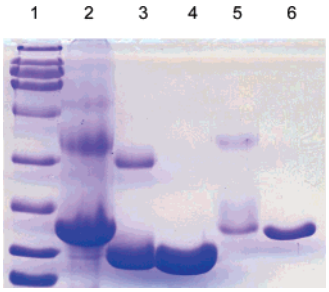


Figure 3. SDS-PAGE analysis of cross-linked protein species and coupling reaction between cross-linked Hb and cross-linked SOD: lane 1, molecular weight standards; lane 2, xlHb + xlSOD; lane 3, 1 + NEM−Hb; lane 4, NEM−Hb; lane 5, 5 + SOD; lane 6, nSOD.

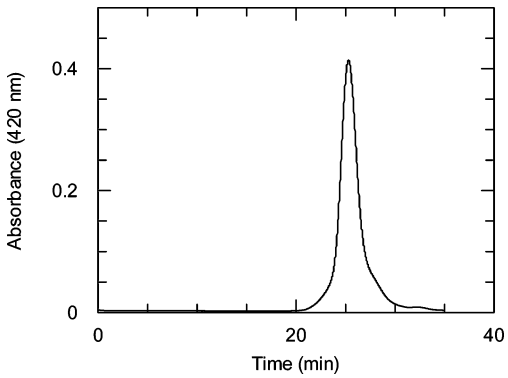


Figure 4. G75 size exclusion chromatogram of the purified conjugate.

(12) Mattson, G.; Conklin, E.; Desai, S.; Nielander, G.; Savage, M. D.; Morgensen, S. *Mol. Biol. Rep.* **1993**, *17*, 167−183.
(13) Winterbourn, C. C. *Methods Enzymol.* **1990**, *186*, 265−72.

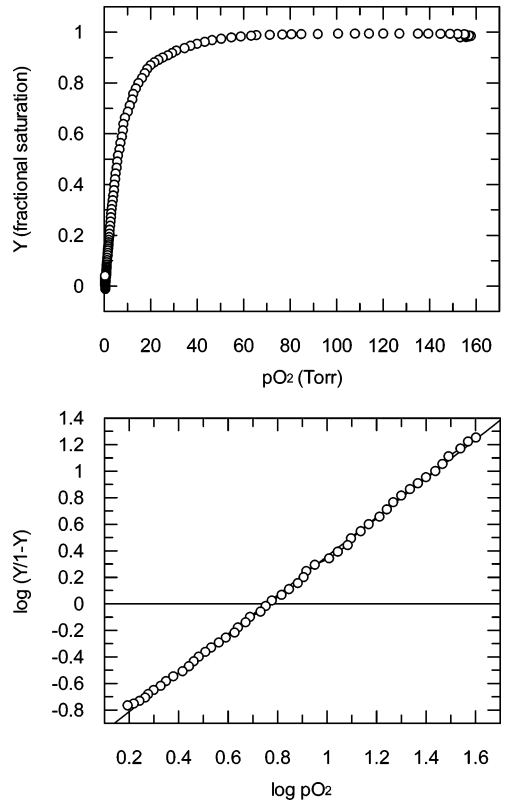


Figure 5. (a) Oxygen binding cruve for the hemoglobin−superoxide dismutase conjugate at 25 °C, pH 7.4, $I = 0.1$ (b) Hill plot for data in the oxygen-binding curve at the P_{50} region. The slope is the Hill coefficient (n_{50}).

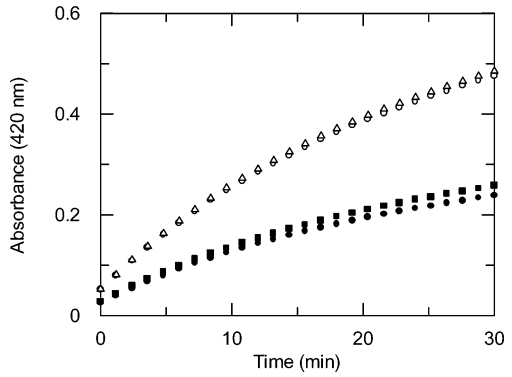
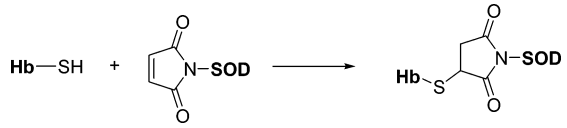


Figure 6. Inhibition of autoxidation of 4.0×10^{-4} M pyrogallol in the presence of Hb−SOD conjugate. Native SOD (●) and Hb−SOD (■) samples contain one unit of enzyme dimer (5.3×10^{-7} M). Control samples of pyrogallol (○) and cross-linked Hb (△) contain no SOD.

Scheme 5. Chemoselective Coupling of Hemoglobin to Superoxide Dismutase through Thiol−Maleimide Chemistry



that catalase inhibited the progress of reaction 3. Under our conditions, it appears that hydrogen peroxide radicals are more potent species in causing oxidative damage to hemoglobin than is the superoxide anion. The rate of metHb formation (about 12%) when catalase is used is probably due to the oxidative damage caused by prior dismutation to peroxide by superoxide anion. Full protection against oxidative damage (over 90%) is observed when both enzymes are present in the assay mixture, mimicking the antioxidant system that is present in the red cells. These findings are consistent with results of Yang and Olsen, who found that the inclusion of native superoxide dismutase inhibited metHb formation by about 50%, while the presence of catalase inhibited metHb formation by about 65%. They observed that oxidative damage was inhibited by about 80% when both antioxidant enzymes were present in the assay.[14]

The hemes derived from hemoglobin within the conjugate bind and release oxygen with about the same affinity as in the native protein; cooperativity is diminished (n_{50} is reduced) but not abolished. Our spectroscopic data suggest that hemoglobin in the conjugate undergoes a normal conformational change (R−T). The alterations of the protein's structure that arise from interactions of the heme upon binding of oxygen appear to be transmitted less efficiently. The general effect of SOD upon solvation of the attached hemoglobin could account for the observation. The enzymic activity of SOD is not affected by operation within the conjugate, indicating that substrate binding and stabilization of the transition state are not affected by the presence of the attached hemoglobin. Since the native protein does not show cooperativity, there is no change that could be expected to compare to that in hemoglobin. Our results indicate that protein−protein interactions within the hemoglobin−SOD conjugate affect the mechanism of allosteric effects without altering relative energy levels. By extension, transient contacts within the red cell would reduce the cooperativity of individual hemoglobins but the longer term and overall allosteric properties would remain.

Conclusions

We described an approach for generating protein−protein conjugates that relies on site-selective chemical modification and chemoselective coupling of proteins. The reagents **1** and **5** react selectively with the lysylamines of hemoglobin and superoxide dismutase, respectively, to generate structurally stabilized proteins. The thiol group on hemoglobin that is introduced by chemical modification serves as a point of attachment for a maleimide-containing superoxide dismutase. The resulting hemoglobin−superoxide dismutase conjugate displays both oxygen-carrying and antioxidant properties, demonstrating that the conjugation process does not abolish the activity of proteins. Successful ligation of superoxide dismutase and hemoglobin demonstrates that this approach should be useful in generating other protein-based materials. Since the material would be added in small quantities to a circulating system, the specific oxygen affinity should not affect the efficacy of the overall system. The need for catalase to be present in order to remove hydrogen peroxide generated by SOD remains an issue for further research. On the basis of the work of D'Agnillo and Chang,[5,6] it would appear to be an important goal.

Experimental Section

Materials. Syntheses were carried out using commercial reagents and anhydrous solvents. Buffers were prepared from doubly distilled deionized water. Synthesized materials were identified using a combination of NMR spectroscopy and high-resolution mass spectrometry.

(14) Yang, T.; Olsen, K. W. *Biochem. Biophys. Res. Commun.* **1989**, *163*, 733−738.

Proton NMR spectra were recorded at 200 or 300 MHz. Carbon NMR spectra were recorded at 75.6 or 100.8 MHz. Phosphorus NMR spectra were recorded at 120 MHz. Melting points are uncorrected. Protein concentrations were determined using a protein assay kit. Hemoglobin A was isolated from red cells donated by the corresponding author and purified. Superoxide dismutase and reagents were purchased. High performance liquid chromatography coupled to mass spectroscopy analysis, MS (ESI), was performed at the Mass Spectroscopy Laboratory at the Molecular Medicine Research Centre, University of Toronto.

4,4′-Dithiobisbenzoic Acid (2). This procedure is a modified version of the procedure described by Evans for preparation of aromatic disulfides.[15] 4-Mercaptobenzoic acid (0.53 g, 3.4×10^{-3} mol) and iodine (0.44 g, 1.7×10^{-3} mol) were dissolved in 10 mL of absolute ethanol. Triethylamine (1.5 mL, 10.2×10^{-4} mol) was added and the solution was left for 16 h. Excess iodine was removed by reduction with 10% sodium thiosulfate. The cloudy solution was concentrated and combined with 60 mL of 0.01 M hydrochloric acid. The white precipitate was collected and dried under vacuum. The product was recrystallized from *N*,*N*-dimethylacetamide and water (0.51 g, 98% yield). Mp: >300 °C. ^{1}H NMR (DMSO-d_6): δ 8.0 (d, 2H, J = 8.0 Hz, ArH); 7.7 (d, 2H, J = 8.1 Hz, ArH). ^{13}C NMR (DMSO-d_6): δ 166.6, 140.8, 130.3, 129.7, 126.1. MS (ESI negative): 306, calculated; 305, found for the singly deprotonated species.

***N*,*N*′-Diisophthalyl-4,4′-disulfanlydibenzamide (4).** **2** (0.43 g, 1.6×10^{-3} mol) was refluxed in excess thionyl chloride to generate 4,4′-dithiobisibenzoyl dichloride (**3**). This was combined with 5-aminoisophthalic acid (0.72 g, 4.0×10^{-3} mol) in 20 mL of *N*,*N*-dimethylacetamide and mixed with 4-(dimethylamino)pyridine (0.05 g, 0.4×10^{-4} mol) according to the general method of Aharoni and Edwards.[16] The reaction proceeded for 16 h under nitrogen and was quenched with water (50 mL), producing a white precipitate. The flask was placed in an ice bath for 2 h and the solid was collected by vacuum filtration. The solid was washed with hot methanol then with hot water and dried under vacuum. This was recrystallized from hot *N*,*N*-dimethylformamide and water, followed by washing with diethyl ether to give the product (0.85 g, 85% yield), **4**. Mp: 295 °C (dec). ^{1}H NMR (DMSO-d_6): δ 10.6 (s, 2H, CONH), 8.6 (s, 4H, ArH), 8.2 (s, 2H, ArH), 8.0 (d, 4H, J = 8.4 Hz, ArH), 7.7 (d, 4H, J = 8.4 Hz, ArH). ^{13}C NMR (DMSO-d_6): δ 166.5, 165.0, 139.7, 133.3, 131.7, 128.9, 126.4, 125.1, 124.7. MS (ESI nagative): 634 (calculated), 632 (for the doubly deprotonated species).

***N*,*N*′-Bis[bis(sodium methyl phosphate)isophthalyl)-4,4′-disulfanlydibenzamide (1).** The tetraacid chloride of **4** was prepared by refluxing **4** (0.27 g, 3.8×10^{-4} mol) in excess thionyl chloride for 4 h. This was converted to the tetrakis dimethyl phosphate. The solution was stirred for 16 h in dry tetrahydrofuran under nitrogen with 0.28 g (1.9×10^{-3} mol) of sodium dimethyl phosphate (prepared in dry acetone from sodium iodide and trimethyl phosphate[17]). The solvent was removed in vacuo, leaving a yellow oil (0.46 g, 4.3×10^{-4} mol). This was dissolved in dry acetone and mixed with 0.39 g (2.6×10^{-3} mol) of sodium iodide in dry acetone to remove one methyl group from each phosphate. The yellow solution was left overnight. A pale yellow precipitate was collected and washed with dry acetone to give **1** (0.23 g, 56% yield). Mp: 250 °C (dec); IR (KBr): C=O 1717 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 10.7 (s, 2H, CONH), 8.7 (s, 4H, ArH), 8.2 (s, 2H, ArH), 8.0 (d, 4H, J = 6.8 Hz, ArH), 7.7 (d, 4H, J = 8.4 Hz, ArH), 3.4 (d, 12H, J^P = 7.0 Hz, OCH3). ^{13}C NMR (DMSO-d_6): δ 166.5, 165.1, 139.8, 131.7, 130.1, 128.9, 126.3, 125.0, 124.6, 52.6 (OCH_3). ^{31}P NMR (DMSO-d_6): δ −5.9 (decoupled).

4-Maleimidobenzoic Acid. 4-Aminobenzoic acid (2.0 g, 1.5×10^{-2} mol) and maleic acid (1.7 g, 1.5×10^{-2} mol) were dissolved in 30 mL of dioxane and refluxed for 18 h in the presence of phosphorus pentoxide (3.0 g, 2.2×10^{-2} mmol).[18] The reaction mixture was then filtered while hot. The cooled filtrate was poured over ice to precipitate the product, which was collected by vacuum filtration. A second crop of crystals was obtained by concentrating the filtrate. The product was washed with cold water and dried under vacuum (2.9 g, 94% yield). Mp: 193−195 °C. ^{1}H NMR (DMSO-d_6): δ 8.0 (d, 2H, J = 8.6 Hz, ArH), 7.5 (d, 2H, J = 8.5 Hz, ArH), 7.2 (s, 2H, CH). ^{13}C NMR (DMSO-d_6): δ 169.5, 166.7, 135.5, 134.9, 129.9, 129.5, 126.1, MS (ESI negative): 217 (calculated), 216 (found for singly deprotonated species).

5-(4-(2,5-Dioxo-2*H*-pyrrol-1(5)-yl)benzamido)isophthalic Acid (6). 4-Maleimidobenzoic acid (0.27 g, 1.2×10^{-3} mol) was converted to the acid chloride (Scheme 4) by refluxing in excess thionyl chloride for 12 h.[19] Excess thionyl chloride was removed in vacuo to give 4-maleimidobenzoyl chloride (0.28 g, 1.2×10^{-3} mol). The acid chloride (0.28 g, 1.2×10^{-3} mol) was dissolved in 5 mL of *N*,*N*-dimethylacetamide with 4-(dimethylamino)pyridine (0.02 g, 2.0×10^{-4} mol) and mixed with 5-aminoisophthalic acid (0.22 g, 1.4×10^{-3} mol) dissolved in 10 mL of *N*,*N*-dimethylacetamide. The reaction proceeded for 48 h under nitrogen. The reaction was terminated by addition of 100 mL of water. The white precipitate, **6**, was collected by vacuum filtration, washed with cold acetone and 95% ethanol (3 × 10 mL), and dried under vacuum (0.36 g, 90% yield). Mp: >250 °C. ^{1}H NMR (DMSO-d_6): δ 10.7 (s, 1H, CONH), 8.7 (s, 3H, ArH), 8.2 (s, 1H, NH), 8.1 (d, 2H, J = 7.9 Hz, ArH), 7.6 (d, 2H, J = 7.2 Hz, ArH), 7.2 (s, 2H, CH). ^{13}C NMR (DMSO-d_6): δ 169.6, 166.6, 165.2, 139.8, 134.8, 134.6, 133.3, 131.7, 128.4, 126.2, 125.0, 124.7. MS (ESI negative): 380 (calculated), 379 (singly deprotonated).

Compound 7. The dichloride of **6** (0.21 g, 5.1×10^{-4} mol) was combined with 2 equiv of aminoisophthalic acid (0.17 g, 1.1×10^{-3} mol) and 4-(dimethylamino)pyridine (0.04 g, 3.3×10^{-4} mol) in 10 mL of *N*,*N*-dimethylacetamide under nitrogen for 48 h. The triamide product, **7**, was precipitated by addition of 250 mL of water. The mixture was kept at 4 °C for 2 h, and the product was collected by vacuum filtration. This was washed with 2-propanol, diethyl ether, and hot methanol (3 × 30 mL, each) and dried under vacuum (0.27 g, 76% yield). Mp: >250 °C. ^{1}H NMR (DMSO-d_6): δ 10.8 (s, 3H, CONH), 8.7 (s, 4H, ArH), 8.6 (s, 2H, ArH), 8.4 (s, 1H, ArH), 8.2 (s, 2H, ArH), 8.1 (d, 2H, J = 7.7 Hz, ArH), 7.6 (d, 2H, J = 7.7 Hz, ArH), 7.2 (s, 2H, CH). ^{13}C NMR (DMSO-d_6): δ 170.3, 167.2, 166.2, 166.0, 140.4, 140.3, 136.0, 135.6, 135.4, 134.0, 132.5, 129.1, 126.9, 125.9, 125.4, 123.7, 122.7. MS (ESI): 706 (calculated), 705 (singly deprotonated species).

Compound 8. The dichloride of **7** (0.04 g, 5.0×10^{-5} mol) was prepared by refluxing in excess thionyl chloride. This was combined with sodium dimethyl phosphate (0.06 g, 4.0×10^{-4} mol, prepared in dry acetone from sodium iodide and trimethyl phosphate) in dry tetrhydrofuran and mixed for 48 h under nitrogen. The mixture was filtered and solvent removed from the filtrate, producing the bis dimethyl phosphate in Scheme 4 as a brown oil (0.1 g, 1.4×10^{-4} mol). This was dissolved in dry acetone and mixed with sodium iodide (0.13 g, 8.7×10^{-4} mol) to give bis methyl phosphate, **5**. The reaction proceeded under nitrogen for 18 h, and the pale yellow precipitate was collected by vacuum filtration. The product was washed with cold dry acetone and dried (0.1 g, 60% yield). Mp: >250 °C. ^{1}H NMR (DMSO-d_6): δ 11.12 (s, 3H, NH), 8.8 (s, 4H, ArH), 8.7 (s, 2H, ArH), 8.4 (s, 1H, ArH), 8.2 (s, 2H, ArH), 8.0 (d, 2H, J = 6.6 Hz, ArH), 7.6 (d, 2H, J = 7.0 Hz, ArH), 7.2 (s, 2H, CH), 3.4 (d, 12H, J^P = 10.8 Hz, OCH_3). ^{13}C NMR (DMSO-d_6): δ 169.6, 166.4, 165.1, 139.9, 139.7, 134.8, 134.6, 133.2, 131.8, 131.7, 128.5, 126.1, 125.1, 124.7, 123.2, 122.3, 52.6. ^{31}P NMR (DMSO-d_6): δ −5.7 (decoupled).

Reaction of Hemoglobin with *N*-Ethyl Maleimide (NEM). Carbonmonoxy hemoglobin (2.0 mL, 3.0×10^{-6} mol) in 0.02 M phosphate,

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0.15 M NaCl, 0.01 M EDTA, pH 7.0 was combined with 5 mol equiv of *N*-ethyl maleimide (0.002 g). The reaction proceeded at room temperature for 1 h with stirring. The solution was then passed through a column of Sephadex G25 (equilibrated with 0.05 M sodium borate buffer, pH 8.0) to remove residual reagent. The NEM-modifed hemoglobin (NEM−Hb) was kept on ice.

Cross-Linking NEM−Hemoglobin with 1. Carbonmonoxy NEM−Hb (2.0 mL, 3.0×10^{-6} mol) was converted to the oxy form by light from a tungsten lamp under a stream of humidified oxygen (2 h). The sample was then placed under a stream of humidified nitrogen for 2 h, converting it to the deoxy form. Cross-linking reagent **1** (6.6 mg, 6×10^{-6} mol) was added to the deoxy NEM−Hb solution as a solid with solution under nitrogen. The reaction proceeded for 16 h at 37 °C. The reaction mixture was then placed under a stream of humidified carbon monoxide for 10 min. Excess cross-linking reagent was removed by passing the sample through a Sephadex G-25 column that had been equilibrated with 0.l M, pH 7.2 MOPS.

Reaction of Cross-Linked Hemoglobin Products with TCEP. Cross-linked carbonmonoxy NEM−hemoglobin (3.0×10^{-6} mol) was passed through a Sephadex G25 column equilibrated with 25×10^{-3} M Tris-HCl, 0.5 M magnesium chloride pH 7.4 buffer. The sample was kept at 4 °C for 18 h and then passed through a column of Sephadex G100, equilibrated with 2.5×10^{-2} M, pH 7.4 Tris buffer containing 0.5 M magnesium chloride. Fractions containing cross-linked hemoglobin tetramers were collected, pooled, and passed through a Sephadex G25 column equilibrated with 20×10^{-3} M, pH 7.2 MOPS. The sample was then passed through a deionizing resin to remove magnesium chloride. Following the elution of the sample with water, the sample was passed through the column equilibrated with TCEP to cleave the disulfide bond of **1** [20−22] (Sephadex G25 column equilibrated with 20×10^{-3} M MOPS, 2×10^{-3} M TCEP pH 6.8 buffer). The sample was kept at 4 °C for 18 h and then passed through a Sephadex G100 column equilibrated with 2×10^{-2} M, pH 6.8 MOPS containing 2×10^{-3} M TCEP (tris(2-carboxyethyl)phosphine). Fractions containing cross-linked hemoglobin activated for conjugation (HbSH species) were collected and concentrated. The purity of the sample was assessed by HPLC. The sample was stored at −80 °C.

Cross-Linking Superoxide Dismutase with 5. Superoxide dismutase from bovine erythrocytes (3.0 mg, 9.6×10^{-8} mol) was dissolved in 1.0 mL of 0.01 M, pH 7.2 phosphate. Cross-linking reagent **5** (0.5 mg, 3.8×10^{-7} mol) was added to the enzyme solution as a solid. Reaction proceeded for 1 h at room temperature with stirring. Excess reagent was removed by selective precipitation of the enzyme with 2 mL of cold acetone.[23] The precipitated enzyme was collected by centrifugation at 5000*g* for 15 min at 4 °C. The pellet was resuspended in 0.01 M phosphate buffer, pH 7.0.

Coupling Cross-Linked Hb to Cross-Linked SOD. Cross-linked SOD (9.6×10^{-8} mol) and 0.5 equiv of CO−HbSH (4.8×10^{-8} mol), prepared as described above, were mixed in 2×10^{-2} M MOPS, 2×10^{-3} M TCEP pH 6.8 and kept for 2 h at room temperature and then at 4 °C for 18 h. The solution was passed through a column of Sephadex G100 that had been equilibrated with the same buffer. The first-eluting fractions were collected, pooled, and concentrated until the sample eluted as a single peak on a G75 Superdex HR 10 × 30 gel filtration column under partially dissociating conditions for hemoglobin (25×10^{-2} M Tris-HCl, 0.5 M magnesium chloride, pH 7.4).[24] The sample was then dialyzed against phosphate buffer, $I = 0.1$, pH 7.4, using a 100 kDa membrane.

Chromatographic Analysis of Hemoglobin Species. The structures of modified hemoglobins were analyzed by the method of Jones.[25] Analytical reverse-phase HPLC using a 330 Å pore C4 Vydac column (250 × 4.6 mm) was used to monitor the extent of globin chain modification in the reaction with **1**. Modified and native globin chains were separated and isolated by reverse-phase HPLC using a preparative 330-Å pore C4 Vydac column (250 × 12 mm). The solvent gradients were 49% A (20% acetonitrile in water, 0.1% TFA) and 51% B (60% acetonitrile in water, 0.1% TFA) from 0.5 to 60.5 min, 35% A and 65% B from 60.5 to 80.5 min, 14% A and 86% B from 80.5 to 90.5, and 100% B from 95 to 100 min. The effluent was monitored at 220 nm. The isolated species were analyzed by size exclusion chromatography on Superdex G75 HR (gel filtration). The column was calibrated using molecular weight standards: blue dextran (MW 2 000 000), β-amylase (MW 206 000), yeast alcohol dehydrogenase (MW 141 000), BSA (MW 66 000), and carbonic anhydrase (MW 32 000). The effluent was monitored at 280 and 414 nm.

The modification of SOD was analyzed by HPLC using a C4 Vydac reverse-phase column[26] with 330-Å pore size (250 × 4.6 mm) and solvent gradients starting with 75% A (0.1% H_3PO_4 in water) and ending with 100% B (95% acetonitrile, 0.1% H_3PO_4 in water) in 15 min. Chemically modified SOD was isolated by C4 reversed phase or by analytical anion exchange AX300 HPLC using solvent gradients starting with 100% A (15×10^{-3} M Tris, pH 8.0) and ending with 100% B (15×10^{-3} M Tris, 0.15 M NaOAc, pH 8.0) in 30 min.

Molecular Weight Determination of Hemoglobin Species. SDS-PAGE analysis was used for the reaction of hemoglobin with **1** and SOD with **5**, as well as with the coupling reaction between cross-linked NEM−SOD dismutase and cross-linked (SH) hemoglobin. Samples were prepared for SDS-PAGE analysis by a procedure modified from that of Malinowski and Fridovich.[27] A sample of protein ($\sim(2-5) \times 10^{-6}$ g/mL) was prepared in a loading buffer of 0.01 M, pH 7.1 phosphate, 6.0 M urea, 1.0% SDS, 0.05 M EDTA, 1.0% glycerol, 1.0% β-mercaptoethanol, and 0.5% bromophenol blue. The samples were placed in boiling water for 10 min and then loaded onto polyacrylamide gels (separating gel, 12.5% acrylamide; stacking gel, 5% acrylamide in Tris-HCl) followed by electrophoresis in a dual-slab cell apparatus at 200 mV. The gels were stained with 0.1% Coomassie Blue R-250, scanned, and analyzed.

Enzymatic Hydrolysis of Native and Cross-Linked Hemoglobin β Chains. Native and modified β globin chains were separated using a preparative 330-Å Vydac C4 reverse-phase column (250 × 12 mm) and lyophilized. Approximately 0.5 mg of globin chains was dissolved in 0.1 mL of 8 M urea and mixed for 4 h at room temperature. TPCK-treated trypsin solution (4% of globin weight used) in 0.8 ammonium bicarbonate buffer (pH 8.5) was then added. The resulting sample was diluted with the bicarbonate buffer to a final concentration of 2 M urea. The sample was kept at room temperature for 24 h. It was then placed in a boiling water bath for 2 min and cooled to room temperature. This was mixed with a solution of endoproteinase Glu-C from *Staphylococcus aureus* (2% of globin weight used), followed by addition of 0.8 M ammonium bicarbonate to 0.8 mL total volume. The sample was then left at room temperature for 72 h. The hydrolysates were filtered through an 0.45 μm filter before injecting onto the C18 HPLC column.

Peptide fragments were separated by reverse-phase HPLC using a Vydac C18 column (93 × 4.7 mm) and eluted using a gradient starting with 100% in water with 0.1% TFA, and ending with 100% acetonitrile with 0.1% TFA, in a modification of the method described by Shelton.[28] The effluent was monitored at 214 and 280 nm.

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Enzymatic Hydrolysis of Native and Cross-Linked SOD. Lyophilized protein (approximately 3.4 mg) was dissolved in 8 M urea. The sample was placed in a boiling water bath for 10 min and then cooled to room temperature, followed by the dilution with 0.8 M ammonium bicarbonate buffer (pH 8.5) to the final urea concentration of 2 M. TPCK-treated trypsin solution (4% of protein weight used) was then added. This was incubated at room temperature for 24 h. The sample was then heated in a boiling water bath for 2 min and then allowed to cool to room temperature. The hydrolysates were filtered through a 0.45 μm filter before injecting onto the C18 column.

Analysis of Oxygen-Binding/Releasing Properties of Hemoglobin-Containing Species. Oxygen-binding/releasing measurements were conducted using a modified version of the method described by Imai.[29] A sample of a hemoglobin-containing species in phosphate buffer (I = 0.1 M ionic strength, pH 7.4) was irradiated with visible light under humidified oxygen on ice for 2 h, followed by addition of catalase solution (0.8×10^{-3} g/mL) and equilibration of the sample at room temperature for 1 h. Absorbance was recorded at 560 nm. The sample was deoxygenated or oxygenated at 25 °C under humidified nitrogen or air, respectively. Data analysis was performed by converting the observed absorbance to a partial saturation (Y):

$$Y = (A_{obsd} - A_{oxy})/(A_{oxy} - A_{deoxy})$$

The apparatus for determining oxygen-binding properties was contained in the sample compartment of a computer-interfaced UV−vis spectrophotometer. The apparatus also contains an amperometric oxygen probe connected to an oxygen monitor whose output is digitized and connected to the same computer. The flow of pure gases from reagent tanks was controlled by a high-accuracy flow meter. The computer was programmed to display and record absorbance and oxygen concentration simultaneously. Gas mixtures were varied steadily by adjusting the flow valve and tank pressures.

Assay of SOD Activity and Its Protection of Hemoglobin. This was done by the method described by Marklund.[30] The assay measures the ability of enzyme to inhibit autoxidation of 1,2,3-trihydroxybenzene (pyrogallol), which can be followed at 420 nm. Solutions of superoxide dismutase and pyrogallol were prepared in a pH 8.2 buffer of 0.05 M Tris-cacodylic acid containing 1.0×10^{-3} M diethylenetriaminepentaacetic acid. The enzymatic reaction was initiated by addition of pyrogallol.

The autoxidation of pyrogallol was used as a source of superoxide anion radical, and its effects upon hemoglobin within the conjugate were monitored. The final concentrations of protein and pyrogallol in the assay mixture were 5×10^{-5} and 2×10^{-4} M, respectively. All measurements were conducted in air-equilibrated, pH 8.2, 5.0×10^{-2} M Tris-cacodylic acid, 1.0×10^{-3} M diethylenetriaminepentaacetic acid buffer at 20 °C. The reaction was initiated by the addition of pyrogallol to the hemoglobin solution. The formation of metHb from oxyHb was followed at 630 nm.

Circular Dichroism Analysis. The deoxygenated conjugate was prepared in pH 7.2, 1.0×10^{-2} M phosphate buffer by passing humidified nitrogen over the oxyhemoglobin−SOD conjugate for 4 h at 37 °C. The oxygenated conjugate was prepared from the deoxygenated conjugate by exposing the sample to a stream of humidified oxygen for 15 min. Spectra were recorded with a computer-interfaced spectropolarimeter in a quartz cuvette.

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Supporting Information Available: A table of peptides of the digest of native superoxide dismutase. This material is available free of charge via the Internet at http://pubs.acs.org.

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