Affinity Labeling of Hemoglobin with 4,4′-Diisothiocyanostilbene-2,2′-disulfonate: Covalent Cross-Linking in the 2,3-Diphosphoglycerate Binding Site†

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ABSTRACT: The bifunctional reagent 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) reacts with hemoglobin to give various products whose properties are dependent on the ligation state of the protein during the reaction. A major product obtained after reaction of (carbonmonoxy)hemoglobin with DIDS was a high oxygen affinity derivative [P_{50} = 1.4 mmHg, control P_{50} = 6 mmHg; 50 mM [bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane (Bis-Tris), pH 7.4, 0.1 M Cl^-, 25 °C] which contained two molecules of DIDS per tetramer resulting from adduct formation at each β-chain amino terminus. In contrast, a major product of the reaction of deoxyhemoglobin with DIDS consisted of hemoglobin which had incorporated one molecule of DIDS per tetramer and was cross-linked between the β-chain amino termini. This cross-linked hemoglobin was found to have a greatly decreased O_2 affinity (P_{50} = 28 mmHg). Inhibition of the T to R transition due to the structural constraint produced by cross-linking the β amino termini is likely to be a major factor in the decreased O_2 affinity of this product. The structural and functional properties of this molecule make it a potential candidate for a cell-free blood substitute.

Covalent modification of hemoglobin (Hb) with affinity labels directed toward the organic phosphate binding site has been accomplished with a number of compounds (Benesch et al., 1972, 1975; Walder et al., 1979). In some cases, such modifications have been performed in order to achieve a specific molecular engineering objective, e.g., to produce a modified HbS that will be resistant to polymerization (Klotz et al., 1981). Another such goal has been the preparation of a cell-free modified hemoglobin solution which would be suitable for clinical use as an emergency blood replacement. A large amount of effort has been focused on development of such a solution because of the potential advantages it would hold over whole blood transfusion, including elimination of the need for cross-matching donor and recipient and minimization of the risk of transmitting infectious agents [for review, see DeVenuto (1982)]. Modification of native Hb is neccessary for this purpose because of two critical problems associated with the infusion of native Hb: (1) high intrinsic oxygen affinity of Hb outside the milieu of the erythrocyte with its allosteric effectors, principally 2,3-diphosphoglycerate (2,3-DPG), and (2) rapid renal clearance of dissociated $\alpha\beta$ dimers, which, unlike tetramers, are small enough to pass through the glomerulus and be excreted. In consideration of these problems, the molecular engineering for a cell-free, Hb-based blood substitute involve the synthesis of a nondissociating molecule with low oxygen affinity. The present work deals with the synthesis and isolation of a Hb modified with the bifunctional reagent 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS, Figure 1) which exhibits these properties.

Materials and Methods

Materials. Human hemoglobin was isolated as previously described (Shih et al., 1980). DIDS was obtained from Fluka (Ronkonkoma, NY) and was used without further purification. The compound was stable for at least 3 months under storage at −20 °C. Trypsin was from Worthington. Pharmacia was the source for CM-Sephadex ion-exchange and Sephadex G-25 resins.

Reaction of Hb with DIDS. A 10-mL aliquot of 0.5 mM hemoglobin solution in 50 mM Tris-HCl, pH 7.8, was deoxygenated by being flushed with nitrogen in a round-bottom flask on a rotary apparatus (Shih et al., 1982) for approximately 3 h at 0 °C. A 0.15-mL aliquot of 50 mM DIDS was degassed under vacuum and introduced anaerobically into the reaction vessel with a syringe and plastic tubing to give a final concentration of 0.75 mM. The reaction was carried out protected from light under continuous nitrogen flushing for 60 min at 0 °C, after which time the mixture was immediately chromatographed on a 5 × 30 cm Sephadex G-25 column equilibrated with 50 mM Tris–maleate, pH 6.2. Reaction of DIDS with liganded hemoglobin was performed under identical conditions except that (carbonmonoxy)hemoglobin was used and nitrogen flushing was omitted.

Chromatography of Reaction Products. The reaction mixture was analyzed by cation-exchange high-performance liquid chromatography (HPLC) on a poly(aspartic acid)-coated silica column (PolyCAT-A, Custom L.C. Inc., Houston, TX) following a modification of the procedure of Ou et al., (1983). Buffers were 40 mM Bis-Tris containing 4 mM KCN, pH 6.5 (A), and 40 mM Bis-Tris, containing 0.2 M NaCl and 4 mM KCN, pH 6.8 (B). Modified hemoglobins were separated with a linear gradient from 0% B to 56% B in 24 min and then to 100% B in 10 min at a flow rate of 1 mL/min. Column eluant was monitored at 419 mm, and peaks were integrated with an IBM System 9000 computer.

Preparative cation-exchange chromatography of DIDS-cross-linked hemoglobin was performed on a DEAE-Sephacel column equilibrated with 0.2 M glycine, pH 7.8, with a gradient of 20–250 mM NaCl followed by rechromatography on a CM-Sephadex column equilibrated with 50 mM Tris–maleate with a pH gradient from 6.7 to 7.4. Non-cross-linked disubstituted hemoglobin was purified by chromatography on

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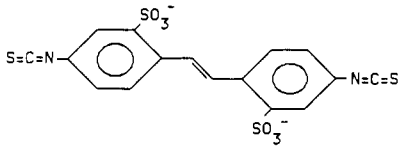

FIGURE 1: Structure of 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS).

a CM-Sephadex column equilibrated with 50 mM Tris–maleate with a pH gradient from 6.2 to 7.4. All ion-exchange buffers were equilibrated with CO gas, and separations were performed at 4 °C. Globins from ion exchange purified components were isolated by reverse-phase HPLC on a Vydac large-pore C_4 column according to the procedure of Shelton et al. (1984).

Trypsin digestion was performed on isolated α- and β-globins for 8 h in 0.1 M NH_4HCO_3 buffer, pH 8.5, at a trypsin concentration of 1:50 (w/w). Tryptic peptides were separated by reverse-phase HPLC on a Vydac C_{18} large-pore column following a modification of the method of Shelton et al. (1985). A gradient between 0.1% trifluoroacetic acid (TFA) (A) and 0.1% TFA in acetonitrile (B) ran from 0 to 13.6% B in 20 min, then to 34% B in 50 min, and then to 100% B in 5 min at a flow rate of 1 mL/min. Peptides subjected to amino acid analysis were first rechromatographed with the same gradient between 10 mM ammonium acetate, pH 6.0, and acetonitrile. An IBM LC/9533 system equipped with dual in-line detectors for sequential monitoring at 214 and 344 nm was used for peptide detection. Amino acid analysis of isolated peptides was performed by reversed-phase HPLC with PITC precolumn derivatization (Heinrikson & Meredith, 1984).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Ion-exchange purified, modified hemoglobins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in a 15% gel according to the procedure of Laemmli (1974). The gel was fixed in 50% methanol and 10% acetic acid and stained with Coomassie blue R-250. Cross-linked bovine hemoglobins (Sigma) were used as molecular weight markers.

Functional Studies. Oxygen equilibrium studies on DIDS-modified hemoglobins were performed in 50 mM Bis-Tris, pH 7.4, containing 0.1 M Cl^- at 25 °C according to an automated recording method (Imai et al., 1981). A Cary 219 spectrophotometer and a polarographic oxygen electrode (Beckman Instruments No. 39065) were employed. Data aquisition and reduction were performed with a PDP 11/VO3 computer (Digital Equipment Corp.) (Shih & Jones, 1986). The concentration of hemoglobin tetramer was 15 μM.

RESULTS

After 0.5 mM (carbonmonoxy)hemoglobin was reacted with 0.75 mM DIDS, the resulting mixture was subjected to cation-exchange HPLC (Figure 2, top). This reaction led to the appearance of only one modified product in significant yield. This product eluted earlier than native Hb on cation-exchange HPLC. Preparative low-pressure ion-exchange chromatography on CM-Sephadex with Tris–maleate buffer was used to isolate this component, designated Hb(DIDS)$_2$-R, for further study. Cation-exchange HPLC demonstrated this product to be chromatographically homogeneous (Figure 2, bottom). In contrast to (carbonmonoxy)hemoglobin, the reaction of 0.5 mM deoxyhemoglobin with 0.75 mM DIDS led to formation of a number of products which also eluted earlier than native Hb on cation-exchange HPLC (Figure 3, top). A major component from this reaction was isolated by low-pressure ion-exchange chromatography on DEAE-Sephacel

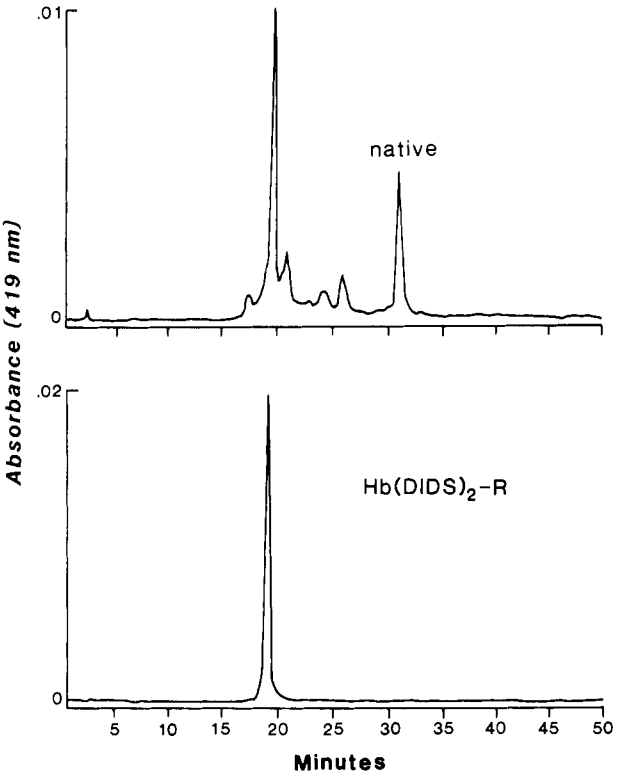

FIGURE 2: Cation-exchange HPLC of DIDS–(carbonmonoxy)-hemoglobin reaction mixture before (top) and after low-pressure ion-exchange purification of Hb(DIDS)$_2$-R (bottom).

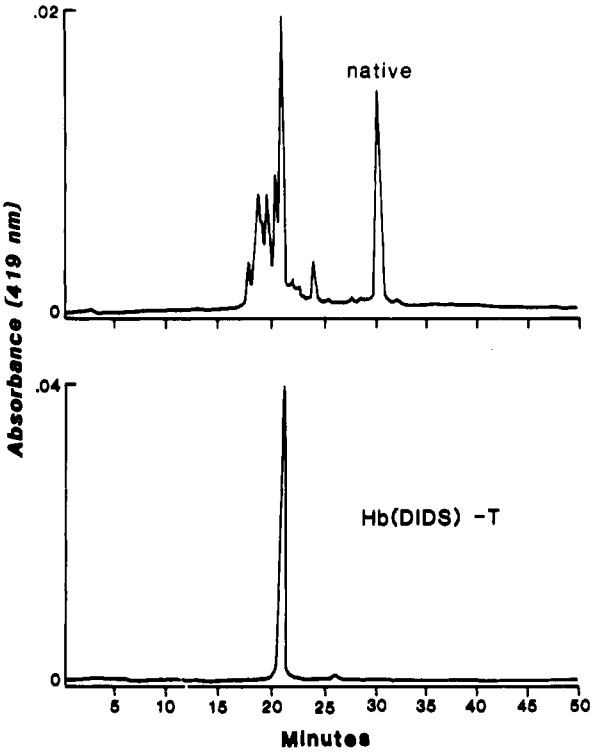

FIGURE 3: Cation-exchange HPLC of DIDS–deoxyhemoglobin reaction mixture before (top) and after low-pressure ion-exchange purification of Hb(DIDS)-T (bottom).

followed by rechromatography on CM-Sephadex. This purified component, obtained in a yield of approximately 5% of the starting material, was designated Hb(DIDS)-T (Figure 3, bottom). In addition to Hb(DIDS)-T, a product chromatographically identical with Hb(DIDS)$_2$-R was formed in the deoxy reaction.

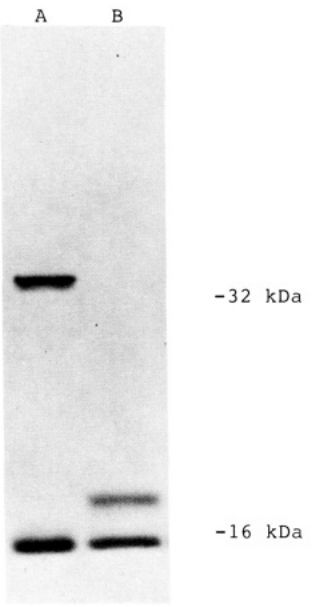

FIGURE 4: SDS–polyacrylamide gel electrophoresis of Hb(DIDS)-T (lane A) and Hb(DIDS)$_2$-R (lane B). Molecular weight markers were native and cross-linked bovine globins (Sigma).

The two ion exchange purified hemoglobin derivatives were subjected to polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol and sodium dodecyl sulfate (Figure 4). In addition to a band running in the position of native globin monomer, a band appeared in the Hb(DIDS)-T product which migrated in the position of a dimer, indicating the occurrence of a covalent intersubunit cross-link (Figure 4, lane A). A new band also appeared in Hb(DIDS)$_2$-R which migrated slightly slower than native monomer (Figure 4, lane B). Reverse-phase C_4 HPLC was performed in order to resolve each of the DIDS-modified components into its constituent globin chains (Figure 5). Both Hb(DIDS)$_2$-R and Hb(DIDS)-T had α-globin which eluted in the position of native α-globin. The tryptic peptide map of the α globin from each component was also identical with that of native α-globin (data not shown). In contrast, the β-globins from Hb(DIDS)-T and Hb(DIDS)$_2$-R each eluted late relative to native β-globin (Figure 5).

Tryptic digestion of β-globins derived from Hb(DIDS)-T and Hb(DIDS)$_2$-R followed by HPLC mapping revealed differences in peptide patterns from native β-globin (Figure 6). In the case of Hb(DIDS)$_2$-R β-globin, the normal amino-terminal tryptic peptide βt-1 (residues 1–8) was missing from the hydrolysate. Additionally, a new peptide appeared in the hydrolysate (Figure 6B, arrow). This peptide absorbed strongly at 344 nm, an absorbance maximum for DIDS. No tryptic peptides from native hemoglobin exhibited this absorption characteristic. Amino acid analysis of this peptide revealed it to have a composition identical with that of normal βt-1 except for the absence of the amino-terminal residue Val-1 (Table I). HPLC of the tryptic hydrolysate of β-globin from Hb(DIDS)-T revealed that the normal amino-terminal peptide, βt-1, was missing from this digest as well. A new peptide appearing in this hydrolysate also exhibited an absorbance maximum at 344 nm but eluted later than the modified peptide from Hb(DIDS)$_2$-R (Figure 6C, arrow). This peptide had an amino acid composition which was also identical with that of βt-1 except for the absence of the amino-terminal valine residue (Table I).

By measuring the absorbance at 344 nm, it was possible to quantitate the amount of DIDS relative to the moles of amino acids for both modified peptides. Assuming an extinction

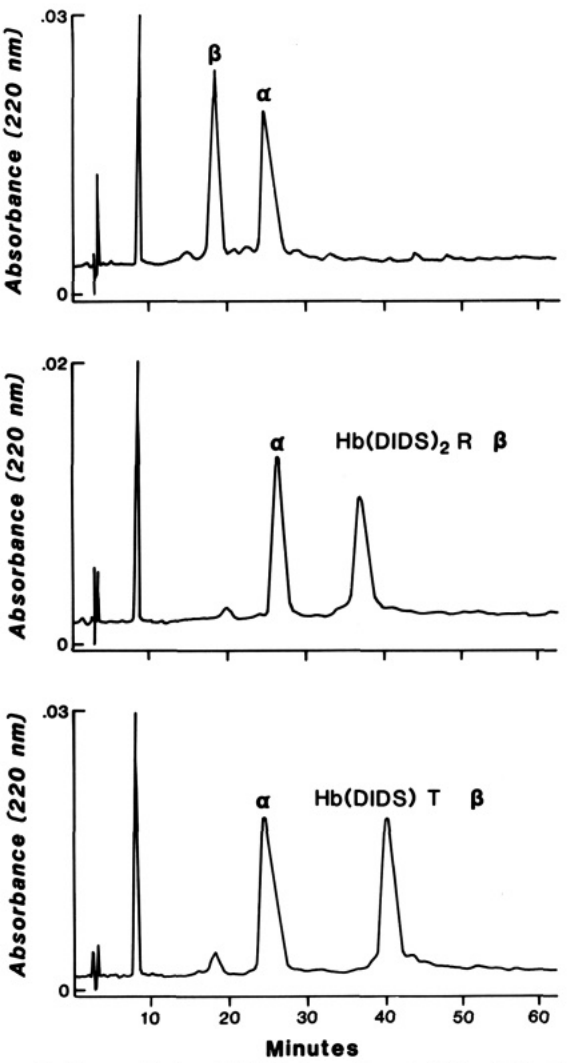

FIGURE 5: Reversed-phase HPLC of constituent globin chains from native globin (top), Hb(DIDS)$_2$-R (middle), and Hb(DIDS)-T (bottom).

Table I: Amino Acid Compositions of Modified Amino-Terminal β-Chain Peptides[a]

	βt-1 (native)	βt-1 [Hb(DIDS)-T]	βt-1 [Hb(DIDS)$_2$-R]
Val	1	0.12	0.07
His	1	1.02	1.02
Thr	1	1.03	1.03
Pro	1	1.00	1.04
Glu	2	1.86	1.94
Leu	1	1.12	1.06
Lys	1	0.95	0.92
DIDS[b]	(0)	0.60	1.28

[a] Amino acid analysis of modified peptides from Hb(DIDS)-T and Hb(DIDS)$_2$-R. Analyses were done on 500–1000 pmol of peptide as described under Materials and Methods. [b] Molar ratios of DIDS were determined from peptide absorbance at 344 nm prior to hydrolysis assuming an ϵ_{344} of 30.5 mM^{-1}.

coefficient of 30.5 mM^{-1} for DIDS, the recovery after hydrolysis for the modified peptide from Hb(DIDS)$_2$-R was 0.8 mol of βt-1 per mole of DIDS and 1.7 mol of βt-1 per mole of DIDS for the modified peptide from Hb(DIDS)-T (Table I).

The oxygen equilibrium curves for Hb(DIDS)-T and Hb(DIDS)$_2$-R are shown in Figure 7. Hb(DIDS)-T has a significantly decreased oxygen affinity relative to HbA (P_{50} = 28 mmHg vs 6 mmHg; Figure 7). In addition, the subunit cooperativity is reduced (n_{max} = 2.1). Addition of 2,3-DPG

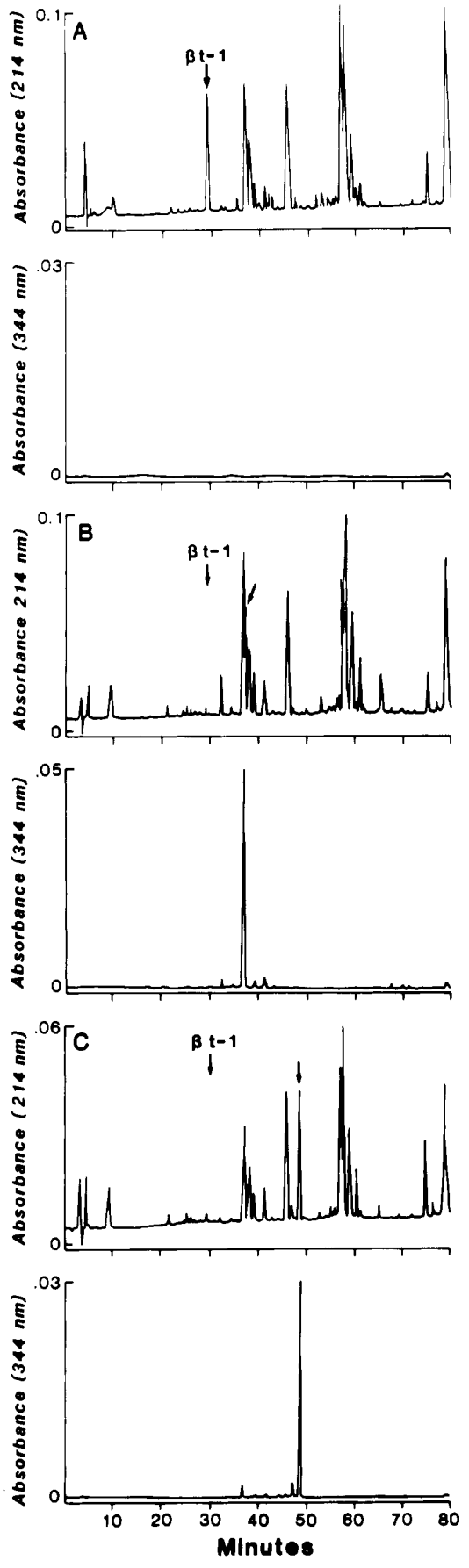

FIGURE 6: C_{18} HPLC tryptic peptide maps of β-globins purified from (A) native Hb, (B) Hb(DIDS)$_2$-R, and (C) Hb(DIDS)-T. Absorbance was monitored simultaneously at 214 (top) and 344 nm (bottom). Arrows indicate appearance of DIDS-modified peptides derived from βt-1.

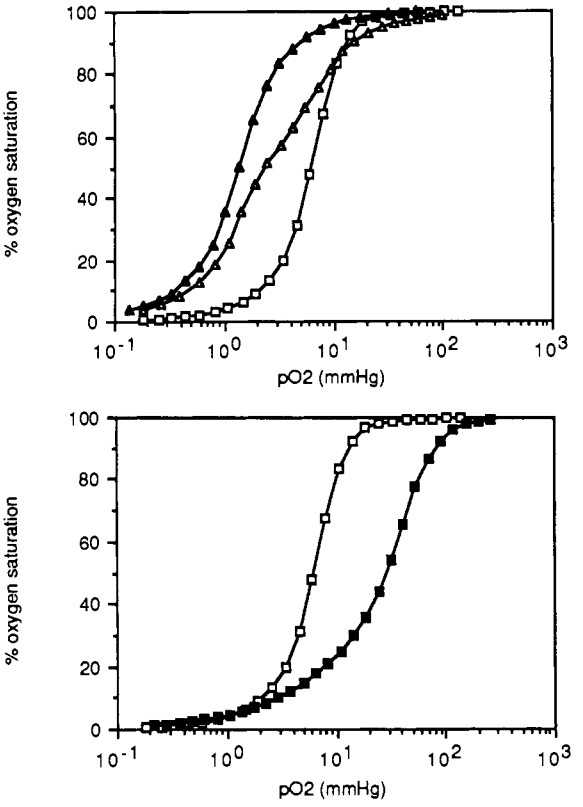

FIGURE 7: Oxygen equilibrium curves for DIDS-modified hemoglobins. (Top) (□) HbA; (▲) Hb(DIDS)$_2$-R (first equilibrium); (△) Hb(DIDS)$_2$-R (second equilibrium). (Bottom) (□) HbA; (■) Hb(DIDS)-T.

had no effect on the position or shape of the equilibrium curve (data not shown). The stability of Hb(DIDS)-T was assessed by measuring its absorption spectrum before and after the oxygen equilibrium determination. Its stability was comparable to that of HbA. Hb(DIDS)$_2$-R exhibited unusual behavior during the oxygen equilibrium determination. Upon recording of an initial deoxygenation of oxyHb(DIDS)$_2$-R, the P_{50} was found to be decreased relative to that of HbA (P_{50} = 1.4 mmHg; Figure 7). However, upon reoxygenation and recording of a second deoxygenation, the curve shifted somewhat to the right and became markedly biphasic, suggesting that a structural change was occurring upon deoxygenation. This was supported by cation-exchange HPLC of Hb(DIDS)$_2$-R after reoxygenation, which revealed a new peak eluting slightly earlier than Hb(DIDS)$_2$-R, as well as by SDS–PAGE which revealed a new band migrating at the position of a dimer (data not shown).

Discussion

The specificity of affinity labeling of hemoglobin in the organic phosphate binding site is based on electrostatic interactions between the protein and ligand analogue as well as reactivity of specific residues in the binding site. The structure of the organic phosphate binding site, which contains four reactive primary amines, has been mapped by high-resolution X-ray diffraction for both the oxy and deoxy derivatives (Perutz et al., 1968; Fermi, 1975). Molecular modeling studies indicate that the distance between the functional moieties in DIDS is approximately 16 Å, very close to the distance between the β-chain amino termini in the T state (Perutz et al., 1968). A major product of the reaction of deoxyhemoglobin with DIDS, designated Hb(DIDS)-T, was purified and partially characterized. Tryptic peptide mapping, modified peptide amino acid analysis, and SDS–PAGE of Hb(DIDS)-T

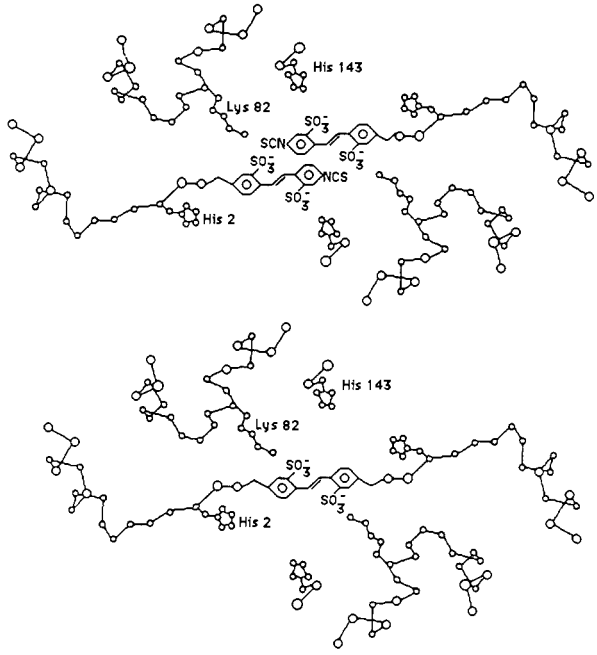

FIGURE 8: Postulated structures for $Hb(DIDS)_2$-R (top) and Hb-(DIDS)-T (bottom).

indicate that this species is cross-linked between the β-chain amino termini. A model for the structure of Hb(DIDS)-T is shown in Figure 8 (bottom). During the normal structural transition of deoxyhemoglobin to oxyhemoglobin, the β termini move apart to a distance of 20 Å (Fermi, 1975). The structural constraint introduced by the 16-Å DIDS bridge between the β-chain N-termini would thus be expected to inhibit the transition to the R state where the termini are 20 Å apart. Indeed, Hb(DIDS)-T was found to have a greatly decreased O_2 affinity compared to Hb A under the conditions tested (50 mM Bis-Tris, pH 7.4, 0.1 M Cl^-, 25 °C; P_{50} of Hb(DIDS)-T = 28 mmHg, P_{50} of HbA = 6 mmHg). Addition of 2,3-DPG had no effect on oxygen affinity of this product, presumably due to blockade of the central cavity by the DIDS molecule. Although X-ray crystallography of Hb(DIDS)-T will be necessary to determine the detailed structural effects of cross-linking the β-chain N-termini, the increased P_{50} and decreased Hill constant of Hb(DIDS)-T support the hypothesis that the molecule is structurally constrained, effectively shifting the allosteric equilibrium toward a T-like structure. A similar phenomenon has been described for hemoglobin modified with 2-nor-2-formylpyridoxal phosphate (nfPLP), which has been shown by X-ray crystallography to cross-link between residues Lys-82β_1 and Val-1β_2 (Arnone et al., 1977).

In contrast to Hb(DIDS)-T, the major product obtained after reaction of (carbonmonoxy)hemoglobin with DIDS was a non-cross-linked hemoglobin, $Hb(DIDS)_2$-R, containing two molecules of DIDS per tetramer. Peptide mapping and SDS–PAGE suggest that this product contains DIDS mono-adducts at each β-chain amino terminus. Presumably the greater distance between the β-chain amino termini in the liganded (R) state prevents cross-link formation from occurring. A model of the proposed structure of $Hb(DIDS)_2$-R is shown in Figure 8 (top). This product had increased O_2 affinity relative to control (P_{50} = 1.4 mmHg).

The partial conversion of $Hb(DIDS)_2$-R to form a cross-linked product following deoxygenation suggests the possibility of an unreacted isothiocyanate from the N-terminal β adduct reacting with another amino group in the region, possibly Lys-82, to form an intermolecular cross-link. The structure of this product is as yet unconfirmed but is under investigation.

In summary, the results presented in this paper demonstrate the feasibility of molecular engineering of hemoglobin by a site-directed reagent with structural properties appropriate for cross-linking specific residues in the organic phosphate binding site in order to perturb the allosteric equilibrium in a predictable manner. Under the experimental conditions described, Hb(DIDS)-T exhibits a P_{50} close to that of whole blood and, in addition, is cross-linked. These properties make Hb-(DIDS)-T a potential candidate as a cell-free blood substitute.

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REFERENCES

Arnone, A., Benesch, R. E., & Benesch, R. (1977) *J. Mol. Biol. 115*, 627.

Benesch, R. E., Benesch, R., Renthal, R. D., & Maeda, N. (1972) *Biochemistry 11*, 3577.

Benesch, R., Benech, R. E., Yung, S., & Edalji, R. (1975) *Biochem. Biophys. Res. Commun. 63*, 1123.

DeVenuto, F. (1982) *Crit. Care Med. 10*, 238.

Fermi, G. (1975) *J. Mol. Biol. 97*, 237.

Heinrikson, R. L., & Meredith, S. C. (1984) *Anal. Biochem. 136*, 65.

Imai, K. (1981) *Methods Enzymol. 76*, 438.

Klotz, I. M., Haney, D. N., & King, L. C. (1981) *Science (Washington, D.C.) 213*, 724.

Laemmli, U. K. (1974) *Nature (London) 227*, 680.

Ou, C.-N., Buffone, G. J., & Reimer, G. L. (1983) *J. Chromatogr. 266*, 197.

Perutz, M. F. (1970) *Nature (London) 228*, 737.

Perutz, M. F., Muirhead, H., Cox, J. M., Goaman, L. C. G., Matthews, F. S., McGandy, E. L., & Webb, L. E. (1968) *Nature (London) 219*, 29.

Shelton, J. B., Shelton, J. R., & Schroeder, W. A. (1984) *J. Liq. Chromatogr. 7*, 1969.

Shelton, J. B., Shelton, J. R., & Schroeder, W. A. (1985) *Hemoglobin 9*, 325.

Shih, D. T., & Jones, R. T. (1986) *Methods Hematol. 15*, 125.

Shih, D. T., Imai, K., Tyuma, I., & Hayashi, A. (1980) *Hemoglobin 4*, 125.

Shih, D. T., Jones, R. T., & Johnson, C. S. (1982) *Hemoglobin 6*, 153.

Walder, J. A., Zaugg, R. H., Walder, R. Y., Steele, J. M., & Klotz, I. (1979) *Biochemistry 18*, 4265.