# Functional Comparison of Specifically Cross-Linked Hemoglobins Biased Toward the R and T States

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ABSTRACT Selected functional and spectroscopic properties of two human hemoglobin ($HbA_0$) derivatives that were site-specifically cross-linked in the cleft between $\beta$-chains where 2,3-bisphosphoglycerate normally binds have been determined to assess the effects of the cross-linking on the behavior of the protein. Trimesoyl tris(3,5-dibromosalicylate) (TTDS) cross-links Hb between $\beta$82Lys residues. The resulting TTDS-Hb exhibits a slower rate of oxygen dissociation and an increased rate of carbon monoxide association than observed for $HbA_0$. The electron paramagnetic resonance (EPR) spectrum of TTDS-HbNO does not exhibit the hyperfine structure that is indicative of significant conformational change despite the fact that the 2,3-bisphosphoglycerate binding site is occupied by the cross-linking reagent. The reactivity of the $\beta$93Cys residues of TTDS-Hb is only slightly decreased relative to that of $HbA_0$. On the other hand, cross-linking Hb between Lys82 and the amino-terminal $\beta$1Val group with trimesoyl tris(methyl phosphate) (TMMP) increases the rate of oxygen dissociation and reduces the rate of CO association relative to the rates observed for $HbA_0$. In addition, the EPR spectrum of the TMMP-HbNO exhibits the three-line hyperfine structure that results from disruption of the proximal His-Fe bond of the $\alpha$-chains, and the accessibility of the $\beta$Cys93 residues in this derivative is decreased fourfold. The present results are consistent with the conclusion that the quaternary structure of TTDS-Hb is shifted toward the R state whereas the quaternary structure of TMMP-Hb is shifted toward the T state and provides additional evidence that the identity of the residues involved in intramolecular cross-linking of hemoglobin within the 2,3-bisphosphoglycerate binding site between $\beta$-chains can have a significant influence on the conformational and functional properties of the protein.

## INTRODUCTION

The development of site-specific intramolecular cross-linking reagents for the modification of hemoglobin has provided a new and informative means of evaluating the functional and spectroscopic consequences of the conformational change of hemoglobin that occurs upon ligand binding (Winslow, 1992). Modifications of this type are also advantageous for the development of hemoglobin-based blood substitutes because they prevent the dissociation of hemoglobin tetramers into dimers (Bunn and Jandl, 1968), and they afford the ability to vary the affinity and cooperativity of ligand binding in a relatively systematic fashion (Benesch and Benesch, 1981). Specificity of hemoglobin cross-linking has been achieved through use of polyanionic cross-linking reagents that are recognized by the specific cationic binding loci in hemoglobin. For example, various aspirin derivatives have been used for cross-linking hemoglobins through the $\alpha$-subunits at position $\alpha$Lys99 (Vandegriff et al., 1989; Snyder et al., 1987) or through the $\beta$-subunits at position $\beta$Lys82 (Walder et al., 1979; Zhang and Olsen, 1994). Organic phosphates have been shown to secure a linkage between the amino-terminal groups of the $\beta$-chains (Benesch et al., 1972). Although cross-linking between $\beta$-chains generally increases the oxygen affinity of hemoglobin, carboxymethylation of the $CO_2$ binding sites at the $\beta$-chain amino termini decreases oxygen affinity (Fantl et al., 1987). Cross-linking has also been shown to influence the functional properties of hemoglobin by blocking the amino acid side-chains responsible for the chloride and/or Bohr effects (Manning, 1991)

Of particular interest, Kluger and co-workers (1992a,b) have recently developed bifunctional cross-linking reagents such as trimesoyl tris(3,5-dibromosalicylate) (TTDS) and trimesoyl tris(methyl phosphate) (TMMP) to react specifically with amino acid residues at the interface between the two $\beta$-chains that are normally involved in binding allosteric effectors such as 2,3-bisphosphoglycerate (Fig. 1). As established by these investigators, TTDS preferentially cross-links $\beta$82Lys-$\beta$82′Lys, and TMMP preferentially cross-links the $\epsilon$-amino group of $\beta$82Lys with the $\alpha$-amino group of $\beta$1′Val. Interestingly, these initial experiments indicate that cross-linking of hemoglobin with TTDS increases the affinity of the protein for oxygen whereas cross-linking with TMMP decreases the affinity for oxygen. Related cross-linkers exhibit a systematic and intermediate influence on the oxygen-binding properties of hemoglobin in which the relative affinity for oxygen and the cooperativity of oxygen binding vary with the length of the cross-linking reagent introduced at the interface of the two $\beta$-chains (Kluger et al., 1996).

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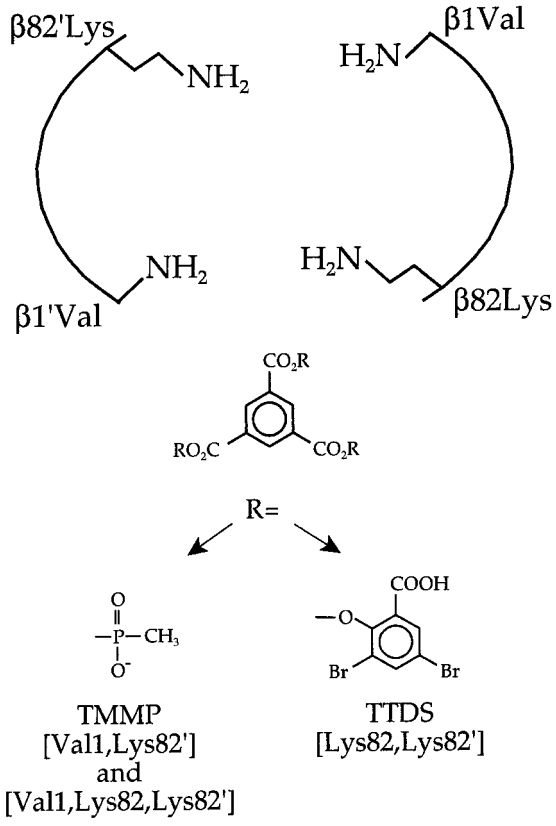


FIGURE 1 Bisphosphoglycerate binding site of human hemoglobin highlighting the amino acid residues involved in the regioselective cross-linking. The substituents of the trimesic acid linker are shown with their respective products.

To evaluate the functional consequences of these cross-linking reagents further, we have evaluated the effect of cross-linking human hemoglobin with TTDS and TMMP on several conformationally-linked functional and spectroscopic properties of the protein. The results indicate that although these reagents modify the same general region of hemoglobin, the modified hemoglobins they produce are functionally inequivalent and that these derivatives provide useful new structural probes of hemoglobin function.

## MATERIALS AND METHODS

### Protein purification and modification

Human $HbA_0$ was purified from out-dated blood bank blood in highly purified form by anion and cation exchange chromatography at Hemosol, Inc. Cross-linking of $HbA_0$ with trimesoyl tris(3,5-dibromosalicylate) (TTDS) (Kluger et al., 1992a) and with trimesoyl tris(methyl phosphate) (TMMP) (Kluger et al., 1992b) was performed by methods similar to those described previously. Purification of the cross-linked products was achieved by modification of the method described by Kluger et al. (1992b) for purification of TMMP cross-linked hemoglobin. TMMP-Hb was a 2:1 mixture of Hb cross-linked through two sites (Val1 and Lys82′) or three sites (Val1, Lys82, and Lys82′). These two adducts exhibit identical three-dimensional structures as determined by x-ray diffraction analysis (Schumacher et al., 1995) and identical oxygen-binding properties (Kluger et al., 1992b; Jones et al., 1993), so they can be regarded as identical to each other for current purposes. Hemoglobin concentrations were determined from the molar absorptivities tabulated by Antonini and Brunori (1971).

### Autoxidation kinetics

Autoxidation of oxyhemoglobin (20 $\mu$M heme; >95% $HbO_2$) in 50 mM bis(2-hydroxyethyl)imino tris(hydroxymethyl)methane (bis/Tris) buffer (pH 7.0, 37°C) was monitored with a Cary model 3 spectrophotometer equipped with a jacketed cell holder and a Lauda model RC3 circulating water bath. Protein samples were placed in a sealed cuvette, and spectra were collected at defined time intervals under computer control. The resulting data were analyzed by singular value decomposition (Hendler and Shrager, 1994; Cashon and Alayash, 1995) with the program SPECFIT (Spectrum Software Associates, Chapel Hill, NC) to obtain a spectral matrix to reduce baseline drift effects. The absorption changes were fit to a single exponential function by nonlinear least-squares analysis to obtain rate constants. Each rate constant was derived from the average of six independent measurements.

### Oxygen-binding measurements

Oxygen-binding affinity was measured (37°C, pH 7.4) with a modified TCS Hemox OEC analyzer (TCS Scientific, New Hope, PA) that measures the partial pressure of oxygen polarographically and the oxygenation status of hemoglobin spectrophotometrically. Responses of the Clark oxygen electrode (YSI, Yellow Springs, OH) and spectrophotometer were acquired by a personal computer with an A/D converter. Before analysis, carbonylhemoglobin samples were converted to oxyhemoglobin by illumination under a stream of pure oxygen. The oxyhemoglobin samples were then diluted to 0.1 g/dl into a buffer (37°C) consisting of 4 ml of Hemox solution (135 mM NaCl, 30 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid, 5 mM KCl, pH 7.4, at 37°C), 10 $\mu$l of anti-foaming agent, and 10 $\mu$l of stabilizing agent (0.001% hexamethylphosphoramide) (TCS Scientific). The oxygenated derivative was deoxygenated by bubbling nitrogen through the sample until the $P_{O2}$ reached a minimum value. The $P_{50}$ ($\pm$2 mm Hg) and Hill coefficient ($n_H$ $\pm$0.1) were determined from Hill plots constructed from data collected during deoxygenation of oxyhemoglobin.

### Oxygen dissociation kinetics

Rate constants for dissociation of oxygen from oxyhemoglobin derivatives were determined with an OLIS RSM-1000 rapid-scanning stopped-flow spectrophotometer (OLIS, Bogart, GA). Oxyhemoglobin solutions (20 $\mu$M in heme) prepared in bis/Tris buffer (50 mM, pH 7.4) were deoxygenated by mixing with anaerobically prepared sodium dithionite solution (concentration after mixing was 1.5 mg/ml), and the absorbance changes in the 400–630-nm region were monitored. For each measurement, 400 spectra were recorded, analyzed by singular value decomposition and fit to a single exponential function by the method of least squares with software provided by OLIS. Rate constants were determined from the average of five data sets.

### Carbon monoxide association kinetics

Carbonylhemoglobin (5 $\mu$M heme) solutions (50 mM bis/Tris buffer, pH 7.4) were prepared in an anaerobic quartz cuvette with a dual manifold vacuum line by thorough degassing of a solution of oxyhemoglobin followed by exposure to CO gas. The resulting sample was placed in the water-jacketed cell holder of a flash photolysis apparatus (OLIS) with a design similar to that of the laser photolysis system described by Sawicki and Morris (1981). This instrument employs a Phase-R DL1200 flashlamp pumped dye laser (now LumenX, New Durham, NH) and an ethanol solution of rhodamine dye (Allied Chemicals, Morristown, NJ). The rebinding of CO after photodissociation was monitored by the change in

absorbance at 436 nm; the resulting data were fit to the sum of two independent first-order rate functions by the method of least squares with the program SCIENTIST (MicroMath, Orem, UT).

### Electron paramagnetic resonance (EPR) spectroscopy

A dual-manifold vacuum line and anaerobic cuvette assembly were used to prepare solutions of nitrosylhemoglobin by anaerobic exposure of deoxygenated hemoglobin (1 mM in heme, 50 mM bis/Tris buffer, pH 7.0) to NO gas that had been passed through a degassed column (24 × 2.5 cm) of potassium hydroxide pellets. The resulting nitrosylhemoglobin solutions were transferred to anaerobic EPR tubes for acquisition of EPR spectra at 77 K. EPR spectra were obtained at X-band frequencies (~9.5 GHz) with a Bruker model ESP 300E spectrometer equipped with a Hewlett-Packard model 5352B frequency counter and a finger dewar.

### Sulfhydryl reactivity

The reactivity of the $\beta$93Cys residue in native and cross-linked forms of hemoglobin was studied by monitoring the reaction of these proteins with 4,4′-dithiodipyridine (DTDP) (Aldrich Chemical Co., Milwaukee, WI) (Taketa and Morell, 1969). In these measurements, the protein solution (2.5 ml, 10 $\mu$M in heme) in bis/Tris buffer (50 mM, pH 7.0) or *N*-tris(hydroxyethyl)methyl-3-aminopropane sulfonic acid buffer (50 mM, pH 8.4) was mixed with 0.2 ml of DTDP solution (12.5 mM in the same buffer as the protein). For reactions involving deoxy-Hb, a dual-manifold vacuum line was used to deoxygenate the samples in a gas-tight cuvette by exposure to a vacuum and then flushing with purified nitrogen gas. The reaction of deoxy-Hb samples was initiated by addition of nitrogen-purged DTDP with a gas-tight syringe. The initial pseudo-first-order rate process monitored at 325 nm comprised >85–90% of the total absorption change. The total number of thiol groups reacted was determined from a molar absorptivity for DTDP of $\Delta\epsilon_{325\ nm} = 1.98 \times 10^4\ M^{-1}$ (Grassetti and Murray, 1967).

## RESULTS

### Autoxidation kinetics

The rate of autoxidation in the absence of added anions was determined for three forms of Hb (Table 1). Under these experimental conditions, $HbA_0$ autoxidizes in a first-order manner with a rate constant of 0.12 $h^{-1}$. The corresponding rate constant for autoxidation of TTDS-Hb ($k$ = 0.078(5) $h^{-1}$) and TMMP-Hb ($k$ = 0.062(5) $h^{-1}$) were both significantly smaller than that of the native protein.

### Oxygen affinity and dissociation

The affinities of the cross-linked hemoglobins for oxygen determined at equilibrium are shown in Table 1. These results obtained at 37°C are consistent with those reported by Kluger and co-workers (1992b) at 25°C. The kinetics of oxygen dissociation from native and cross-linked hemoglobins was studied by monitoring the reaction of the corresponding oxygenated derivatives with excess sodium dithionite by stopped-flow spectrophotometry. A representative reaction trace illustrating the spectroscopic changes that occur between 400 and 630 nm (20-ms intervals) is provided in Fig. 2. The rate constants for dissociation of oxygen (Table 2) correlate with the oxygen-binding affinities (expressed as $P_{50}$ (mm Hg)) if it is assumed that cross-linking has no effect on the rate constant for binding of oxygen to the modified hemoglobins. As indicated by these results, a slight decrease is observed in the rate constant for oxygen dissociation from TTDS-Hb ($P_{50}$ = 13) and a ~2-fold decrease is observed for those of TMMP-Hb ($P_{50}$ = 35) relative to those of $HbA_0$ ($P_{50}$ = 14). The temperature dependences of these rate constants was also determined (Fig. 2, inset), and linear least-squares fits of these data to the Arrhenius relationship provided the following activation enthalpies: $HbA_0$, 22.6(4) kJ $mol^{-1}$; TTDS-Hb, 21.7(8) kJ

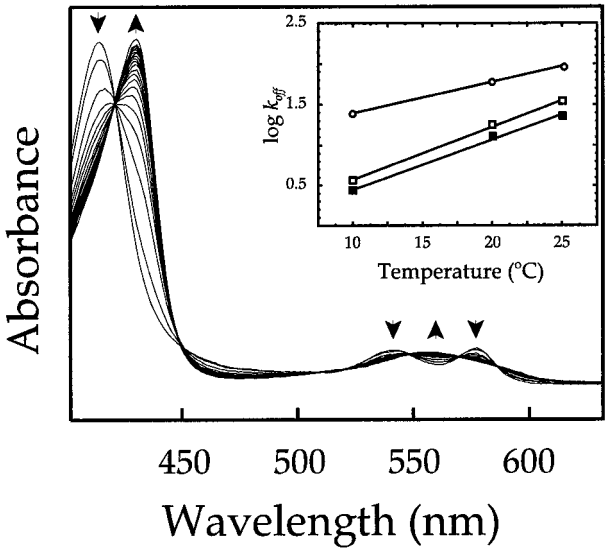


FIGURE 2 Rapid-scanning electronic spectra (400–630 nm) obtained at 20-ms intervals after mixing dithionite with native and cross-linked forms of oxyhemoglobin. Deoxygenation was achieved by reaction of 20 $\mu$M oxy-Hb in 50 mM bis/Tris buffer (pH 7.4) with a final concentration of sodium dithionite of 1.5 mg/ml. The arrows indicate the direction of change during the reaction. The inset shows the dependence on temperature of the oxygen dissociation rate constants for $HbA_0$ (□), TTDS-Hb (■), and TMMP-Hb (○).

**TABLE 1 Oxygen-binding properties and autoxidation rates of native and cross-linked hemoglobins**

| Protein | $P_{50}$ (mmHg) at 37°C | Hill constant ($n_H$) | Autoxidation rate $k$ ($h^{-1}$) |
|---|---|---|---|
| $HbA_0$ | 14 | 2.6 | 0.12 (2) |
| TTDS-Hb | 13 | 2.1 | 0.078 (5) |
| TMMP-Hb | 35 | 2.5 | 0.062 (5) |

**TABLE 2 Rate constants for $O_2$ dissociation from native and cross-linked hemoglobins**

| | $k_{off}$ ($s^{-1}$) | | | |
|---|---|---|---|---|
| Protein | 10°C | 20°C | 25°C | 37°C |
| $HbA_0$ | 3.7 (2) | 17.7 (2) | 34.6 (2) | 145 (3) |
| TTDS-Hb | 2.7 (2) | 12.8 (3) | 22.7 (8) | 95 (4) |
| TMMP-Hb | 25 (3) | 61 (1) | 94 (3) | ND |

ND, not determined, as rate exceeded experimental limit.

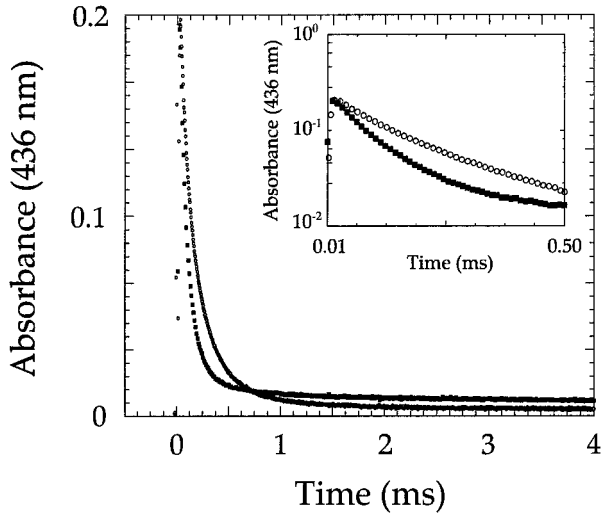


FIGURE 3 Kinetics of CO binding to TTDS and TMMP cross-linked hemoglobins in 50 mM bis/Tris buffer (pH 7.4) in the presence of 1 mM carbon monoxide at 20°C. The fit of the data at 436 nm for TTDS-Hb (■) and TMMP-Hb (○) is shown in the inset.

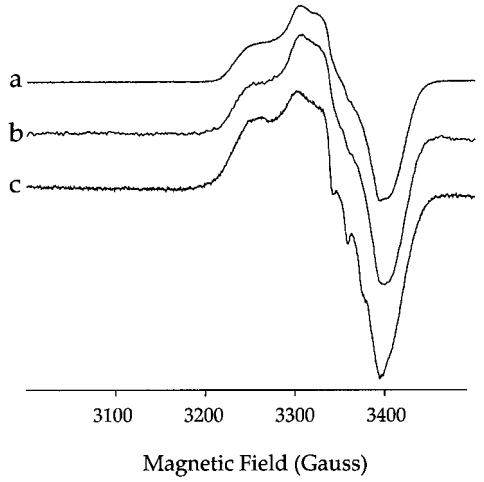


FIGURE 4 X-band EPR spectra of nitrosyl hemoglobin derivatives at 77 K (50 mM bis/Tris buffer, pH 7.0). (*a*) $HbA_0$; (*b*) TTDS-Hb; (*c*) TMMP-Hb.

$mol^{-1}$; TMMP-Hb, 13.17(4) kJ $mol^{-1}$. The rate constant for dissociation of oxygen from TMMP-Hb at 37°C was greater than could be determined by the stopped-flow method.

## CO reassociation kinetics

The rate constants for binding of carbon monoxide to native and cross-linked hemoglobins were determined by monitoring the change in absorbance at 436 nm after photolysis under anaerobic conditions at pH 7.4 (20°C and 25°C) in bis/Tris buffer (50 mM) saturated with CO (i.e., 1 mM). Representative results obtained for the two cross-linked hemoglobins are illustrated in Fig. 3. For both $HbA_0$ and TTDS-Hb, the CO binding kinetics are best fit by the sum of two exponential functions whereas the reassociation of CO with TMMP-Hb is best fit by a single exponential function (Fig. 3, inset). The rate constants determined from these analyses are summarized in Table 3.

## EPR and electronic spectroscopy

EPR spectra of the nitrosyl derivatives of native and cross-linked derivatives of $HbA_0$ are shown in Fig. 4. Interestingly, the intensity of the three-line hyperfine signal exhibited by the nitrosyl derivative of TMMP-Hb was significantly increased as expected for a protein shifted toward the T state structure (Szabo and Perutz, 1976).

The electronic absorption spectra of the nitrosylhemoglobin derivatives change in a manner that is consistent with the changes observed in the EPR spectra of these derivatives. Specifically, the extinction coefficient of the Soret band of TMMP-Hb is decreased ~20% and broadened relative to that of $HbA_0$ (data not shown). This observation is consistent with the EPR spectrum in that it is characteristic of a pentacoordinated heme iron resulting from weakening of the proximal His-Fe bond (Kon, 1975; Hille et al., 1979; Maxwell and Caughey, 1976; Perutz et al., 1976). On the other hand, the electronic absorption spectrum of nitrosyl TTDS-Hb is identical to that of nitrosyl $HbA_0$.

**TABLE 3 Rate constants ($M^{-1}s^{-1}$) for CO association with native and cross-linked hemoglobins**

| Protein | 20°C | 25°C |
|---|---|---|
| $HbA_0$ | $k_1 = 10.10\ (4) \times 10^6$ | $k_1 = 10.90\ (6) \times 10^6$ |
| | $k_2 = 1.70\ (3) \times 10^5$ | $k_2 = 2.10\ (3) \times 10^5$ |
| TTDS-Hb | $k_1 = 13.18\ (2) \times 10^6$ | $k_1 = 14.20\ (8) \times 10^6$ |
| | $k_2 = 3.83\ (1) \times 10^5$ | $k_2 = 4.10\ (6) \times 10^5$ |
| TMMP-Hb | $k_1 = 3.47\ (4) \times 10^6$ | $k_1 = 3.79\ (6) \times 10^6$ |

## Sulfhydryl reactivity

The influence of pH and ligand binding on the sulfhydryl reactivity of the native and cross-linked hemoglobins is shown in Table 4. From the results shown in Fig. 5, it is apparent that both the TTDS and TMMP cross-linked Hbs exhibit a two- to fivefold decrease in reactivity with this reagent relative to that of $HbA_0$. Two reactive thiol groups/tetramer are observed in both the cross-linked and the native forms of $HbA_0$. As previously observed (Taketa and Morell, 1969) and confirmed here for native hemoglobin, conversion of the cross-linked hemoglobins to the carbonyl derivative slightly decreases the reactivity with DTDP relative to that of the corresponding oxyhemoglobin derivatives. Furthermore, deoxygenation of the cross-linked hemoglobins decreased reactivity of the reactive thiol as previously observed for the native protein (Riggs, 1961; Morell et al., 1962; Benesch and Benesch, 1962). At alkaline pH (8.4) the rate constants for reaction of these proteins with DTDP is

**TABLE 4 Initial rate constants ($k_{app}$ ($min^{-1}$)) for reaction of 4,4′-dithiodipyridine with $HbA_0$ and cross-linked hemoglobin derivatives**

| Protein | pH 7.0, 20°C | pH 7.0, 30°C | pH 8.4, 20°C | SH:Hb |
|---|---|---|---|---|
| $HbA_0$ | | | | |
| Oxy | 0.32 (4) | 0.39 | 1.53 | 2.2 |
| CO | 0.25 (2) | | | |
| Deoxy | 0.15 (2) | | | |
| TTDS-Hb | | | | |
| Oxy | 0.20 (2) | 0.25 | 0.60 | 2.0 |
| CO | 0.14 (1) | | | |
| Deoxy | 0.02 (5) | | | |
| TMMP-Hb | | | | |
| Oxy | 0.10 (3) | 0.13 | 0.40 | 1.8 |
| CO | 0.12 (2) | | | |
| Deoxy | 0.06 (2) | | | |

increased to the same degree (three- to fivefold relative to those at pH 7.0) as observed for native $HbA_0$.

## DISCUSSION

Cross-linked hemoglobin derivatives have attracted considerable attention for their potential application as blood substitutes. In addition, however, structurally characterized cross-linked hemoglobin derivatives provide a unique opportunity to explore the functional properties of hemoglobin and the mechanism of cooperative ligand binding in a fashion different from that permitted by the investigation of hemoglobin variants. The present work concerns functional properties of two such cross-linked hemoglobins that have previously been argued to exhibit opposite conformational biases toward the R and T states.

In general, hemoglobins with decreased affinity for oxygen autoxidize more rapidly (Vandegriff, 1995; Shikama, 1998), consistent with the conclusion resulting from the study of variants that steric accessibility of the heme binding pocket is a factor (Brantley et al., 1993). Clearly, for cross-linked Hbs, the dimer-tetramer equilibrium and the greater susceptibility of $\alpha\beta$-dimers to autoxidation relative to the $\alpha_2\beta_2$ tetramer (Zhang et al., 1991) are not considerations, and this consideration would contribute to the greater stability of both cross-linked proteins to autoxidation relative to $HbA_0$. For TTDS-Hb studied here, fumarate-cross-linked Hb (Cashon and Alayash, 1995), glutaraldehyde-cross-linked Hb (Guillochon et al., 1986), and polyethylene glycol-cross-linked Hb (Rogers et al., 1995), the correlation between affinity for oxygen and susceptibility to autoxidation noted above is apparent. However, TMMP-Hb possesses both a decreased affinity for oxygen and a decreased rate of autoxidation relative to $HbA_0$. Other factors that may contribute to this unusual behavior include the identity of the protein residues that are cross-linked (Cashon and Alayash, 1995) and the electrochemical properties of the heme iron of TMMP-Hb.

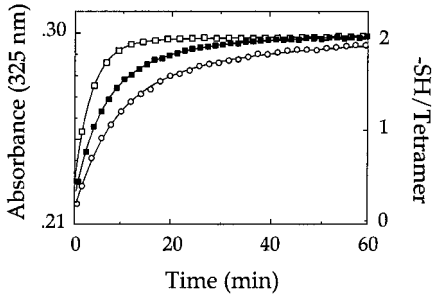


FIGURE 5 Kinetics of DTDP reaction with native and cross-linked hemoglobins. Disulfide exchange was measured at 25°C in 50 mM bis/Tris buffer, pH 7.0. The final concentrations are DTDP (930 $\mu$M) and Hb (9.3 $\mu$M heme). The initial first-order reactions are shown for $HbA_0$ (□), TTDS-Hb (■), and TMMP-Hb(○).

A potential functional consequence of cross-linking hemoglobin is modification of the kinetics of oxygen dissociation from oxyhemoglobin (Table 2). The activation enthalpies noted above for the three proteins studied here are quite similar to each other. For this reason, it is not clear that the smaller value observed for TMMP-Hb reflects a significant mechanistic difference. Nevertheless, it is interesting to note that the values for oxygen dissociation from the two R-state derivatives are identical to each other and that the only potential difference is exhibited by the derivative that is biased toward the T state. If this apparent difference is valid, it could reflect a smaller conformational barrier to oxygen dissociation from this derivative. Unambiguous interpretation of these effects is limited by the likely possibility that entropy-enthalpy compensation effects (Exner, 1964; Lumry and Rajender, 1970) may be involved.

The effects of cross-linking hemoglobin are also manifested in the electronic structure of the heme group. EPR spectra of nitrosylhemoglobins provide information concerning changes in the environment of the heme iron atom that reflect the overall conformational state of the protein (Kon and Kataoka, 1969; Salhany et al., 1975). In the absence of oxygen, NO will bind to the heme iron of deoxyhemoglobin to form nitrosylhemoglobin, which exhibits a rhombic EPR spectrum (Kon, 1968). In the presence of allosteric effectors such as 2,3-bisphosphoglycerate and inositol hexaphosphate, a three-line hyperfine structure centered at $g = 2.009$ is observed (Rein et al., 1972) in the spectrum of this derivative that has been more specifically attributed to selective weakening of the proximal His-Fe bond of the $\alpha$-chains (Hille et al., 1979; Perutz, 1979). This finding and the current results for TMMP-HbNO are fully consistent with the three-dimensional structure of TMMP-Hb reported by Schumacher et al. (1995) in which the proximal His residue in TMMP-HbCO occupies a position that is intermediate between its position in the T and R states. The intensity of the three-line hyperfine structure observed for nitrosylhemoglobins from several ruminant mammals has been shown to correlate inversely with the affinity of these hemoglobins for oxygen in the absence of allosteric effectors (Ascenzi et al., 1992).

The ligand-binding properties of the hemoglobin subunits are inequivalent. The biphasic kinetics observed during CO

recombination has previously been attributed to the greater affinity of the β-chains for CO (Sharma et al., 1991). The rate constant for CO binding changes in response to alterations in the proximal side of the heme-binding pocket and the energy barrier required for the movement of the central metal atom of the heme into the plane of the porphyrin (Coletta et al., 1988). The current results suggest that cross-linking of hemoglobin imposes a differential heme strain energy on the α- and β-subunits. The weaker His-Fe bond detected in the EPR spectrum of nitrosyl-TMMP-Hb (Fig. 3) indicates that the heme iron of this derivative is farther out of the plane than is the case for either $HbA_0$ or TTDS-Hb and that this displacement influences the CO binding kinetics. Stabilization of the T state in the TMMP-Hb tetramer decreases the rate constants for CO binding to the β-chains and/or decreases in the difference in CO binding kinetics of the α- and β-chains so that under the conditions of our experiment, the kinetics are monophasic. This observation is in accord with results of Perrella and co-workers (1992) who found that the α-subunits bind CO 1.5 times faster than the β-subunits upon the addition of inositol hexaphosphate to $HbA_0$. This finding implies that the overall sequence by which the hemoglobin chains cooperate is changed and a new kinetic pathway of carbon monoxide binding is established. High-resolution carbon monoxide binding experiments and investigation of the intermediates (Huang and Ackers, 1996) suggest that a switch-over point occurs when two ligands are bound to hemoglobin. Stabilizing the T state of Hb through formation of TMMP-Hb could have the same effect and provide insight into the changes in the subunits that affect the overall mechanism of ligand binding to hemoglobin.

Additional information concerning the conformational consequence of cross-linking hemoglobin was sought by determining the reactivity of the β93Cys residue, a well established reflection of hemoglobin conformational state (Riggs, 1961; Morell et al., 1962; Benesch and Benesch, 1962). Reactivity of this residue is greatly impeded in the deoxygenated protein owing to formation of six salt bridges that restrict access of exogenous reagents to this thiol group (Dickerson and Geis, 1983, and references therein). The identity of sulfhydryl reactivity we observe for deoxy-$HbA_0$ and TMMP-HbCO is fully consistent with the structural results of Schumacher et al. (1995) in which the position of the β93Cys residue in TMMP-HbCO is shown to be identical to that in deoxy-$HbA_0$. Interestingly, the sulfhydryl reactivity of the deoxygenated cross-linked proteins correlates with the reactivity of the oxygenated proteins.

The conformational and electronic modulation of hemoglobin function that result from chemical cross-linking depend not only on the length of the cross-linker but also on the identity of the amino acid residues within the 2,3-bisphosphoglycerate site with which the cross-linking reagent reacts. The structure of TMMP-Hb (Schumacher et al., 1995) demonstrates that hemoglobin cross-linked with this reagent exhibits a conformational state intermediate between the limiting R and T states in the crystalline state. The present study indicates that in solution, this protein exhibits more T-state-like behavior than the closely related TTDS-Hb. This latter derivative also appears to be intermediate in conformation but somewhat more similar to the R state. Prediction of the functional consequences of site-specific cross-linking of hemoglobin is currently hampered by limited availability of diverse functional information concerning such derivatives of the protein. With additional studies of the type provided here for other related cross-linked hemoglobin derivatives, it should be possible to achieve this challenging goal.

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