MOLECULAR ENGINEERING OF HEMOGLOBIN: AFFINITY LABELING WITH BIFUNCTIONAL HETEROTROPIC LIGAND ANALOGS

by

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A DISSERTATION

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ABBREVIATIONS

2,3-DPG	2,3-diphosphoglyceric acid
ATP	adenosine triphosphate
o-ATP	periodate-oxidized adenosine triphosphate
glc-1-P	glucose-1-phosphate
o-glc-1-P	periodate-oxidized glucose-1-phosphate
PRPP	phosphoribosyl pyrophosphate
o-PRPP	periodate-oxidized phosphoribosyl pyrophosphate
NAD	nicotinamide adenine dinucleotide
o-NAD	periodate-oxidized nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
o-NADP	periodate-oxidized nicotinamide adenine dinucleotide phosphate
DIDS	trans-4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
HPLC	high performance liquid chromatography
$C_4(C_{18})$HPLC	high performance liquid chromatography with butyl-(octadecyl-) silane packed columns
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate
M_r	apparent molecular weight (from SDS-PAGE)
n	Hill constant
P_{50}	partial oxygen pressure (mm Hg) at which the fractional saturation of hemoglobin equals 50%
Å	angstroms
Hb	hemoglobin
di-ATP Hb	hemoglobin covalently incorporating two molecules of o-ATP per tetramer
$Hb(DIDS)^T$	T-state hemoglobin crosslinked with one molecule of DIDS incorporated per tetramer
$Hb(DIDS)_2^T$	T-state hemoglobin crosslinked with two molecules of DIDS incorporated per tetramer
$Hb(DIDS)_2^R$	R-state hemoglobin (un-crosslinked) with two molecules of DIDS incorporated per tetramer

ABSTRACT

Human hemoglobin has been covalently modified using a number of site-directed bifunctional reagents in order to attempt synthesis of a crosslinked tetramer with reduced oxygen affinity. Such a semisynthetic molecule would have the minimum physical chemical characteristics required for an acellular hemoglobin-based blood substitute. One molecular engineering scheme utilized for these studies was reductive alkylation with periodate-oxidized ring-opened dialdehydes derived from molecules such as nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, phosphoribosylpyrophosphate, glucose-1-phosphate, and adenosine triphosphate. Although crosslinking of hemoglobin β subunits was accomplished with a number of these reagents, the oxygen affinities of the crosslinked species were increased. Under strongly reducing conditions (in the presence of sodium borohydride), periodate-oxidized adenosine triphosphate was demonstrated to react specifically with residues in the organic phosphate binding site to produce a non-crosslinked modified hemoglobin molecule with a significantly reduced oxygen affinity. Only one reagent studied was found to have the structural characteristics appropriate to crosslink hemoglobin and simultaneously lower the oxygen affinity to a significant extent. 4,4'-diisothiocyano-stilbene-2,2'-disulfonate (DIDS) has a rigid biphenyl structure with an intramolecular bifunctional radius approximately equal to the distance between the amino termini of the β subunits in the low affinity T structure. A major product of the reaction of DIDS with deoxyhemoglobin consisted of Hb which had incorporated 1 molecule of DIDS per tetramer and was crosslinked between the β chain amino termini. This crosslinked hemoglobin was found to have a greatly decreased O_2 affinity in 50mM bis-Tris pH 7.4, 0.1 M Cl^- at 25° (P_{50} = 28 mm Hg, control P_{50} = 6 mm Hg). These characteristics make this chemically modified derivative of hemoglobin a potential candidate as a blood substitute.

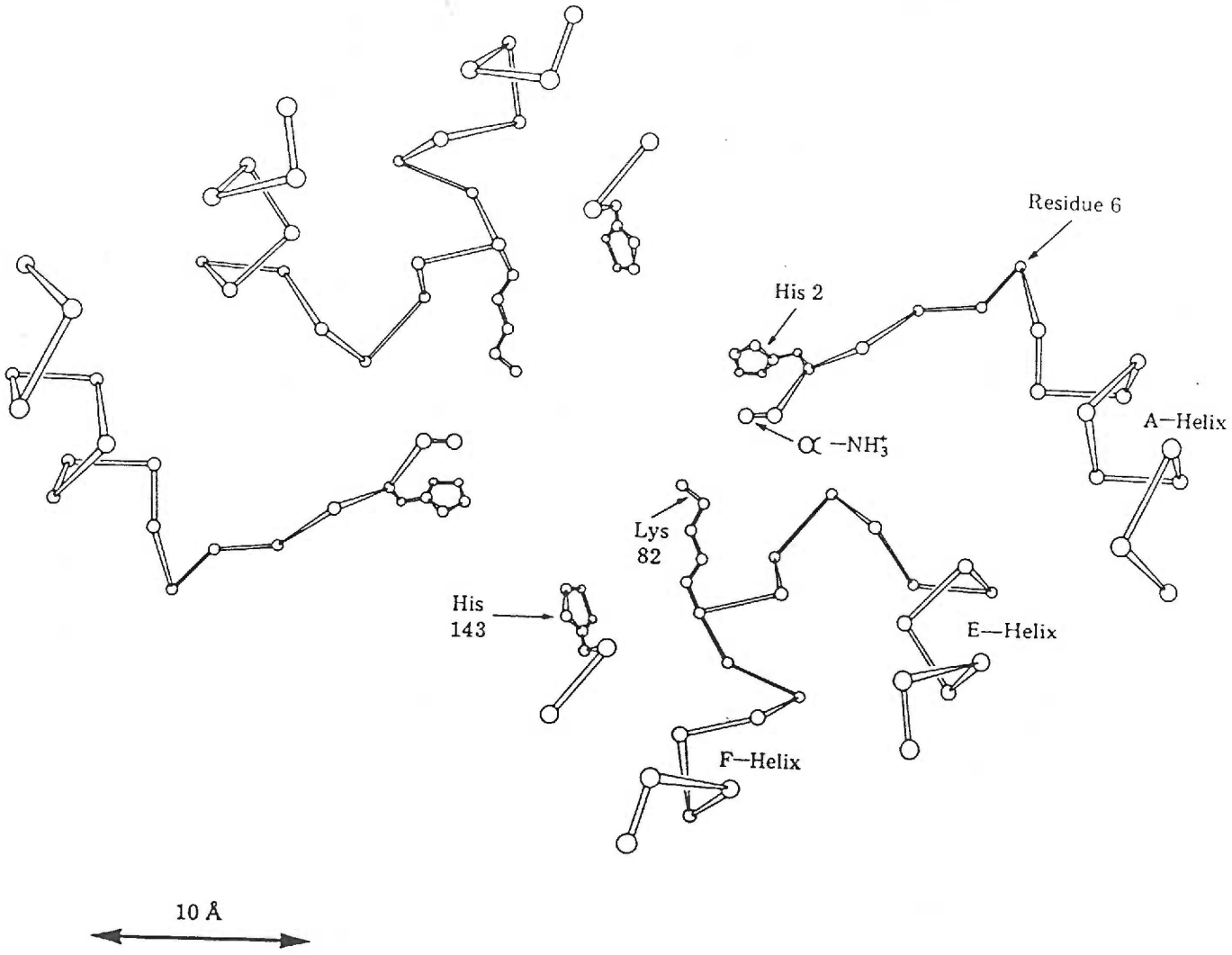
INTRODUCTION

Perspectives and rationale

This dissertation is concerned with molecular studies of hemoglobin covalently modified with various bifunctional heterotropic ligand analogs. These analogs are molecules that bind to and react with residues in the symmetric central cavity of the protein between the β chain amino termini (figure 1). This cavity, the organic phosphate binding site, can bind various allosteric effector molecules such as 2,3-diphosphoglycerate (2,3-DPG), inositol hexaphosphate (IHP), and adenosine triphosphate (ATP). These allosteric effectors alter the functional properties of hemoglobin *in vivo* and *in vitro* (1-3,15). One objective of these studies is to gain insight into the structural basis of functional modulation by heterotropic ligand binding in the organic phosphate binding site, a phenomenon which is still incompletely understood. By using affinity bifunctional ligand analogs to covalently crosslink residues in this binding site, information can be gained about the effects of specific structural perturbations in this region on molecular functions such as oxygen affinity and subunit cooperativity. As will be described below, this information can be used to engineer specifically designed alterations in protein structure and function. An allied objective of these studies involves applying these protein modification techniques with bifunctional ligands to synthesize a hemoglobin-based blood substitute. This will involve engineering a chemically modified hemoglobin with altered structural and functional characteristics; specifically, a crosslinked molecule with permanently lowered oxygen affinity.

There are several potential advantages a cell-free hemoglobin-based blood substitute would hold over whole blood for transfusion. Some of these include elimination of the need to crossmatch donor and recipient, potentially longer shelf-life than whole blood, and reduced

Figure 1. Skeletal diagram of the 2,3-DPG binding site.


Residue 6
His 2
α –NH3+
A—Helix
Lys
82
His
143
E—Helix
F—Helix
10 Å

risk of transmission of infectious agents (8). In addition, hemoglobin solutions are oncotically active and can expand plasma volume at the same time that they carry oxygen. They are also capable of easily permeating microcapillary beds. These features make such solutions attractive candidates for situations where rapid initial treatment of hypovolemia and tissue hypoxia is required (6-9). However, in order to circumvent certain problems discussed below which are associated with infusing solutions of native hemoglobin, structural and functional alterations of the molecule will be necessary.

Aside from unknown potential problems such as possible adverse effects on renal, hepatic, reticuloendothelial and immune functions, there are two significant and immediate problems blocking the application of an acellular hemoglobin solution as a blood substitute (6-9). The first is rapid renal clearance of the protein from the plasma. Native oxyhemoglobin exists in equilibrium between tetramer and dimer (with a very small amount of monomer), and although the tetramer is too large to undergo glomerular filtration, the dimer (and monomer) are rapidly filtered and excreted from the plasma. This results in a circulatory half-life of free hemoglobin of only 2-4 hours, too short a time to be clinically useful (111). The second major problem is the high intrinsic oxygen affinity of native hemoglobin outside the milieu of the erythrocyte with its allosteric effectors such as 2,3-DPG. While the P_{50} of intracellular hemoglobin is approximately 27 mm Hg, this value is drastically decreased in an environment such as plasma which lacks the allosteric effector 2,3-DPG (112). This increase in oxygen affinity would significantly interfere with oxygen delivery to the tissues. These obstacles present an interesting challenge and have stimulated a large amount of interest in the development of molecular engineering strategies to overcome them. In general, these strategies hinge upon two objectives: 1) increasing the effective molecular size of hemoglobin to prevent or slow renal clearance, and 2) decreasing the high intrinsic oxygen affinity to facilitate oxygen delivery to tissues. The

focus of the work described in this dissertation is on the molecular engineering of human hemoglobin utilizing bifunctional heterotropic ligand analogs whose structural characteristics are adapted for crosslinking residues in the 2,3-DPG binding site in a manner which will perturb the tertiary and quaternary structure so that the R to T state equilibrium is shifted toward the T state. The resulting molecule would then have the properties deemed neccessary for a hemoglobin-based, cell-free blood substitute, i.e. , it would have a low oxygen affinity and be non-dissociating.

Background

1. Affinity labeling of proteins

Chemical modification has traditionally been a very powerful tool for studying proteins (4,5). The development of site-directed or affinity modification techniques has greatly increased the power of this approach, not only for studying protein structure and function, but also for altering a protein's properties in a deliberate manner (10-12). A detailed knowledge of a protein's structure often will suggest a rational approach for specifically altering a functional property by the introduction of a discrete structural change. This technique has been elegantly exploited by the use of site-directed mutagenesis to introduce specific amino acid replacements in proteins (13,14).

Affinity labeling is a method to covalently modify specific regions or residues in a protein. The specificity of this modification technique arises from the binding of the affinity label to a discrete site on the protein and subsequent modification of an amino acid residue through formation of a covalent bond. There are thus two distinct steps involved in affinity labeling of proteins: specific binding, and covalent bond formation. A critical aspect in the affinity labeling of a protein is therefore the nature of the specific interaction between

label and protein. Many different types of binding sites on proteins have been recognized, including enzyme active sites, allosteric binding sites, substrate or cofactor binding sites, and carrier sites such as fatty acid and drug binding sites on albumin. Hemoglobin is a protein which is involved in binding several classes of molecules. As the primary oxygen carrying substance in the erythrocyte, hemoglobin must of course bind and release oxygen, but in addition, it can also bind and release a variety of heterotropic ligands such as carbon dioxide, chloride ions, protons, and organic phosphates (78,79). Each of these ligands is an allosteric effector with a discrete binding site or sites on the protein and each exerts an influence on hemoglobin function by reversibly binding and causing specific structural alterations. The focus of the work described in this dissertation is on ligand analog binding and covalent modification in the organic phosphate binding site.

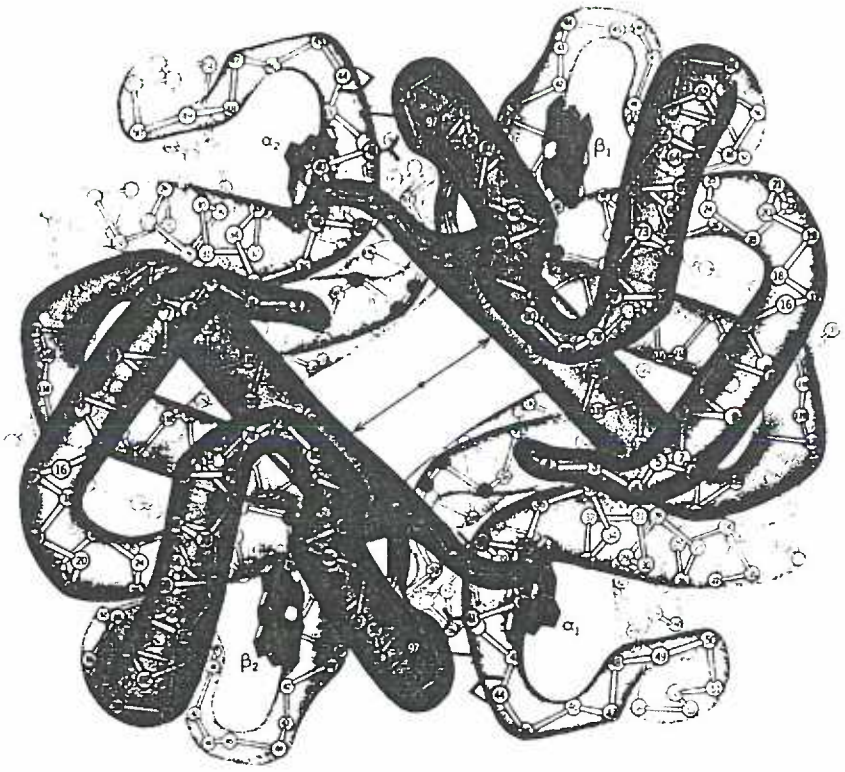
2. The organic phosphate binding site of hemoglobin

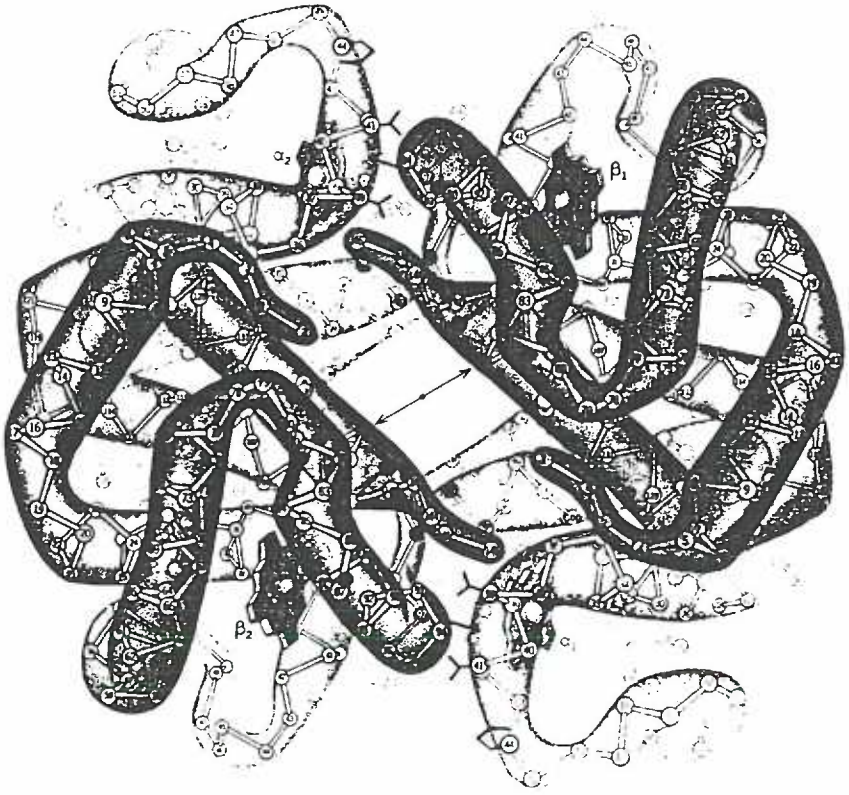
In 1967 Chanutin & Churnish and Benesch & Benesch independently discovered that the oxygen affinity of human hemoglobin is significantly lowered in the presence of the red cell metabolite 2,3-diphosphoglycerate (1,2). It was subsequently demonstrated that this effect was due at least in part to the binding of 2,3-DPG to deoxyhemoglobin with a higher affinity than to oxyhemoglobin (16). At physiological salt concentrations, the ligand was found to bind in the ratio of 1 mole 2,3-DPG per mole deoxyhemoglobin tetramer (113). X-ray crystallography of the ligand-protein complex showed that hemoglobin has a specific binding site for 2,3-DPG located in the symmetric cleft between the β chains which forms the opening to the central cavity in the molecule (17). This cleft is lined with the cationic residues Lys-82, His-2, His-143, and the amino termini of the β chains (figure1). These residues interact electrostatically with the negatively charged 2,3-DPG molecule, whose concentration in the red cell is approximately 5 mM, close to that of hemoglobin. Other negatively charged molecules, especially phosphates, are capable of

binding to this site and decreasing the oxygen affinity of hemoglobin. Examples include adenosine triphosphate (58), inositol hexaphosphate (16), and orthophosphate (18). Non-phosphoryl polyanions such as benzene pentacarboxylate (19) and inositol hexasulfate (20) have been shown to have similar effects on oxygen affinity. In general, the potency of a compound in reducing the oxygen affinity of hemoglobin is related to the amount of negative charge it carries.

The basis of differential binding of polyanions to hemoglobin appears to depend upon conformational changes which occur in the molecule when it switches from the oxy, or R state, to the deoxy, or T state (37). These changes involve movement of the β chains so that they are several angstroms further apart in the T state than in the R state (figure 2). In the $\beta_1\beta_2$ crevice the H helices and the EF corners are spread out in the T state and the β chain amino termini are drawn more closely into the cleft. These structural changes in the cleft region apparently result in a better stereochemical fit for T state heterotropic ligand binding, increasing the binding constant significantly (approximately 100-fold in the case of 2,3-DPG) (59). This results in a shift in the allosteric equilibrium towards the T state and a concomitant right-shift in the oxygen equilibrium curve. This shift of the R-T equilibrium toward the low oxygen affinity T state was originally postulated to be responsible for the alteration in oxygen affinity observed in the presence of 2,3-DPG (15). In terms of the allosteric theory of Monod, Wyman, and Changeux (43), this would be reflected by an increase in the value of the allosteric constant L ($[Hb^T] / [Hb^R]$). However, more detailed analyses of the effects of 2,3-DPG on oxygen equilibria have indicated that the mechanism of the oxygen equilibrium shift must involve more than differential binding and a concomitant shift in equilibrium position due to a mass action effect (47,48,50). In addition to this indirect effect, DPG exerts a direct effect on hemoglobin's oxygen affinity, resulting in a decrease in the value of the MWC allosteric constant c (K^T/K^R) (96). This finding is contrary to the assumptions of the MWC two-state

Figure 2. Structural transition between T state (top) and R state (bottom) in the $\beta_1\beta_2$ cavity.


α2
β1
α1
β2


α2
β1
β2

model in which heterotropic effects are explained solely by displacements in allosteric equilibrium between protein forms (changes in L). The presence of 2,3-DPG alters the MWC constant c primarily by decreasing K^T, with little effect on K^R. These results imply that there is a direct structural perturbation of the molecule which results in a lower affinity binding of oxygen to the T state.

X-ray diffraction analysis of deoxyhemoglobin complexed with 2,3-DPG indicates that there are indeed subtle but definite changes in the protein's structure (17). The amino-terminal helices of the β chains are drawn closer together in the ligand-protein complex, presumably to maximize electrostatic interactions between the cationic residues in the cleft and the bound DPG molecule. Additionally, the carboxy-terminal helices of the β chains are pushed apart, away from the central cavity and towards the heme groups. The magnitude of these changes is on the order of 1-2 Å. 2,3-DPG has a negligible effect on K^R, the oxygen affinity of the R state protein. This is in accord with experimental evidence indicating that the bound 2,3-DPG molecule is ejected from the binding site following binding of the third molecule of oxygen to the tetramer (114), coinciding with the shift in conformation of the protein from the T to the R state. The heterotropic effector inositol hexaphosphate, on the other hand, exerts an influence on both K^R and K^T. IHP binds preferentially to deoxyhemoglobin but still binds strongly to oxyhemoglobin and influences its structure as well (93).

Several alternative allosteric models have been proposed which incorporate the idea of a direct influence mediated by an induced structural perturbation following 2,3-DPG binding. The simplest of these invoke a 3-state model; R, T, and an additional "S" state which arises when DPG binds to the T state tetramer (47). Other more general and complex models such as multi-affinity state and induced fit models have been proposed to describe the allosteric effect of 2,3-DPG (48-50). Although a detailed explanation of the mechanism

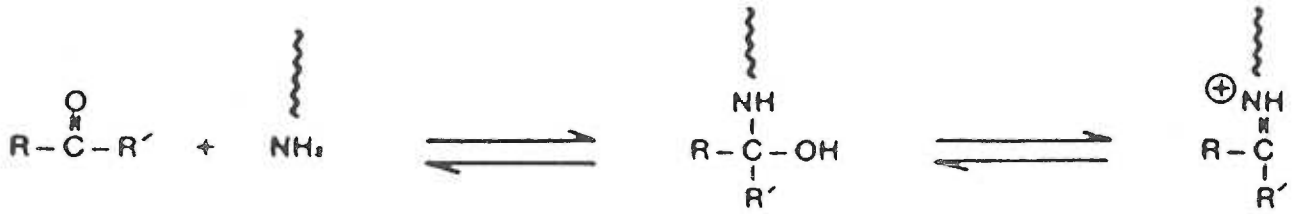
is still lacking, it is apparent that heterotropic ligand binding to the central cavity must mediate direct functional effects by structurally perturbing the site. This phenomenon is clearly important to the concept of covalently modifying the site in order to reduce the oxygen affinity of hemoglobin. By covalently modifying the binding site in a manner which causes structural perturbations similar to those which occur during polyanion binding, it should be possible to permanently decrease the oxygen affinity of hemoglobin.

3. Site-specific modifications of hemoglobin

A. Glycosylation and reductive alkylation

In vivo glycosylation of proteins is a widely occurring process which may or may not be enzyme-mediated. In general, enzyme-mediated glycosylations tend to involve the specific amino acid residues serine, asparagine, and hydroxylysine. Such glycosylations generally serve specific functions such as targeting proteins for particular secretory pathways or protecting a protein from proteolytic degradation. Non-enzymatic glycosylation of proteins can also occur to form a wide variety of adducts, generally involving condensation of a reducing sugar with amino groups from the protein. The general carbonyl-amine reaction is diagrammed in figure 3. The amino group from a lysine residue or an alpha amino terminus acts as a nucleophile and condenses with an aldehyde to form an aldimine, or Schiff base intermediate (21). This reaction is reversible and in equilibrium with free aldehyde and amine. Because the reaction is a nucleophilic condensation, it occurs only with unprotonated amino groups with a free lone electron pair. For this reason the rate of reaction is very sensitive to both the pK of the amino group and the pH of the reaction medium. However, the position of the equilibrium is not significantly affected by varying the nucleophilic strength of the amine (21). The rate of the carbonyl-amine reaction generally shows a pH dependence resulting in a bell-shaped curve because at alkaline pH amine addition to the carbonyl group is fast and the dehydration

Figure 3. Carbonyl-amine reaction leading to Schiff base formation (top) and reduction of Schiff base to secondary amine by borohydride.



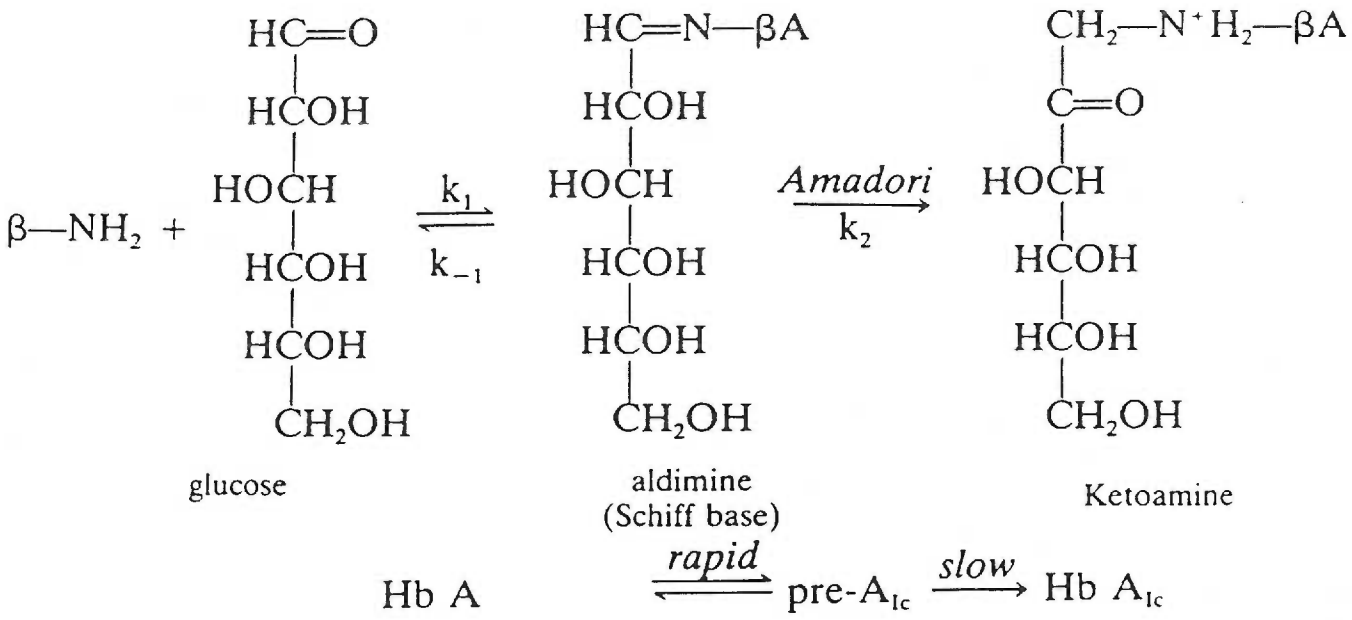

NaBH4

step, which is acid-catalyzed, is rate limiting (figure 3). As pH decreases, however, the amine becomes protonated and the concentration of the tetrahedral intermediate decreases (21). The Schiff base imine is in equilibrium with free amine and carbonyl. This equilibrium is readily reversible unless the imine is reduced to a stable secondary amine with a reducing agent such as sodium borohydride.

Non-enzymatic glycosylation of hemoglobin takes place inside the red cell leading to a number of modified species which can be resolved chromatographically (22-24). Many of these minor components have not been structurally characterized. A number of chromatographically resolved modified hemoglobins have been given designations such as Hbs A_{Ia1}, A_{Ia2}, A_{Ib}, and A_{Ic}. HbA_{Ic} is the most abundant glycosylated hemoglobin, normally present as about 4% of total hemoglobin. HbA_{Ic} has been shown to have a structure involving a glucose adduct with the β chain amino termini (25). Because there is a hydroxyl group on the carbon adjacent to the carbonyl, the unstable Schiff base intermediate can undergo an Amadori rearrangement to yield the stable ketoamine product (figure 4). Because this reaction is non-enzymatic, the concentration of HbA_{Ic} is dependent solely on the concentration of glucose in the erythrocyte and the age of the cell. HbA_{Ic} has been shown to be elevated two to threefold in patients with uncontrolled diabetes (26). Glucose can also form adducts in smaller amounts with α chain amino termini as well as ε-amino groups from lysine residues (27).

There are a number of other sugars in addition to glucose in the red cell although in much lower concentration. However, of particular interest are sugar phosphates which would be predicted to have some affinity for binding in the β cleft. Indeed, both of the carbohydrate containing hemoglobins Hb A_{Ia1} and Hb A_{Ia2} have been shown to contain covalently incorporated phosphate (24). It has also been shown that reducing hexose phosphates react more rapidly and with

Figure 4. Reaction outline of Hb glycosylation and Amadori rearrangement.


β—NH2 +
HC=O
HCOH
HOCH
HCOH
HCOH
CH2OH
k1
k−1
HC=N—βA
HCOH
HOCH
HCOH
HCOH
CH2OH
Amadori
k2
CH2—N+H2—βA
C=O
HOCH
HCOH
HCOH
CH2OH
glucose
aldimine
(Schiff base)
Ketoamine
Hb A
rapid
pre-A1c
slow
Hb A1c

greater specificity than glucose *in vitro* (28,29). The phosphate group allows the sugar to function as an affinity label by conferring specificity for binding to the organic phosphate binding site, followed by formation of a Schiff base between the carbonyl group and the β chain amino terminus. It was found that glucose-6-phosphate reacted much more rapidly with deoxyhemoglobin than with oxyhemoglobin, consistent with the binding of other organic phosphates to deoxyhemoglobin with higher affinity than to oxyhemoglobin (29). Additionally, the presence of 2,3-DPG considerably slowed the reaction rate. In contrast to glucose-6-phosphate, the rate of reaction with glucose was independent of the ligation state of the hemoglobin molecule (29). The reason for the relative specificity of the reaction of glucose with the β chain amino termini is not entirely clear but may relate to the relatively low pK of the amino terminal groups of the β chains in comparison with the α chains which would confer a greater nucleophilicity.

The functional properties of glycosylated hemoglobins have been investigated both because of clinical interest in their effects as well as the information they can give about functional modification due to site-specific modification. Hb A_{Ic} has an oxygen affinity very close to that of Hb A in the absence of organic phosphates (39). However, addition of 2,3-DPG (39) or IHP (40) has significantly less of an effect on the oxygen affinity of Hb A_{Ic} compared to Hb A. It is possible that disruption of organic phosphate binding occurs as a result of steric interference caused by glycosylation of the β chain amino termini. Another factor may be the reduction in pK of the β chain terminal ketoamine moiety. This results in a loss of positive charge, reflected in ion exchange chromatographic behavior, which could decrease the binding of organic phosphates by lessening the strength of the ionic interaction between ligand and binding site.

Another naturally occurring modified hemoglobin occurs as a result of Schiff-base adduct formation between endogenous acetaldehyde and

the amino termini of both chains (31). These acetaldehyde-protein adducts are reversible unless reduced with a reagent such as borohydride.

B. Carbamylation

In addition to glycosylation and acetylation reactions, other types of post-translational modifications of hemoglobin may occur in vivo. Among these are carbamylation, in which hemoglobin reacts with cyanate (in equilibrium with urea) to form irreversible adducts with both α and β amino termini (30). These carbamylated products have increased oxygen affinity (45), a fact that led to the demonstration that carbamylation of hemoglobin S was effective at inhibiting sickling (115).

C. Acetylation

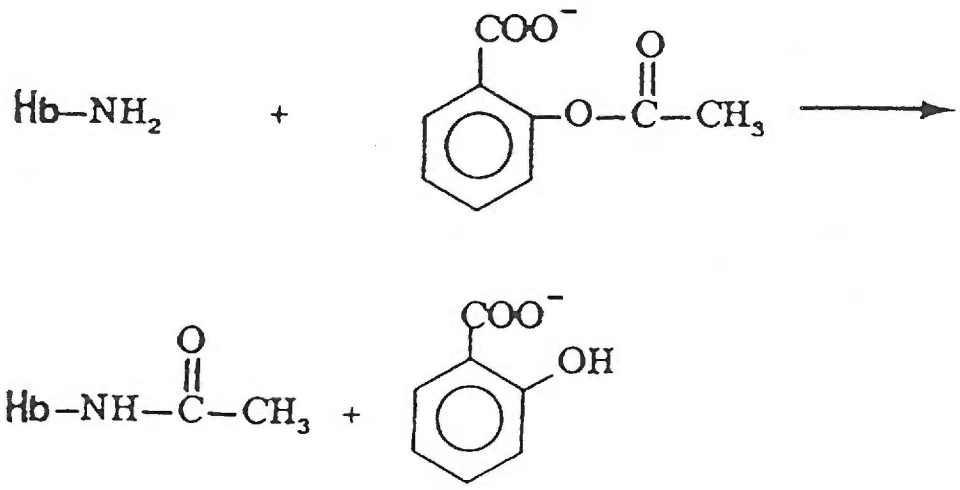
Still another modification of hemoglobin demonstrated to occur both *in vivo* and *in vitro* is acetylation. This can occur both enzymatically and non-enzymatically. Many cellular proteins are α–NH_2 acetylated by an acetyltransferase which utilizes acetyl-CoA as a substrate (32). Enzyme-catalyzed acetylation is dependent in part on the primary structure of the amino terminus (33); proteins whose N-terminal residue is alanine, serine, and to a lesser extent, glycine, are more readily acetylated than others (33). Four mammalian hemoglobins are known to be acetylated: human and primate HbF_1, cat hemoglobin, and the mutant human hemoglobin Hb Raleigh (34-36,38). Each of these hemoglobins has an N-terminal amino acid substitution which promotes acetylation to a variable extent, depending on the substituted residue. Naturally occurring acetylation of hemoglobins is relatively rare. It may be that evolutionary pressure against residues which promote N-terminal acetylation of hemoglobins has been brought to bear because of the importance of the amino terminal region in anion binding (33).

Non-enzymatic acetylation of hemoglobin has also been demonstrated

to occur when hemoglobin is incubated with various acetylating reagents in vitro. Among these reagents is aspirin which has been shown to non-specifically acetylate hemoglobin at various residues according to the mechanism outlined in figure 5. Incubation of whole blood or hemoglobin solutions with [^{14}C] acetylsalicylate leads to incorporation of [^{14}C] acetate into hemoglobin with a concomitant increase in oxygen affinity (46). Because polymerization of Hb S tetramers occurs exclusively in the deoxygenated state, it was reasoned that acetylation might serve as a therapy for sickle-cell anemia by increasing the oxygen affinity of the molecule. However, the reaction rate of aspirin with hemoglobin is relatively low. High concentrations of aspirin (20 mM) and increased temperature were necessary to get measurable incorporation of [^{14}C] acetate (46). In addition the reaction with hemoglobin was non-specific, with acetylation of several lysines on the external surface of the protein occurring, making this modification impractical clinically. A further development in the use of aspirin-based acetylating agents was the finding that by adding electron-withdrawing substituents such as halides to the salicylate leaving group the reaction rate was significantly increased (41). The reaction of hemoglobin with 5 mM O-acetyl-3,5,- dibromosalicylate at pH 7.2, 37°, led to modification yields of 70-80% (41). In addition to the electron withdrawing effects of the halogen substituent, there is an increased polarizability which was postulated by Klotz et al to lead to an increase in binding as well as reactivity (41).

An alternative strategy for modification of hemoglobin with acetylating agents as a possible therapy for sickle cell disease was the development by Walder and associates of diaspirin derivatives linked by alkyl bridges in order to acylate and crosslink residues in the tetramer (42). Stoichiometric addition of (3,5-dibromosalicyl) succinate and bis (3,5-dibromosalicyl) fumarate (figure 6) to oxyhemoglobin gave high yields of modification (approx. 70%). Additionally, it was found that 40% of the β chains were crosslinked

Figure 5. Reaction scheme showing acetylation of Hb by aspirin.


Hb–NH2
+
COO⁻
O
O–C–CH3
Hb–NH–C–CH3
O
+
COO⁻
OH

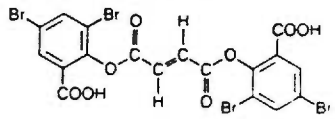
by the reaction as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In contrast, reaction with deoxyhemoglobin led to somewhat lower overall yields of modified product and drastically reduced crosslinking yields (50% modified, <5% crosslinked) (42). The oxygen affinity of the crosslinked hemoglobins was increased, especially at low levels of oxygen saturation, indicating a preferential increase in K^T. The x-ray structural studies of the succinyl and fumaryl crosslinked deoxyhemoglobins revealed a band of positive difference density between Lys 82 β_1 and Lys 82 β_2, indicating a crosslinking bridge between these residues, along with significant perturbation in the structure of the EF corner and F helices where Lys 82 β is located (44). The β cleft structural perturbations observed in the x-ray studies of the deoxygenated crosslinked species are similar to the structural changes seen in the same region of native hemoglobin when it switches from the T to the R conformation. Upon deoxygenation there is an increase in the separation of the β chains which is apparently hindered by the constraint of the crosslinking bridge. The E and F helices at the edge of the binding site are pulled toward the E and F helices from the opposite chain, deforming the structure of the T state protein. Thus, the result of these structural modifications is an increase in oxygen affinity evidently brought about by impeding the R to T transition. When bis (3,5-dibromosalicyl) fumarate is reacted with hemoglobin in the deoxy state, the reagent still reacted with residues in the β cleft as evidenced by competition with 2,3-DPG and IHP, but failed to crosslink between residues on opposing β chains, acting instead as a monofunctional reagent.

Consideration of structural data gathered from x-ray crystallography of native hemoglobin aids in interpretation of the results of the crosslinking experiments with bis (3,5-dibromosalicyl) fumarate and bis (3,5-dibromosalicyl) succinate. Within the β cleft the distance between Lys 82 β_1 and Lys 82 β_2 is 8.1 Å in the deoxy crystal (63). Molecular models of bis (3,5-dibromosalicyl) fumarate and bis

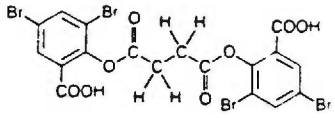
Figure 6. Diagram of structures and reaction schemes for double-headed aspirins with hemoglobin.

Bis (3,5-dibromosalicyl) esters

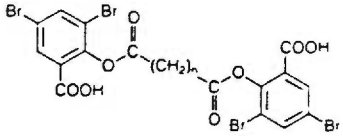
fumarate



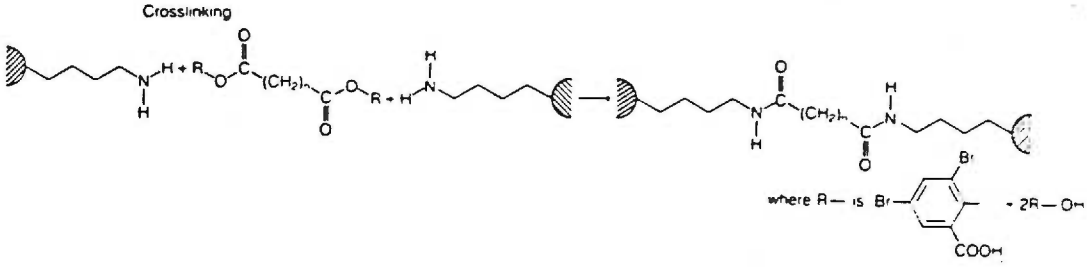
succinate



general case



Crosslinking



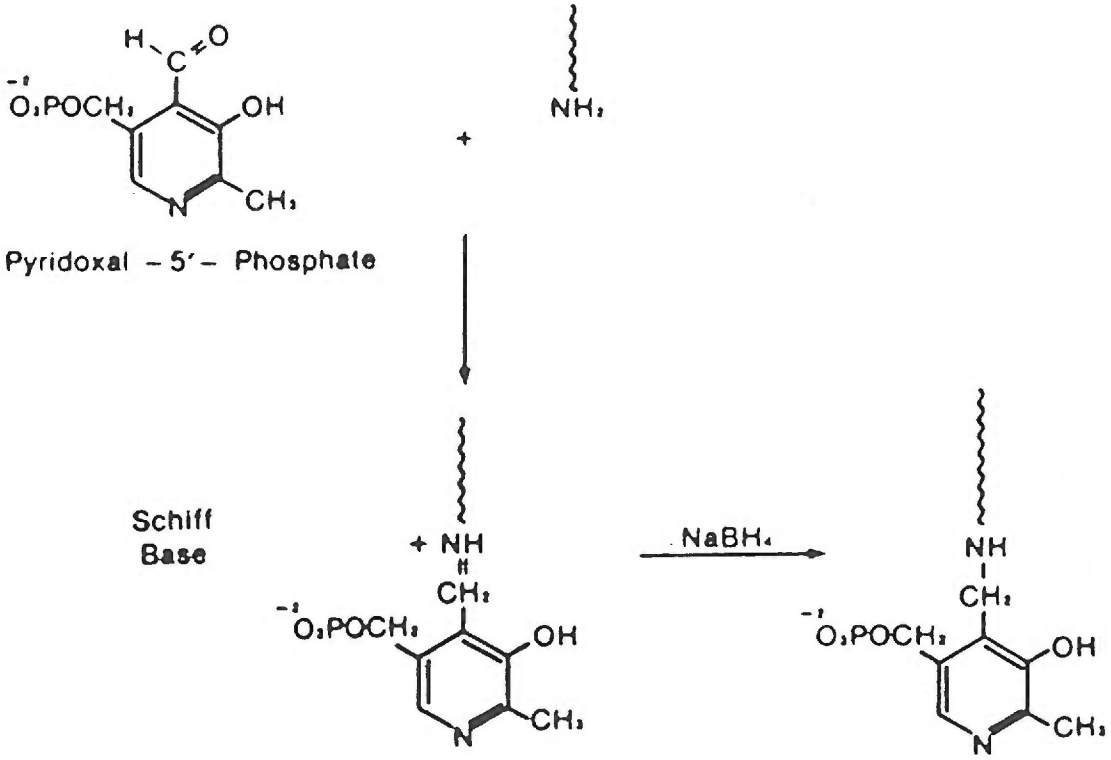
(3,5-dibromosalicyl) succinate indicate that each has a bridging span of 6.8 Å (42). Therefore, in the deoxy conformation, the lysine ε-amino groups are spaced too far apart to allow crosslinking to occur, but in the shift to the oxy conformation, the β chains move closer together and thus allow crosslinking to occur. Hemoglobin which has been crosslinked in the oxy conformation is thus inhibited from switching to the deoxy conformation because of the geometric constraints imposed by the bridging group.

D. Pyridoxylation

Covalent modification of hemoglobin has been accomplished using many different types of agents, but probably the first modification of hemoglobin that could be termed affinity modification came with the discovery in 1972 by Benesch et al. that the vitamin B_6 derivative pyridoxal phosphate (figure 7) has an effect similar to 2,3-DPG on oxygen affinity and that it in fact binds to the same site on hemoglobin as the endogenous organic phosphates (64). Pyridoxal phosphate is a monofunctional organic phosphate which serves as a cofactor for many different enzymes, particularly transaminases (65). Generally pyridoxal phosphate functions catalytically by serving as a carrier of amino groups via Schiff base formation with its free aldehyde group. The cofactor cycles back and forth between the free aldehyde form and the aminated pyridoxamine phosphate form, transferring amino groups between amino acids and keto acids and forming a Schiff base with a lysine residue on the enzyme when it is not complexed with a substrate amino group (66).

The nucleophilic substitution reaction leading to the ketimine or Schiff base complex with aldoses is also the basis of the covalent modification of hemoglobin by pyridoxal phosphate. Although the transient imine complex is unstable and is in equilibrium with the free aldehyde and amino group, it is susceptible to reduction to yield the stable secondary amine. This step requires a reducing agent such as sodium borohydride. When pyridoxal phosphate is added to

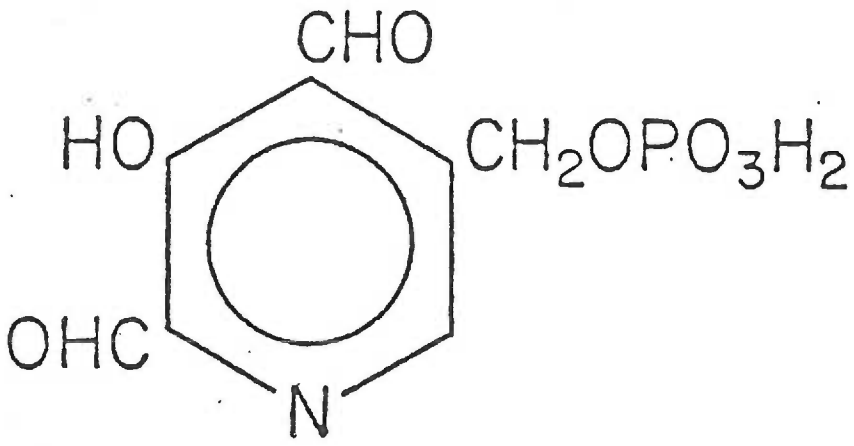
Figure 7. Structure of pyridoxal phosphate and reaction with amino group to form Schiff base subject to reduction by borohydride.


Pyridoxal – 5′ – Phosphate
Schiff Base
NaBH4

deoxyhemoglobin and the resulting complex is reduced with sodium borohydride, the major product consists of hemoglobin covalently modified at the β chain amino termini (67). This modified hemoglobin has a permanently lowered oxygen affinity (log P_{50}=0.90 compared to 0.58 for native protein at 20°, pH 7.3). In addition, the oxygen affinity is unaffected by the addition of 2,3-DPG. X-ray crystallography of the purified complex revealed the structure shown at the top of figure 9. It was found that the ligation state of the molecule exerted a profound effect on the reaction products. When the same reaction was performed with hemoglobin in the oxy state, less specificity in modification was observed and a large amount of hemoglobin modified at the α-chain N-termini was obtained as well (68). In addition to a lower binding affinity for pyridoxal phosphate by the oxy derivative, ligation-induced pK shifts of the α and β N-termini may be responsible for this difference in specificity (69).

In 1973 the synthesis of a bifunctional analog of pyridoxal phosphate, 2-nor-2-formyl pyridoxal phosphate was described (70). This compound contains two aldehyde groups with an approximate spacing of 7.5 Å (figure 8). The analog was designed to introduce stable crosslinks by reduction of the imines formed after reaction of the compound with appropriately spaced amino groups in enzymes which use pyridoxal phosphate as a cofactor. In 1975 Benesch and associates described the reaction of 2-nor-2-formyl pyridoxal phosphate with human hemoglobin to form a derivative which contained crosslinked β chains as evidenced by SDS-PAGE (63). They found that the crosslinked derivative, formed in approximately 50% yield, had a substantially decreased oxygen affinity (log P_{50} = 1.4, control = 0.55; 0.1 M Cl^-, 50 mM bis-Tris pH 7.3). The derivative also had a decreased subunit cooperativity, reflected in a lowered Hill value (n = 1.9, control = 2.7) (63). It was later determined by x-ray crystallography that the crosslink had indeed formed in the polyphosphate binding site between Lys 82 β_1 and Val 1 β_2 (67). Model building studies indicate that the crosslink is formed by the

Figure 8. Structure of 2-nor-2-formyl pyridoxal phosphate.

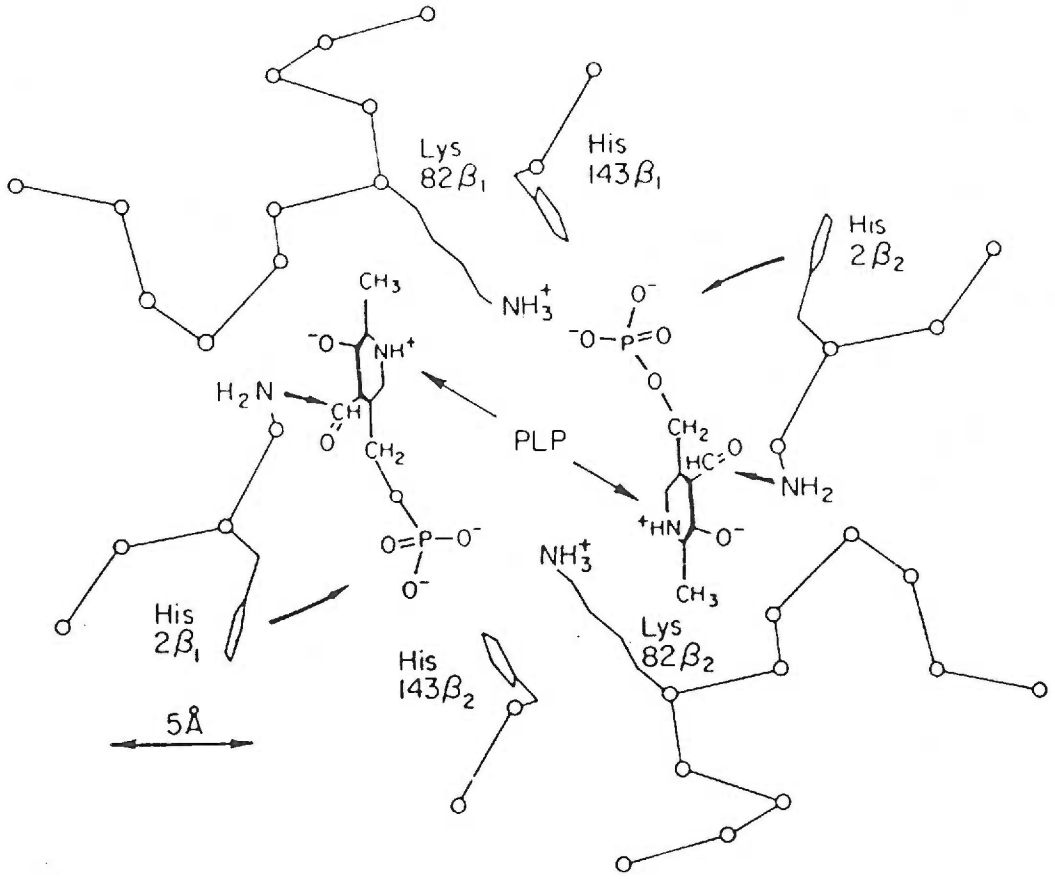

CHO
HO
CH2OPO3H2
OHC
N

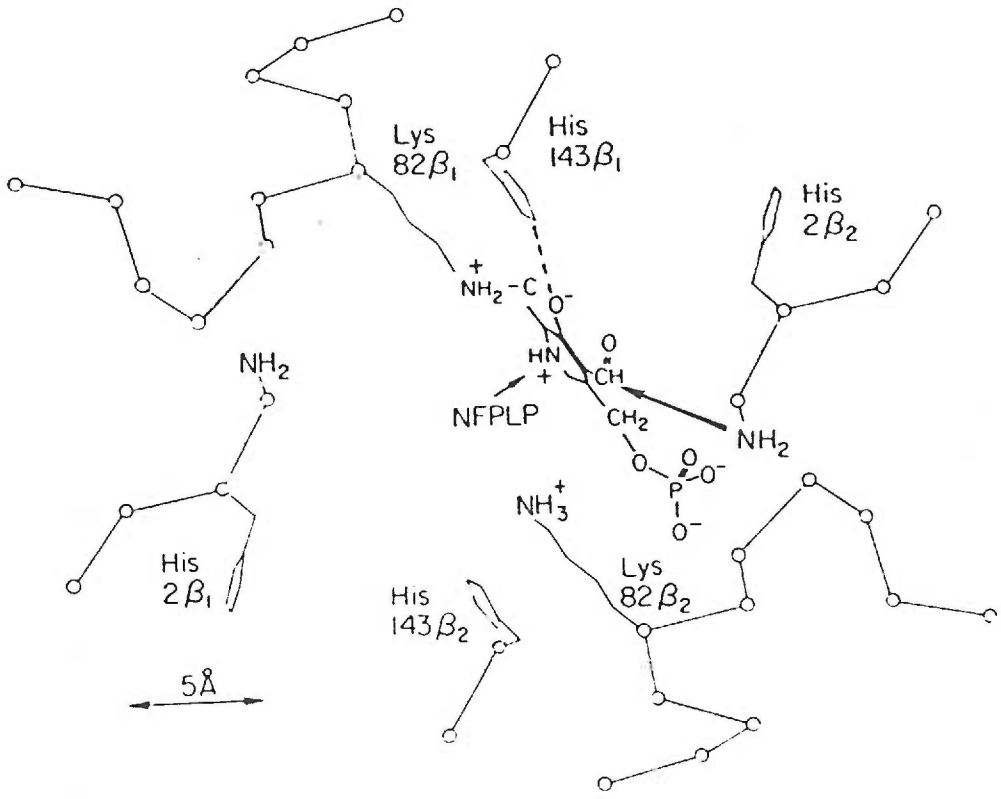
reaction of the 2'-carbonyl from 2-nor-2-formyl pyridoxal phophate with Lys 82 β_1 and the 4'-carbonyl with Val 1 β_2 (Fig. 9). In addition the 3-oxygen interacts with the imidazole group of His 143 β_1, and the phosphate group is apparently stabilized by ionic interactions with Lys 82 β_2, His 143 β_1, and His 146 β_2. Little structural perturbation is required for attachment of the 2'- carbonyl with Lys 82 β_1, but the α-amino terminal helix is drawn approximately 3 Å into the central cavity to form the covalent bond with the 4'-carbonyl (figure 9). This structural shift is very similar to that seen in the deoxyhemoglobin-2,3-DPG complex, where the N-terminal helices are drawn together to facilitate salt bridge formation between phosphate groups on the 2,3-DPG and the terminal α-amino groups (17). These changes are also seen in the deoxyhemoglobin-IHP complex (72).

The structural constraint imposed by crosslinking Lys 82 β_1 and Val 1 β_2 with 2-nor-2-formyl pyridoxal phosphate, whose bridging span is 7.5 Å (63), means that there will be a greater degree of perturbation in the R state structure since the distance between these groups is 16 Å in methemoglobin (73), but is only 11 Å in deoxyhemoglobin (74). It would thus be expected that the crosslinked hemoglobin would exhibit an allosteric equilibrium significantly shifted toward the T state.

Somewhat surprisingly, the crosslinked hemoglobin has a Hill coefficient which, although reduced, still indicates a substantial degree of cooperativity (n = 1.9) (63). This implies that the tetramer is not frozen in a low affinity T-like state, but still functions in a manner well approximated by the two state Monod-Wyman-Changeux model (51). Further support for a conformational switch occurring comes from observation of degradation of the deoxy crosslinked hemoglobin diffraction pattern upon introduction of oxygen to the deoxy crystal (67).

Figure 9. Models derived from x-ray crystallography of di-PLP Hb (top) and nfPLP-crosslinked Hb (bottom).


Lys
82β1
His
143β1
His
2β2
PLP
His
2β1
His
143β2
Lys
82β2
5Å


Lys
82β1
His
143β1
His
2β2
NFPLP
His
2β1
His
143β2
Lys
82β2
5Å

E. Modified hemoglobin-based blood substitutes

Because of the potential advantages discussed above, a great deal of effort has been invested in trying to develop a modified hemoglobin suitable for use as an acellular blood substitute (for reviews, see 97,107-109). Two of the key objectives in the molecular engineering of hemoglobin for this purpose are decreasing the oxygen affinity to compensate for the loss of heterotropic effectors in the plasma, and inhibiting $\alpha\beta$ dimer dissociation to prevent renal filtration. Aside from the strategy of encapsulation of hemoglobin with a heterotropic effector such as 2,3-DPG in liposomes (103), most efforts in this field have focused on generating a covalently stabilized tetramer with an acceptably low oxygen affinity.

Numerous attempts to generate a hemoglobin-based blood substitute have involved crosslinking the molecule with non-specific crosslinking agents such as glutaraldehyde and various imidoesters (for review see 97,107). Problems with these techniques have arisen due to the tendency of non-specific crosslinking to increase the oxygen affinity of hemoglobin, rendering it, in effect, a plasma expander with little oxygen-delivering capacity (104-106). An additional problem with these techniques arises from the heterogeneous nature of the modified hemoglobin solutions, which is pharmacologically undesirable. Similar problems have appeared in attempts to prevent renal filtration by coupling hemoglobin to macromolecules such as dextran (99,100), polyethylene glycol (101), and polyoxyethylene derivatives (102). In every case reported to date, non-specific crosslinking or macromolecular coupling led to an increase in oxygen affinity. One strategy to circumvent this problem involves sequentially modifying hemoglobin first with a reagent to lower oxygen affinity such as pyridoxal phosphate (64) followed by crosslinking with a reagent such as glutaraldehyde (117,118). This approach has been somewhat successful in reducing the increase in oxygen affinity brought about by crosslinking, but the P_{50} values of the pyridoxylated-polymerized hemoglobins are still much lower than

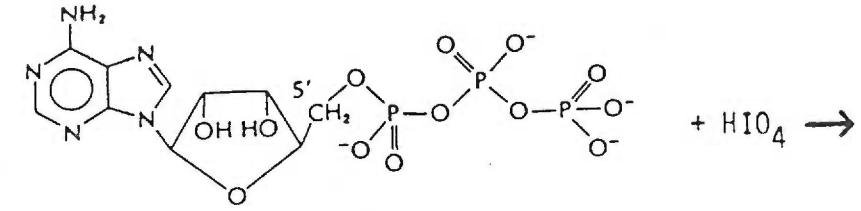
optimal (116-118). In addition, the problem of heterogeneity of the modified solution remains.

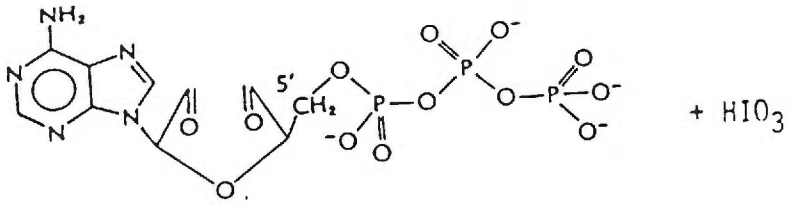
It is clear that the use of a single compound to accomplish the objectives of crosslinking and maintaining low oxygen affinity would be the ideal solution to the problem, but the lack of a suitable reagent for this purpose remains an obstacle. The structural and functional properties of nfPLP-crosslinked hemoglobin (63), the only monoderivatized low-affinity crosslinked hemoglobin described to date, are compatible with those envisioned for a hemoglobin-based acellular blood substitute (7-9), but the synthesis of nfPLP is extremely difficult and not considered commercially practicable (98).

The use of periodic acid to cleave vicinal glycols (1,2-diols) to corresponding dialdehydes has been applied to the synthesis of reactive bifunctional affinity ligands (52-56,62). The periodate oxidative cleavage reaction of 1,2, diols is thought to involve a cyclic intermediate complex which breaks down with splitting of the C-C bond and concomittant oxidation of the hydroxyl groups to aldehydes which are not oxidized further (57). This reaction chemistry can be applied to synthesize bifunctional crosslinkers from molecules with ring-containing diols such as ribonucleotides. Figure 10 shows the synthesis and structure of periodate-oxidized ATP (o-ATP), the 2',3'-dialdehyde derivative of adenosine triphosphate (52,56). The ring-opened dialdehyde structure is theoretically capable of reacting with appropriately spaced amino groups in a protein binding site to form diimine crosslinks which are further susceptible to reduction to form stable secondary amines. This compound has been used to affinity label various enzymes which utilize ATP as a substrate or cofactor (52,54,55).

Because of the known high affinity of ATP for the organic phosphate binding site of hemoglobin (58,59), an attempt was made to use the periodate-oxidized analog as an affinity label for this site with the

Figure 10. Synthesis of o-ATP by oxidation with periodate.


NH2
OH HO
5'
CH2
+ HIO4


NH2
5'
CH2
+ HIO3

objective of creating an intermolecular crosslink between amino groups on opposing β chains (60). In a preliminary paper, Scannon reported variable yields (60-80%) of modification after reacting oxyhemoglobin with o-ATP (60). He found that the modified products had slightly increased oxygen affinities and included some crosslinked species as identified by gel electrophoresis. However, these products were not quantitated or characterized further (60). In 1983 Greenburg and Maffuid reported on the reaction of o-ATP with deoxyhemoglobin rather than oxyhemoglobin. They reported a yield of modified hemoglobin in the range of 60-70% and noted the appearance of crosslinked products in the reaction mixture. They reported a slight decrease in oxygen affinity of the reaction products. These products were not quantitated, purified, or structurally characterized further.

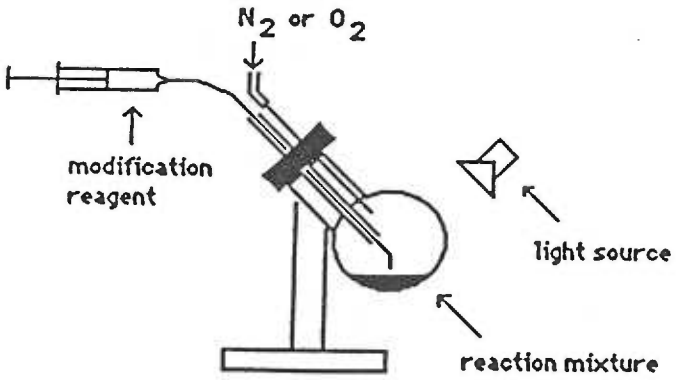
The present work is thus proposed to more fully investigate the modification of hemoglobin with bifunctional affinity ligands such as phosphorylated dialdehydes and to determine the structural and functional characteristics of the reaction products in order to evaluate their suitability as blood substitutes. Information gained from these initial studies will be used to develop an alternative molecular engineering scheme for the simultaneous crosslinking and oxygen affinity attenuation of human hemoglobin to produce a homogeneous product with characteristics appropriate for an acellular blood substitute.

MATERIALS AND METHODS

Hemoglobin preparation

Hemolysates were prepared essentially by the method of Shih et al (110). Fresh heparinized blood was obtained by venipuncture from human volunteers and centrifuged at 600 x g for 20 minutes. Plasma and leukocytes were removed by aspiration and erythrocytes were resuspended in 2-3 volumes 0.9 % NaCl at 0°C. Centrifugation and washing was repeated 3 times. Cells were then lysed by the addition of ice cold deionized water 1:1 v/v, thoroughly mixed and allowed to stand on ice for 10 minutes. Ice-cold saturated NaCl solution was then added to bring the concentration of NaCl to 2%. The hemolysate was then spun at 27,000 x g for 60 minutes to sediment erythrocyte stroma. The supernatant was carefully removed with a syringe and tubing, leaving behind the unlysed cells and stroma. The supernatant was then dialyzed overnight against a large excess of deionized water at 0° (Spectrapor 12,000-14,000 M.W. cutoff membrane tubing, Spectrum Medical Industries, Inc., Los Angeles). After dialysis, the hemolysate was once again centrifuged at 27,000 x g for 60 minutes and the pellet discarded. The hemoglobin was deionized by passage over a mixed-bed resin ion exchange column equilibrated with double-distilled water (80). Hemoglobin A was isolated by ion-exchange chromatography on DEAE-Sephacel equilibrated with 50 mM Tris-Cl pH 7.8 using a gradient of NaCl from 0 to 100 mM at 4°, in the presence of CO. Hemoglobin solutions were stored under CO at 0° in deionized water. Concentrations were determined after conversion to cyanomethemoglobin based on a millimolar extinction coefficient of 11.0 at 540 nm (82). Preparation of deoxyhemoglobin was accomplished by photoirradiation of carboxyhemoglobin in a rotary flask apparatus (81) under a stream of humidified O_2 gas for 60 minutes, followed by flushing with humidified N_2 for 180 minutes. The apparatus used for removing CO and deoxygenating hemoglobin is diagrammed in figure 11.

Figure 11. Diagram of rotary reaction apparatus.


N_2 or O_2
modification reagent
light source
reaction mixture

Preparation of bifunctional modification reagents

1. Synthesis of ring-opened dialdehyde ligand analogs

Nucleotides, pyridine nucleotides, and phosphorylated sugars were obtained from Sigma (St. Louis, MO.). Sodium periodate was from Aldrich (Milwaukee, WI.). Periodate oxidation was performed essentially following methods described by Easterbrook-Smith et al (52), with some modifications. The cis-diol was dissolved in deionized water at a concentration of 90 mM at 0°. In the case of adenosine triphosphate (ATP), phosphoribosyl pyrophosphate (PRPP), and nicotinamide adenine dinucleotide phosphate (NADP), sodium periodate was added to give a concentration of 100 mM. In the case of nicotinamide adenine dinucleotide (NAD) and glucose-1-phosphate (G-1-P), the sodium periodate concentration was 200 mM. Two mole equivalents of oxidizing agent are required for oxidation of NAD and G-1-P because there are 2 1,2-diol moieties in each molecule. After oxidation, o-NAD contains four theoretical free aldehyde functions and o-G-1-P contains two free aldehyde functions with one mole equivalent of formate produced during the oxidation (figure 29). The reaction with periodate was allowed to proceed for 60 minutes protected from light at 0°. The reaction was stopped by the addition of 10 mM ethylene glycol. Periodate-oxidized nucleotides and pyridine nucleotides were purified by chromatography on a Sephadex G-10 column (1.2 x 90 cm) equilibrated with deionized water at 4°. Eluant was monitored at 280 nm, and iodate was assayed qualitatively by precipitation upon addition of an equal volume of saturated $AgNO_3$ to aliquots of column eluant. The leading half of the nucleotide or pyridine nucleotide peak was pooled and assayed by thin layer chromatography on 10 cm x 10 cm polyethyleneimine-cellulose sheets (E. Merck, Darmstadt, W. Germany) using 0.8 M NH_4HCO_3 as the developing solvent. Spots were visualized with UV light. Oxidized reagents remained at the origin, while control non-oxidized compounds migrated with R_f values ranging from 0.3 to 0.5. All

oxidized reagents were stored at -70° in the dark.

2. Other modification reagents

The bifunctional reagents 2,6-pyridine dicarboxylic acid chloride and 5-formyl salicylaldehyde were obtained from Aldrich (Milwaukee, WI). 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) was obtained from Pierce (Rockford, IL), or Fluka (Ronkomkoma, NY). DIDS was stored dessicated and protected from light at 4°. Iodoacetamide and ethylene imine were from Pierce. Pyridoxal phosphate was from Boehringer-Mannheim (Indianapolis, IN).

Reaction of hemoglobin with modification reagents

The following is a general outline of the reaction conditions of hemoglobin with site-directed modification reagents. Specific details of reaction conditions for various modification experiments are given in the results section.

Freshly isolated hemolysate or purified hemoglobin A was used for reaction with various modification reagents. Carbon monoxide was removed by photoirradiation under a stream of humidified O_2 for 60 minutes (81). Hemoglobin was diluted to either 0.25 or 0.50 mM in 50 mM buffer. Four different buffer systems were employed: 1) 50 mM bis-Tris-Cl pH 6.8; 2) 50 mM potassium phosphate pH 7.4; 3) 50 mM Tris-Cl pH 7.8; 3) 50 mM borate pH 9.0.

1. Reactions with deoxyhemoglobin

For modifications of deoxyhemoglobin, the protein was deoxygenated in the rotary apparatus shown in figure 11 by passing a stream of N_2 gas through the flask for 3-4 hours. Modification reagent concentrations ranged from 0.25 mM to 5 mM (molar ratios of reagent to hemoglobin from 1:1 to 10:1). Modification reagents were

introduced for reaction with deoxyhemoglobin by first degassing the reagent solution under vacuum followed by nitrogen bubbling through the solution for 10 minutes. The reagent solution was then immediately drawn into a 1 ml nitrogen-flushed syringe fitted with a needle attatched to a 35 cm length of fine-bore teflon tubing (figure 11). The solution was then introduced anaerobically into the reaction flask to give the desired concentration of reagent. During the course of the reaction, N_2 gas was continuously flushed through the rotary apparatus to maintain the protein in the deoxy state. In the case of the reductive alkylations with the dialdehyde reagents, deoxygenated reducing agent was introduced into the reaction vessel following the introduction of the modification reagent. Sodium borohydride (10 mM) was introduced after a period of time from 30 to 60 minutes after addition of dialdehyde. Sodium cyanoborohydride (10 mM) was added immediately after addition of dialdehyde. Aliquots were removed anaerobically at various times through the teflon tubing attached to the syringe in order to study reaction kinetics. Reaction with all modification reagents was stopped by chromatography on a Sephadex G-25-F column equilibrated with 50 mM buffer at 4°.

Protein, peptide, and amino acid HPLC

1. HPLC system components

One HPLC system was used for protein separations and another for peptide separations and amino acid analysis. The first consisted of an Altex model 332 gradient liquid chromatograph with a model 420 microprocessor system controller, two model 110A single-piston reciprocating pumps with a dynamically stirred gradient mixing chamber, and a model 210 syringe-loading sample injection valve. The detector was a Hitachi model 100-30 variable wavelength (visible/UV) unit equipped with an 8 µl analytical flow cell, connected to a Hewlett Packard model 3392A integrating recorder. Effluent was collected with a Gilson FC-80K micro fractionator. For

amino acid analysis an IBM LC/9533 ternary gradient liquid chromatograph equipped with an IBM LC/9505 autosampler was used. An IBM LC/9522 254 nm fixed-wavelength detector was used to detect phenylthiocarbamyl amino acid derivatives (89). The detector was connected to an IBM System 9000 computer which performed peak integration and served as system controller. For peptide chromatography an IBM LC/9533 HPLC was used with a 214 nm fixed-wavelength LC/9522 detector coupled in line with a variable wavelength LC/9533 detector, allowing sequential monitoring of peaks at two different wavelengths. This was employed for modified peptide detection in cases where a chromophoric ligand was used for covalent modification. Peptides were collected with a Gilson FC-80K micro-fractionator. This system was also interfaced with the IBM 9000 computer for peak integration and system control.

2. HPLC of chemically modified hemoglobins

Analytical cation-exchange HPLC was performed to separate modified hemoglobin species using a poly-aspartic acid 5 μm silica-based column with a 4.6 x 250 mm 300 Å pore size (Polycat-A, Custom LC, Inc., Houston TX), following the procedure of Ou et al. (83) with some modifications. Hemoglobins were separated using a linear gradient between buffer A (40 mM bis-Tris, 4 mM KCN, pH 6.5) and buffer B (40 mM bis-Tris, 4 mM KCN, 0.2 M NaCl, pH 6.8). The gradient ran from 0% B to 56% B in 24 minutes, then from 56% B to 100% B in 10 minutes. Alternatively, analytical cation-exchange HPLC was accomplished with a 4.1 x 250 mm Synchropak CM-300 carboxymethyl-silica based column using the same buffers and gradient. Eluant was monitored at 419 nm, and peaks were integrated by an IBM System 9000 (IBM Instruments, Inc., Danbury, CT.). For rapid qualitative analysis of reaction products, electrophoresis on cellulose acetate sheets of up to eight samples using Tris-EDTA-borate buffer, pH 8.8, was performed (84).

3. HPLC of chemically modified globins

Reversed-phase chromatography on a Vydac C_4 330 Å pore size column (The Separations Group, Hesperia, CA) was performed in order to resolve globin chains (85). Analytical separations (0.1 mg hemoglobin) were achieved with a 4.6 x 250 mm column, while preparative separations (1-5 mg hemoglobin) were done with a 12 x 250 mm column. Eluant was monitored at 220 nm. Globins were analytically resolved utilizing a gradient between buffer A (0.1% TFA, 80% H_2O, 20% acetonitrile), and buffer B (0.1% TFA, 40% H_2O, 60% acetonitrile) of 44% B to 56.5% B in 60 minutes. Preparative separations were achieved with a gradient of 46% B to 55% B in 60 minutes followed by purging with 100% B after each run.

4. HPLC of peptides

Partial enzymatic or chemical hydrolysates of modified globins were chromatographed by reversed-phase HPLC on a 4.6 x 250 mm 330 Å Vydac C_{18} column (The Separations Group, Hesperia, CA. Cat.# 218TP54.6) using a gradient between solvents A (0.1% trifluoroacetic acid in H_2O) and B (0.1% trifluoroacetic acid in acetonitrile). The gradient program ran from 0% B to 13.6% B in 20 minutes, then to 34% B in 50 minutes, and then to 100% B in 5 minutes followed by reequilibration in 100% A. An alternative reversed-phase solvent system was employed to rechromatograph peptides using a similar gradient between 10 mM ammonium acetate buffer, pH 6.0, and acetonitrile. Peptides were collected in tubes and lyophilized. Peptides for amino acid analysis were generally rechromatographed before hydrolysis.

5. Amino acid analysis

Peptides were hydrolyzed in evacuated tubes using 6M HCl vapor at 110° for 22 hours. Amino acid analysis was done by precolumn derivitization of amino acid hydrolysates with phenyl isothiocyanate

(89) followed by reverse-phase HPLC of the phenylthiocarbamyl amino acid derivatives using an IBM model LC/9533 chromatograph and an IBM octadecyl silane column (Cat. # 8635308). Effluent was monitored at 254 nm. Integration and amino acid quantitation were performed with an IBM System 9000 computer.

Ion-exchange liquid chromatography

Preparative-scale chromatography was done by ion exchange on either DEAE-Sephacel or CM-Sephadex ion-exchange resins (Pharmacia, Piscataway, N.J.). Anion-exchange chromatography was done with 50 mM Tris-Cl buffer, pH 7.8, using a linear NaCl gradient. Cation exchange chromatography was done with 50 mM Tris-maleate buffer, pH 6.5, using a gradient of increasing pH and NaCl concentration. All hemoglobins were chromatographed as the carboxy derivative with column buffer run through a pre-column CO trap to equilibrate. After elution, peak fractions were pooled and concentrated in a magnetically stirred pressure ultrafiltration chamber (Amicon Corp., Danvers, MA).

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE) was done according to the procedure of Laemmli (86). A 15% polyacrylamide gel (2.7% crosslinked) was used. Hemoglobins were prepared for electrophoresis by heat denaturation in a buffer containing 65 mM Tris-Cl pH 6.8, 2% SDS, 10% v/v glycerol, and 5% v/v 2-mercaptoethanol. Approximately 20 µg of protein in 10 µl were applied to the gel, and electrophoresed at 20 mA for approximately 8 hours.

Enzymatic and chemical hydrolysis of hemoglobin and globin

1. Trypsin and chymotrypsin

Hydrolysis with TPCK-treated trypsin (Worthington) was carried out at 37° in 80 mM ammonium carbonate buffer, pH 8.5, for 8-12 hours with a ratio of trypsin to protein of 1:50 by weight. In some cases, β globin or chemically modified β globin was aminoethylated with ethylene imine to alkylate cysteine residues in the protein core prior to tryptic hydrolysis (88). Alternatively, specific acetamidation of β–93 cys was accomplished by reacting hemoglobin (0.25 mM) with iodoacetamide (2.5 mM) in 10 mM phosphate buffer pH 7.0 for 2 hours at 20° in the dark (95). This led to virtually complete acetamidation of β-93 cys. The tryptic hydrolysate was lyophilized after digestion and then dissolved in deionized water. Prior to injection onto the HPLC column, the hydrolysate was centrifuged and filtered through a 0.45 μm filter. Enzymatic digestion with chymotrypsin (Worthington) was performed exactly as with trypsin.

2. Partial acid hydrolysis

Chemical cleavage of globin chains by the preferential splitting out of aspartic acid residues using acetic acid was performed after the method of Partridge and Davis (87). Isolated globin chains or whole hemoglobin was refluxed in 0.25 M acetic acid in an evacuated and sealed tube at 100° for 24 hours. The hydrolysate was then lyophilized and redissolved in deionized water, then centrifuged and filtered through a 0.45 μm filter before HPLC.

Spectrophotometry

UV and visible wavelength spectrophotemetry was routinely done with a Gilford model 250 uv/vis spectrophotometer for determining concentrations of hemoglobin and modification compounds. Scanning

spectrophotometry of modified hemoglobins, peptides and modification reagents was done with a Varian Cary model 219 spectrophotometer set at a scan rate of 1.0 nm/sec, period of 1 second, and auto slit gain of 0.30. A Hewlett-Packard diode-array scanning UV/vis spectrophotometer was also used for scanning modified hemoglobins and peptides.

X-ray crystallography

X-ray crystallography was performed by Dr. Max Perutz at the Medical Research Council Laboratory of Molecular Biology in Cambridge, England using a Nonius Cad 4 x-ray diffractometer.

Functional testing

Oxygen equilibria determinations for control and modified hemoglobins under various conditions described in the text were performed by Dr. Daniel T.-B. Shih at the Oregon Health Sciences University Department of Biochemistry. Equilibrium curves were measured with an automatic recording apparatus designed after Imai et al. (90). Data was acquired and processed by a PDP 11/VO3 microcomputer (Digital Equipment Corp.)(91).

RESULTS AND DICUSSION

Reaction of hemoglobin with ring-opened dialdehyde ligands

1. Periodate-oxidized adenosine triphosphate (o-ATP)

A. Binding of o-ATP to hemoglobin

In order to demonstrate specific oxygenation-linked binding of o-ATP to hemoglobin, apparent binding constants for o-ATP were determined for both oxy and deoxyhemoglobin. An Amicon ultrafiltration apparatus (see Materials and Methods) was used to separate bound from free ligand. Concentration of o-ATP in the ultrafiltrate was determined spectrophotometrically assuming an extinction coefficient of 14.9 $mM^{-1} c m^{-1}$ at 258 nm (52). Oxy or deoxyhemoglobin (0.25 mM, 50 mM Tris-Cl pH 7.6, 0°) was mixed with 0.25 mM o-ATP and then immediately subjected to ultrafiltration. Apparent binding constants were determined using the equation:

$$K_d = [\text{o-ATP}]_{free} [\text{Hb}]_{free} / [\text{Hb o-ATP}]_{bound} = 1 / K_a$$

It was found that the o-ATP binding constant for deoxyhemoglobin ($K_a^{deoxy} = 1.1 \times 10^7\ M^{-1}$) was over two orders of magnitude greater than for oxyhemoglobin ($K_a^{oxy} = 6.4 \times 10^4\ M^{-1}$), indicating an oxygen ligation-state dependence on o-ATP binding which parallels other heterotropic ligand binding to the organic phosphate binding site. These values also indicate a high affinity binding, similar to values reported for IHP binding to hemoglobin ($K_a^{deoxy} = 1.7 \times 10^7$, $K_a^{oxy} = 1.1 \times 10^4$) (93), and higher than reported for DPG ($K_a^{deoxy} = 3 \times 10^4$, $K_a^{oxy} = 8 \times 10^2$) (92) or ATP binding ($K_a^{deoxy} = 1.2 \times 10^4$, $K_a^{oxy} = 3.9 \times 10^2$) (59). Although imine formation between o-ATP and amino residues on the protein is theoretically reversible in the absence of reducing agent, such formation would tend to shift the equilibrium towards the bound complex, resulting in a higher apparent binding

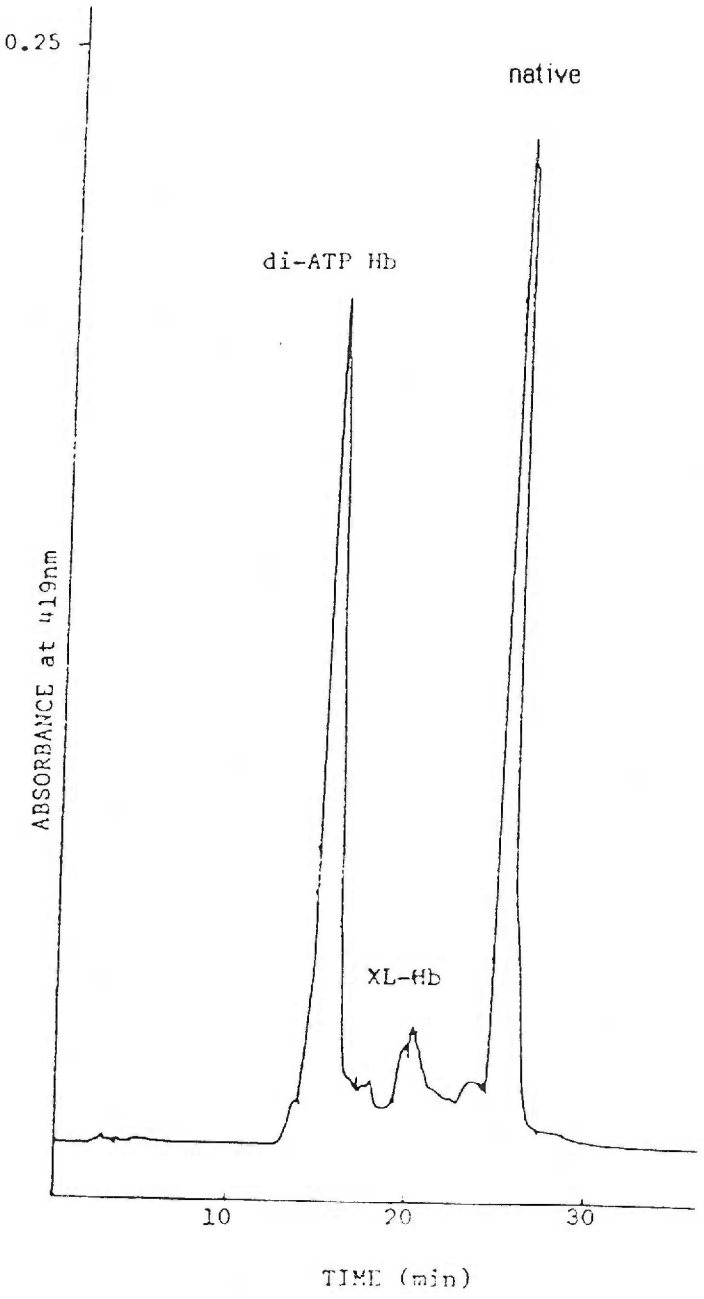
constant.

B. Synthesis of di-ATP Hb

After reacting 0.25 mM deoxyhemoglobin with 0.27 mM o-ATP in 50 mM Tris buffer pH 7.8 at 0° for 45 minutes, followed by reduction with sodium borohydride, the reaction was halted by chromatography on a Sephadex G-25 column equilibrated in reaction buffer, and then chromatographed by cation-exchange HPLC (figure 12). The HPLC elution profile shows the appearance of one major peak (designated fraction 1), in addition to two minor peaks (designated fractions 2 and 3) which eluted in positions intermediate between unmodified hemoglobin and fraction 1. The elution positions of the modified products indicated that they had increased negative charge relative to Hb A, with fraction 1 having the greatest increase followed by fraction 2 and then fraction 3. The time course of the reaction was followed by removing aliquots at various times after addition of sodium borohydride and quantitating the amount of modified hemoglobin by integrating the peaks from cation-exchange HPLC (figure 14). It can be seen that there is an increasing amount of modification with time up to a limit of approximately 50% modified product. Increasing the molar ratio of o-ATP to hemoglobin to 2:1 did not affect the yield of modified products. When the ratio of reagent to protein was increased to 10:1, however, a marked increase in the amount of modification resulted. It appeared that this increase was due to an increase in the amount of non-specific modifications to the protein, as numerous new peaks appeared in the reaction mixture which eluted rapidly from the cation-exchange HPLC column (figure 15). When 2 mM IHP was present in the reaction mixture containing a stoichiometric quantity of o-ATP, modification was blocked, suggesting that the reaction was occurring in the organic phosphate binding site.

Fractions 1,2, and 3 were purified by preparative ion-exchange chromatography on CM-Sephadex with a pH gradient consisting of 50 mM bis-Tris-Cl run from pH 6.6 to 7.2, and were then analyzed by

Figure 12. Cation-exchange HPLC of o-ATP-Hb reaction mixture.


0.25
native
di-ATP Hb
XL-Hb
ABSORBANCE at 419nm
10
20
30
TIME (min)

SDS-PAGE. As shown in figure 13 the major modified product, fraction 1, displayed two bands, one running in the position of unmodified globin (16.5 kD) and a second band running only slightly slower. Fractions 2 and 3, on the other hand, each contained polypeptide which migrated with an M_r of approximately 34,000, demonstrating the presence of an intermolecular crosslink. Analytical reversed-phase C_4 HPLC of fraction 1 was performed to separate α and β globin chains (figure 16). The elution profile of the globins indicated the presence of a peak eluting in the position of native α globin and a second peak eluting slightly faster than native β globin. Further structural information about the modification site(s) was obtained by tryptic mapping of isolated globins from fraction 1 which were purified by preparative reversed-phase C_4 HPLC. Figure 17 shows the tryptic peptide reversed-phase HPL chromatograms of α chains from both native hemoglobin and from fraction 1. No differences appear in the two peptide patterns. Figure 18 shows the reversed-phase HPL chromatograms of β globin tryptic peptides from both native hemoglobin and fraction 1, with an arrow indicating the absence of βt-9 (tryptic peptide 9) in fraction 1 β globin. Tryptic peptide 9 from native β globin, containing residues 67-82, is normally released by proteolysis at the C terminal sides of Lys 66 and Lys 82. The possibility that modification of one of these lysine residues had occurred, blocking enzymatic cleavage and release of βt-9, was thus investigated. Lys 66 β (E10) is a residue which is found in the heme crevice of the molecule relatively far removed from the organic phosphate binding site where o-ATP presumably binds (119). Lys 82 β (EF6), on the other hand, occupies a position in the EF corner of the molecule which borders the organic phosphate binding site (figure 1). This lysine has indeed been shown by x-ray crystallography to be involved in critical salt-bridge formation with various bound polyanions (17, 72), and would presumably be favorably situated for Schiff-base formation to occur between the aldehyde group(s) in a molecule of bound o-ATP and the ε-amino group in the lysine residue.

If the loss of βt-9 from the tryptic hydrolysate of the fraction 1 β

Figure 13. SDS-PAGE of o-ATP modified hemoglobins. Lane 1: standard crosslinked bovine hemoglobin mixture (17K,34K,41K,58K,75K)(Sigma); Lane 2: fraction 3; Lane 3: fraction 2; Lane 4: fraction 1

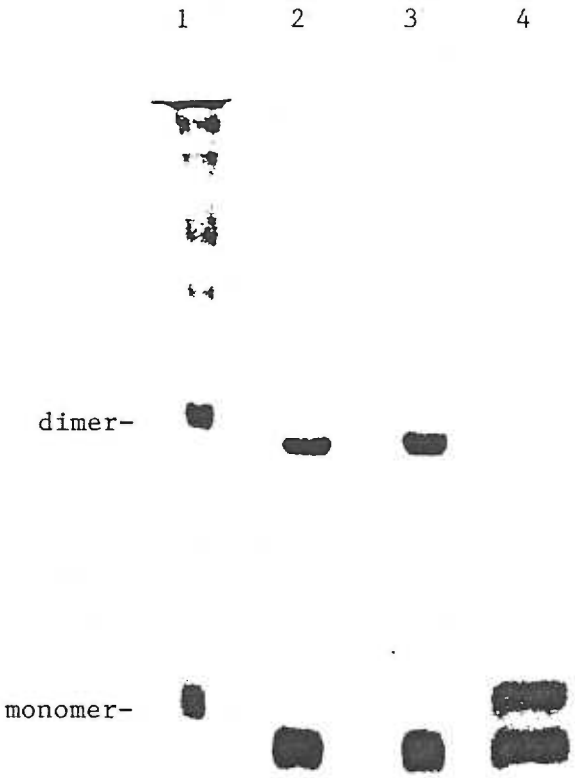

1
2
3
4
dimer-
monomer-

Figure 14. Time course for modification of 0.25 mM hemoglobin with 0.27 mM o-ATP in 50 mM Tris buffer pH 7.8, 0°.

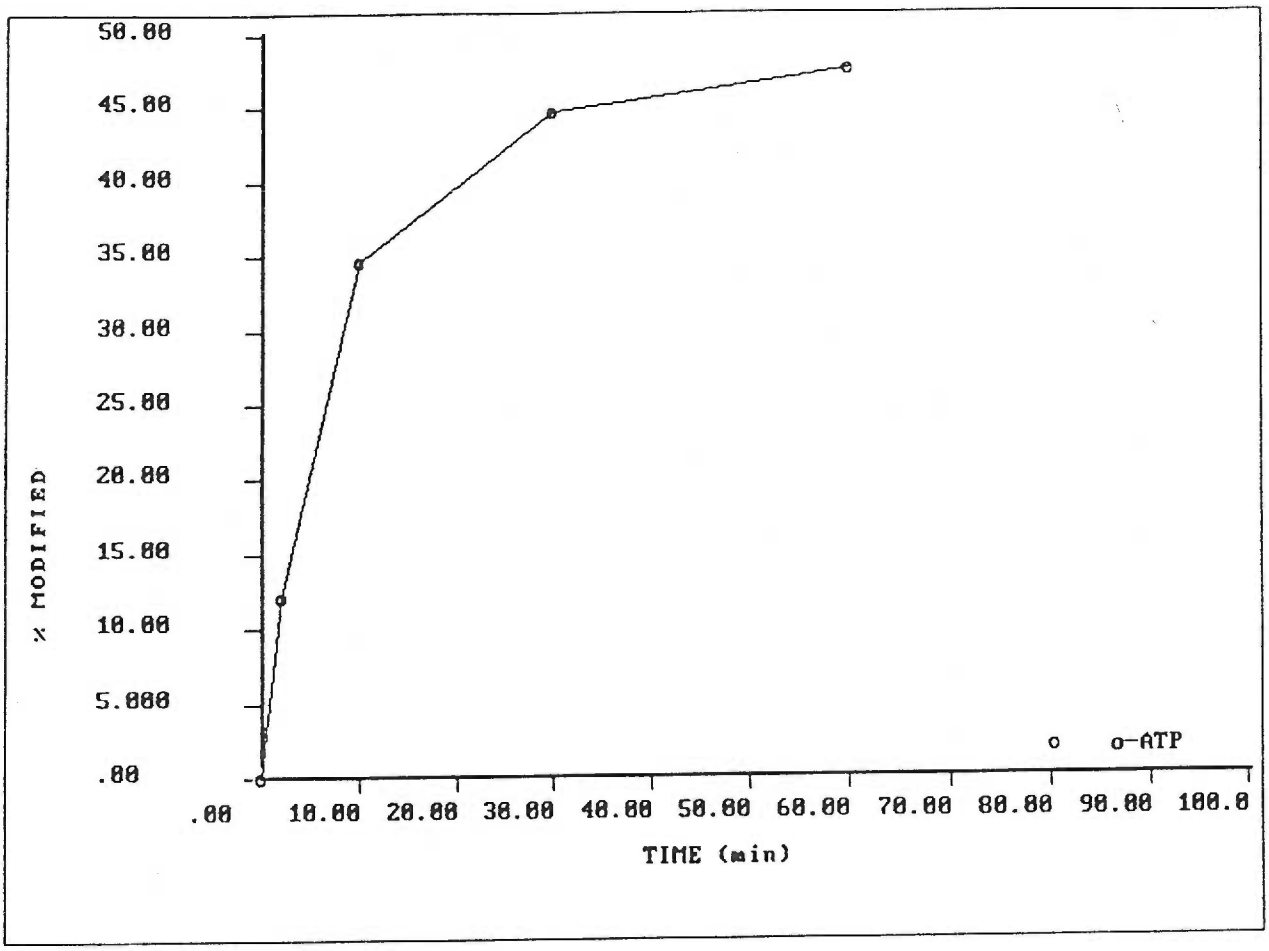

50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.000
.00
% MODIFIED
o o-ATP
.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.0
TIME (min)

Figure 15. Cation-exchange HPLC of poly (o-ATP) Hb after reaction of .25 mM Hb with 2.5 mM o-ATP in 50 mM Tris pH 7.8, 0°.

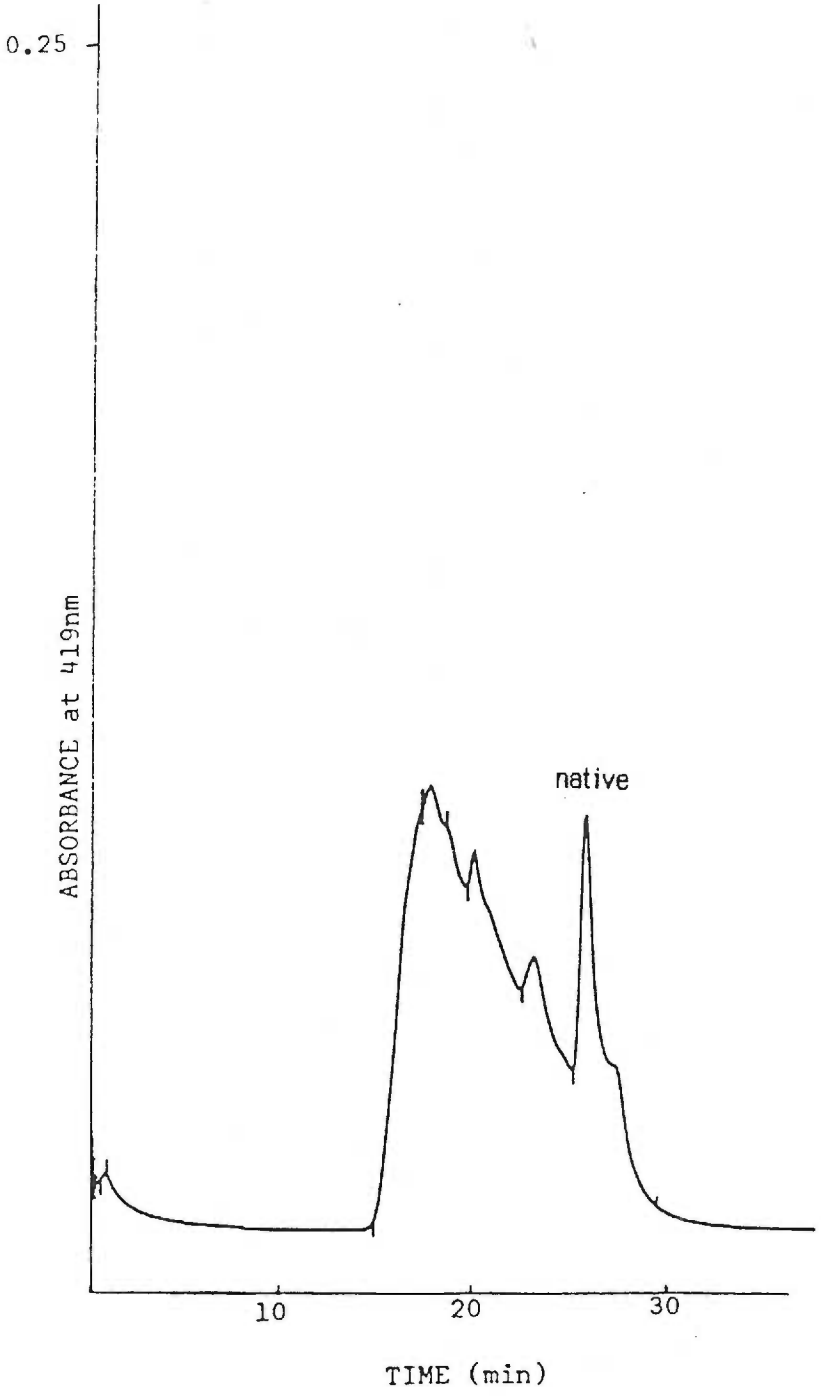
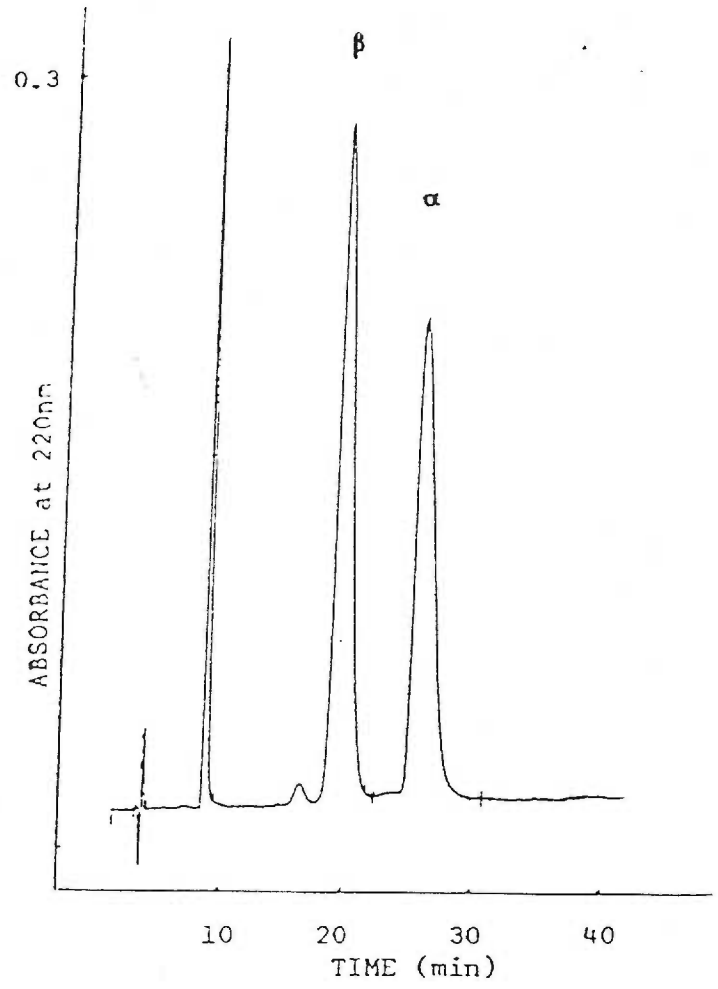

0.25
ABSORBANCE at 419nm
native
10
20
30
TIME (min)

Figure 16. Reversed-phase C_4 HPLC of native Hb (top) and di-ATP Hb (bottom). Peak eluting at 9.5 minutes is heme.


β
α
0.3
ABSORBANCE at 220nm
10
20
30
40
TIME (min)

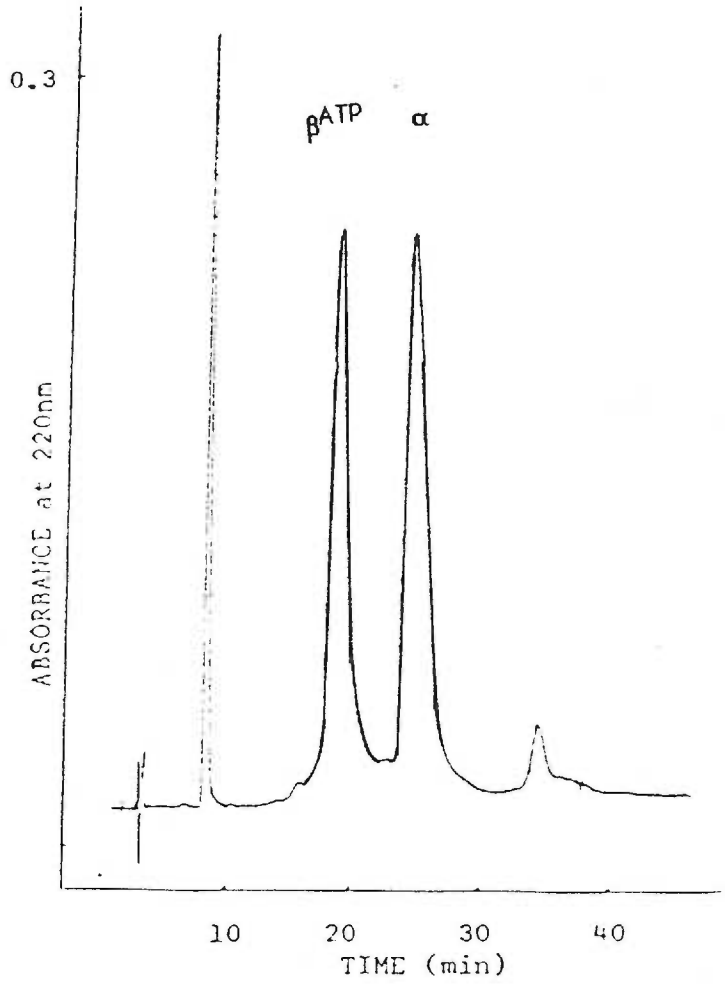
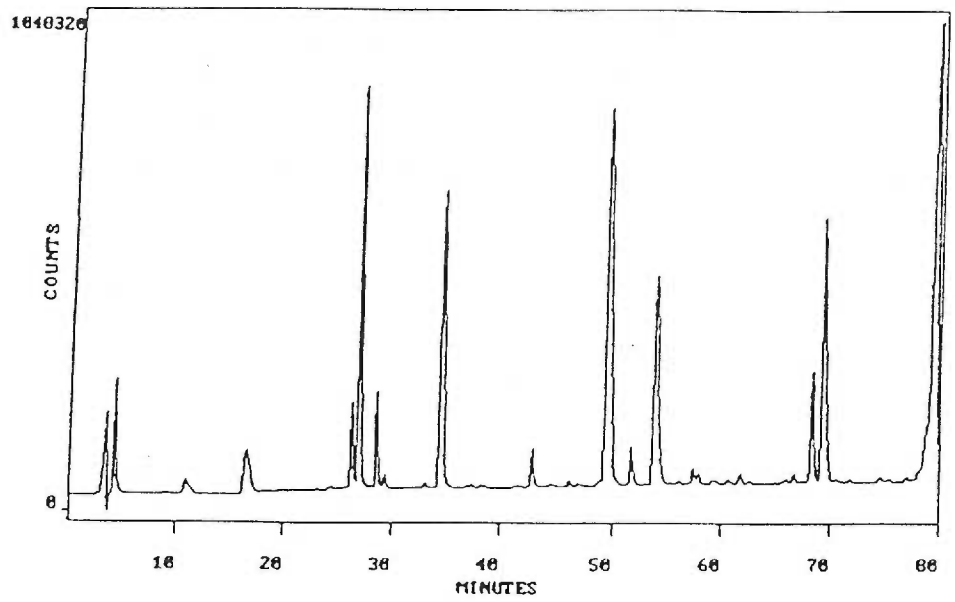

0.3
βATP
α
ABSORBANCE at 220nm
10
20
30
40
TIME (min)

Figure 17. HPLC of tryptic peptides from digestion of α globin from native Hb (top) and di-ATP Hb (bottom). Eluant is monitored at 214 nm.


1840320
COUNTS
0
10
20
30
40
50
60
70
80
MINUTES

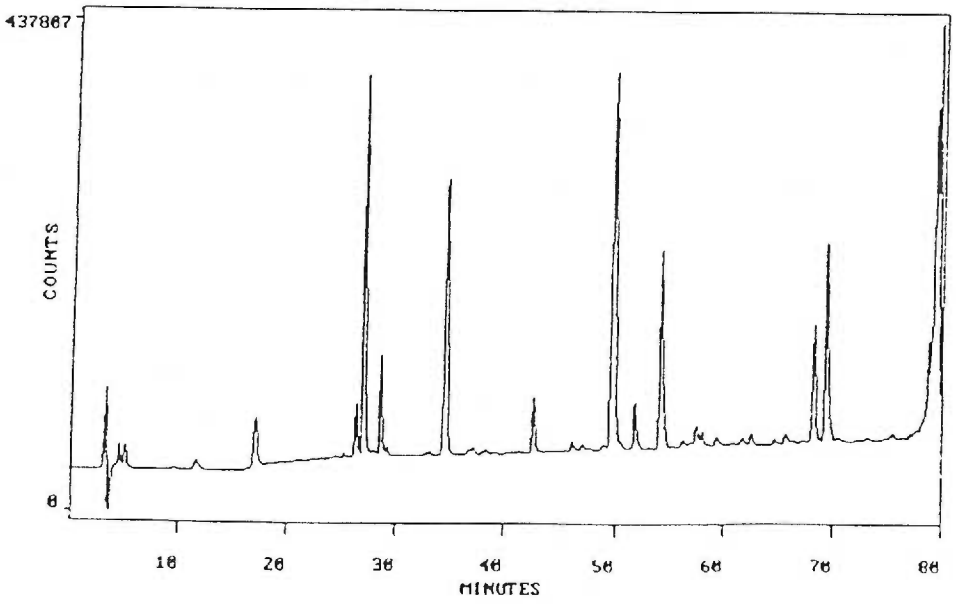
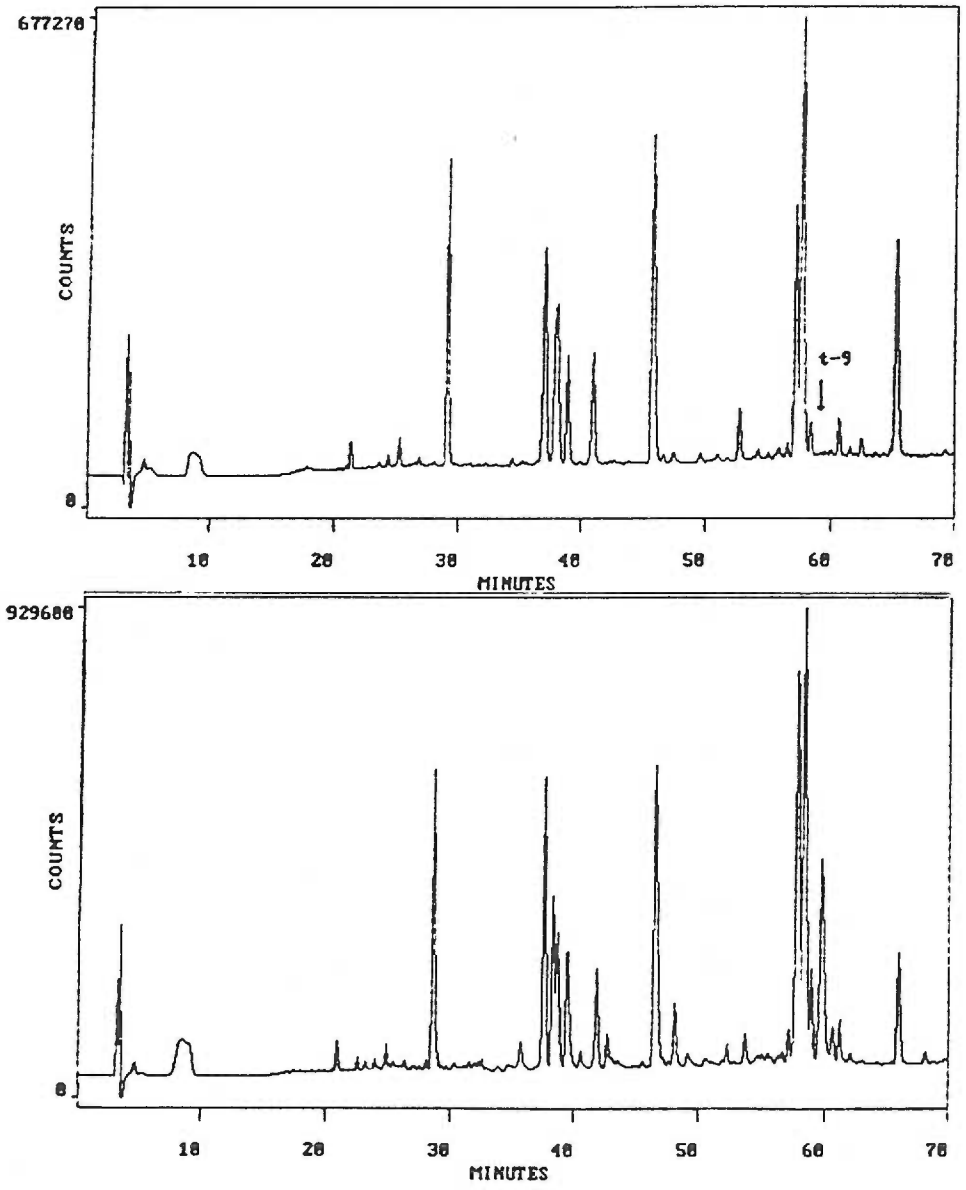

437867
COUNTS
0
10
20
30
40
50
60
70
80
MINUTES

Figure 18. HPLC of tryptic peptides from digestion of β globin from native Hb (top) and di-ATP Hb (bottom). Eluant is monitored at 214 nm.


677270
COUNTS
0
t-9
10
20
30
40
50
60
70
MINUTES
929600
COUNTS
0
10
20
30
40
50
60
70
MINUTES

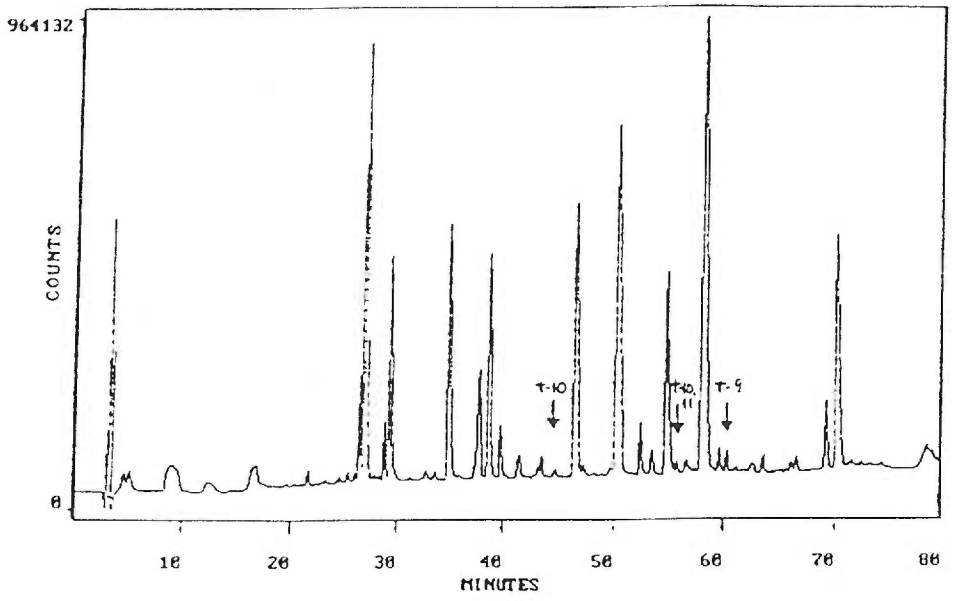
globin were due to inhibition of cleavage at Lys 82 rather than modification of another residue in the peptide, it would require that the adjacent C-terminal peptide, βt-10 (residues 83-95), also be missing from the tryptic hydrolysate. Because unmodified βt-10 is an insoluble peptide it is normally not detected in the tryptic hydrolysate chromatogram (75). It is, however, possible to selectively acetamidate hemoglobin using iodoacetamide at a residue found in βt-10, Cys 93. This modification leads to solubilization of the modified βt-10 after tryptic hydrolysis (95). After acetamidation of fraction 1 followed by trypsin digestion, both peptides βt-9 and βt-10 were absent from the HPL chromatogram, presumably as a large insoluble fragment in the hydrolysate (figure 19). These results demonstrate that there is a blockage of enzymatic cleavage at Lys 82 β presumably resulting from modification of the ε-amino group.

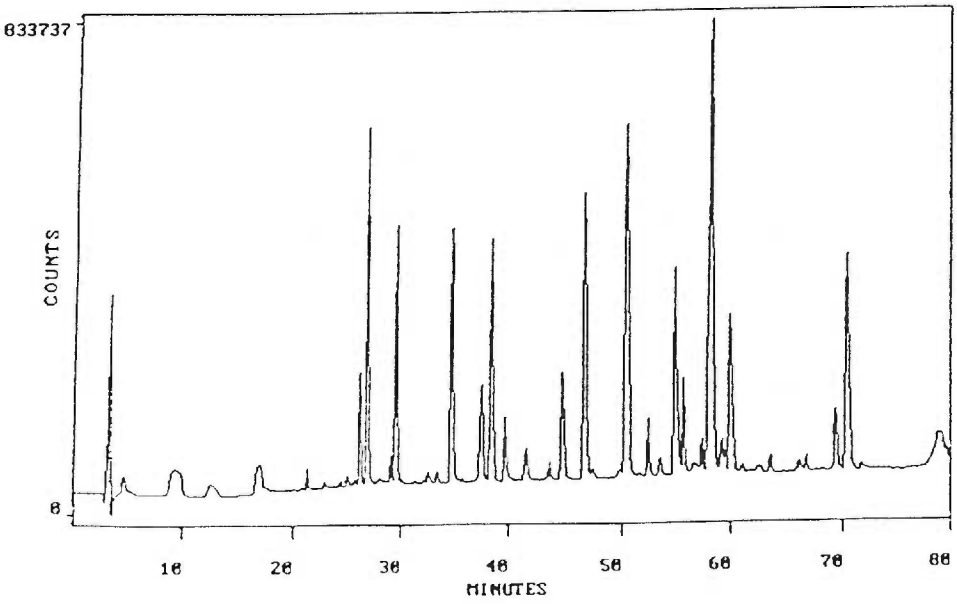
Because there is a complete absence of βt-9 and βt-10 from enzyme digests of fraction 1, cleavage at both lys 82 β_1 and lys 82 β_2 must be blocked, suggesting that both residues are involved in adducts. This implies that there are two molecules of o-ATP bound per tetramer, since there is no intermolecular crosslink between β_1 and β_2 as demonstrated by SDS-PAGE (figure 13). Because o-ATP has a large extinction coefficient at 259 nm (14.9 $mM^{-1}cm^{-1}$) (52), it was possible to obtain an estimate of the number of molecules of o-ATP associated with fraction 1 by spectroscopy. Table 1 gives data on the absorbance at 540 nm and 259 nm of native hemoglobin in comparison with the o-ATP modified forms. Determination of the molar concentration of hemoglobins by measurement of absorbance at 540 nm allowed the calculation of extinction coefficients at 259 nm for both native and modified species. By determining the $\Delta\varepsilon_{259}$ for the modified species, it was possible to show that there are at least 1.7 molecules of o-ATP per tetramer, or 0.85 molecules per β chain in fraction 1. This number is likely to be a low estimate since there is apt to be some shielding of the adenosine ring from the solvent by the protein. Because of the stoichiometric quantity of o-ATP in the reaction mixture and the fact that approximately 50% of the

TABLE 1. Determination of ε_{259} for native and o-ATP modified hemoglobins. Hemoglobin concentration was determined in the cyanmet form on the basis of $\varepsilon_{540}^{CNMetHb} = 44\ mM^{-1}cm^{-1}$ (94). $\varepsilon_{259}^{o\text{-}ATP} = 14.9\ mM^{-1}cm^{-1}$ (52).

Hemoglobin	Abs_{540}(conc.)	Abs_{259}	$\varepsilon_{259} mM^{-1}$	$\Delta\varepsilon_{259} mM^{-1}$
native	0.264 (6.00 μM)	0.618	103.0	(0)
fraction 1	0.269 (6.11 μM)	0.785	128.5	25.5
fraction 2	0.274 (6.23 μM)	0.706	113.2	10.2
fraction 3	0.250 (5.68 μM)	0.650	114.4	11.4

Figure 19. HPLC of tryptic peptides from digestion of iodoacetamide-modified HbA (bottom) and di-ATP Hb (top). Peptides βt-9, βt-10, and βt10-11 are missing from the di-ATP Hb hydrolysate.


964132
COUNTS
0
T-10
T-9
10
20
30
40
50
60
70
80
MINUTES


833737
COUNTS
0
10
20
30
40
50
60
70
80
MINUTES

hemoglobin was modified, the upper limit on the average number of molecules of o-ATP which could be incorporated is two per tetramer. Because of these considerations and the homogeneous appearance of fraction 1 on cation-exchange HPLC (figure 12) and reversed-phase C_4 HPLC (figure 16), it was concluded that fraction 1 consists of di-ATP Hb, with Lys 82 from each β chain involved in an adduct with one molecule of o-ATP.

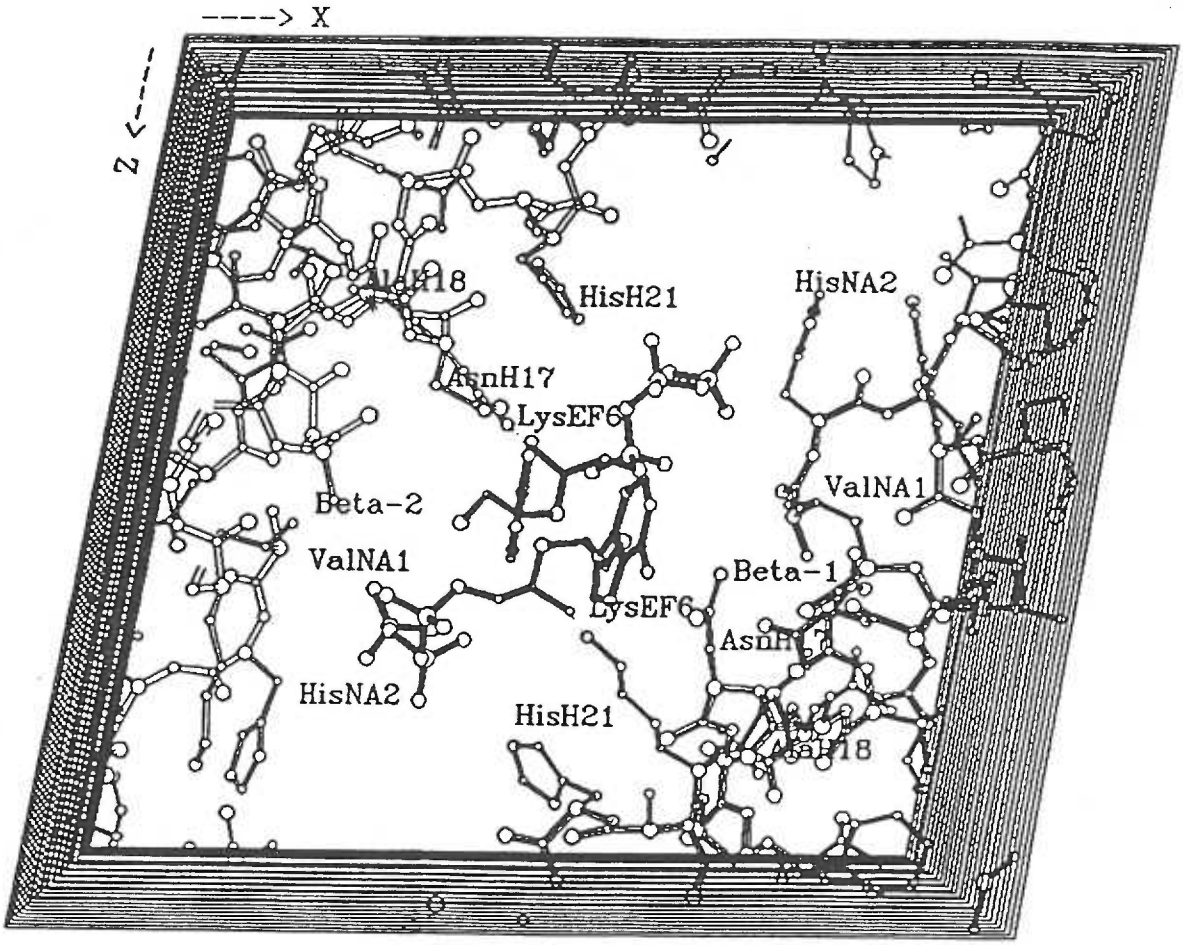
The fact that the yield of di-o-ATP Hb did not exceed 50% even when a two-fold molar excess of o-ATP was added suggested a mechanism for the reaction involving an initial binding step of one molecule of o-ATP per molecule of deoxyhemoglobin, followed by covalent modification of one of the Lys 82 β residues in the tetramer. In the deoxy state in which the reaction proceeds, subunit dissociation is greatly inhibited compared to oxyhemoglobin (120). If binding of one molecule of o-ATP to the β-cleft of deoxyhemoglobin leads to less favorable conditions for a second molecule binding, then the reaction mixture will consist largely of deoxyhemoglobin tetramers which have incorporated a single molecule of o-ATP. After reoxygenation, the following equilibrium is established between tetramer and dimer:

$$2\,\alpha_2\beta\beta^{ATP} \Leftrightarrow 2\,\alpha\beta + 2\,\alpha\beta^{ATP} \Leftrightarrow \alpha_2\beta_2 + \alpha_2\beta_2{}^{ATP}$$

Thus, during ion-exchange chromatography in the presence of oxygen there will be a net separation of the hybrid $\alpha_2\beta\beta^{ATP}$ molecules into $\alpha_2\beta_2$ tetramers and the fraction 1 di-ATP Hb product, $\alpha_2\beta_2{}^{ATP}$, in a one to one ratio, corresponding to the pattern seen during cation-exchange HPLC.

X-ray crystallography of di-ATP Hb done by Dr. M. F. Perutz is in agreement with the structure postulated from biochemical evidence. The electron density map obtained at 2.8 Å resolution allowed construction of a model in which there are two molecules of ATP inserted into the $\beta_1\beta_2$ cleft centered near Lys 82 β_1 and Lys 82 β_2 (figure 20). The planes of the adenosine rings are arranged in a

Figure 20. Molecular model of organic phosphate binding site constructed from x-ray crystallographic study of di-ATP Hb at 2.8 Å resolution.



Scale = 6.9888 mm/Å

abs.scale 2.8Å

parallel "stacked" configuration 3.4 Å apart reminiscent of the base stacking in a DNA helix. The phosphate groups are in positions which could allow ionic interactions with the β chain Val 1 α-amino termini, His 143 residues, and His 2 residues. Although the ribose ring appears to be in an open conformation, at this resolution it is not possible to conclusively state whether the adduct involving the ε-amino group of Lys 82 β is a monofunctional secondary amine involving only one of the aldehyde groups from o-ATP, with the second aldehyde unreacted, or a bifunctional morpholino derivative (54,55) involving both aldehydes from the o-ATP group.

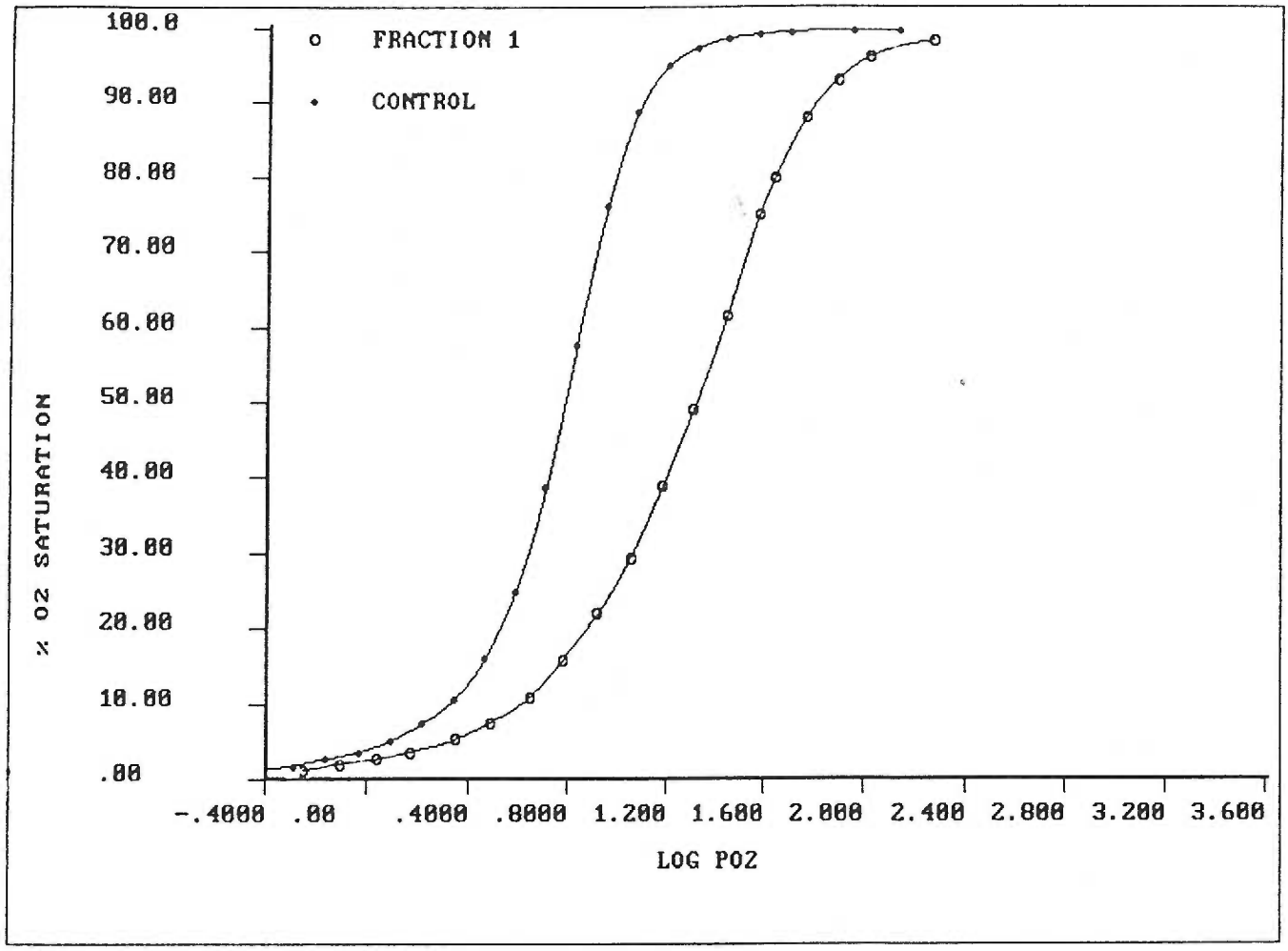
Functional studies with di-ATP hemoglobin reveal that it has a dramatically decreased oxygen affinity compared to native hemoglobin under the conditions tested (pH 7.4, 50 mM Tris, 0.1M Cl^-, 20°) (figure 21). The P_{50} of the molecule is increased from 5.8 to 20.8 mm Hg. Another functional change in di-ATP Hb is its reduced subunit cooperativity compared to unmodified hemoglobin, reflected in the reduced slope of the oxygenation curve (n_{max}=1.9).

C. Synthesis of mono-ATP crosslinked hemoglobin

Because the molecular engineering objectives for a modified hemoglobin blood substitute call for the synthesis of a low oxygen affinity molecule which is crosslinked, efforts were directed at the characterization of fractions 2 and 3 which had been shown by SDS-PAGE to contain intermolecular crosslink(s). Attempts were also made to increase the yield of the crosslinked fractions in the reaction mixture, which was less than 10% of the modified hemoglobin.

Spectroscopic analysis of fractions 2 and 3 indicated that each fraction contained 1 molecule of o-ATP per molecule hemoglobin (Table 1). This was also consistent with the elution from ion-exchange columns of fractions 2 and 3 in positions intermediate between fraction 1 (di-ATP Hb) and native hemoglobin. These data led to the hypothesis that o-ATP was crosslinking β chains in fractions 2

Figure 21. Oxygen equilibrium curve for fraction 1 (di-ATP Hb) 60 μM in 50 mM bis-Tris pH 7.4 0.1 M Cl, 20°.


100.0
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
.00
% O2 SATURATION
FRACTION 1
CONTROL
-.4000
.00
.4000
.8000
1.200
1.600
2.000
2.400
2.800
3.200
3.600
LOG PO2

and 3 by forming adducts with residues in the organic phosphate binding site on opposite chains. Molecular modeling studies of o-ATP showed that when the opened ribose ring is in the extended antiperiplanar configuration then the distance between functional groups is 7.5 nm, equal to the distance between functional groups in 2'-nor-2'-formyl pyridoxal phosphate (nfPLP). NfPLP has been shown by x-ray crystallography to crosslink β chains by forming a bridge involving adducts with Lys 82 β_1 and Val 1 β_2 (67). These residues are 11 Å apart in the deoxyhemoglobin crystal (74), implying that there must be a degree of flexibility allowing crosslinking to occur. It thus seemed reasonable to postulate that o-ATP could be forming a crosslink between these same residues. Alternatively, o-ATP could be crosslinking between Lys 82 β_1 and Lys 82 β_2, whose intermolecular distance is 8.2 Å in the deoxyhemoglobin crystal (63).

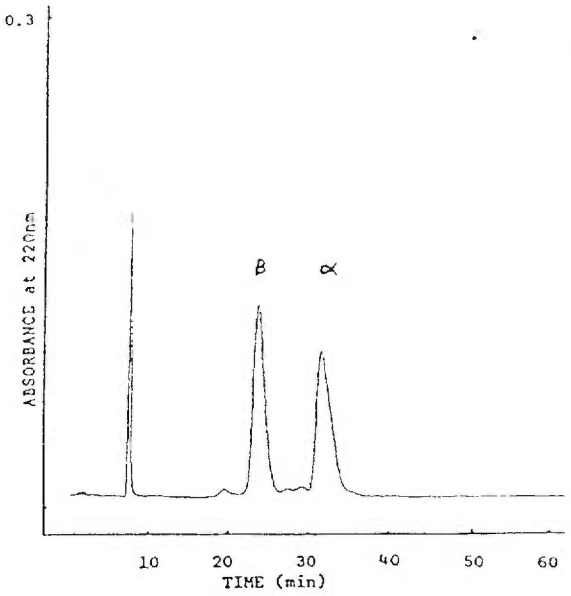
Biochemical evidence from the reaction of o-ATP with hemoglobin has indicated that the major reaction product formed, di-ATP Hb, is a monoadduct with Lys 82 β_1. Since there are two reactive aldehyde groups in o-ATP, either they have both reacted to form a morpholino derivative with the Lys 82 ε-amino group, or only one has reacted and the second free aldehyde remains unreacted. In the second case, it is likely that the free aldehyde group would be reduced to an alcohol after addition of the reducing agent sodium borohydride and would thus be unavailable for further reaction. The reaction of o-ATP with hemoglobin to form the crosslinked products must be a two step process- initial adduct formation on one chain followed by adduct formation on the second chain to form the crosslink. If the rate limiting step in crosslink formation is the formation of the second adduct, then low yields of crosslinked hemoglobin will result if the second free aldehyde is reduced before it can react with the opposite chain. Since the extended-chain antiperiplanar conformation of o-ATP results in the greatest distance between functional groups (7.5 Å), this conformation would seem most favorable for crosslinking residues in the organic phosphate binding site (Lys 82 β_1-Lys 82 β_2 = 8.2 Å; Lys 82 β_1-Val 1 β_2 = 11 Å) (63,74). The extended-chain

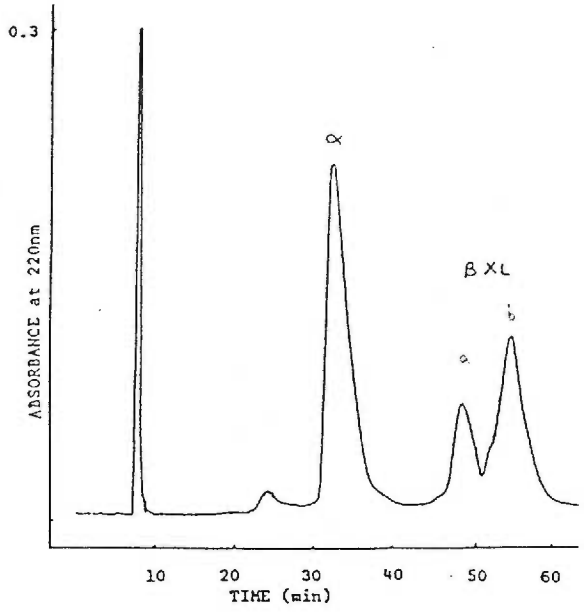
conformation, however, is not likely to be the most energetically favored conformation (56,76). Therefore experiments were designed to try to favor crosslink formation by lengthening the time of reaction before addition of borohydride or by increasing the temperature at which the reaction occurred.

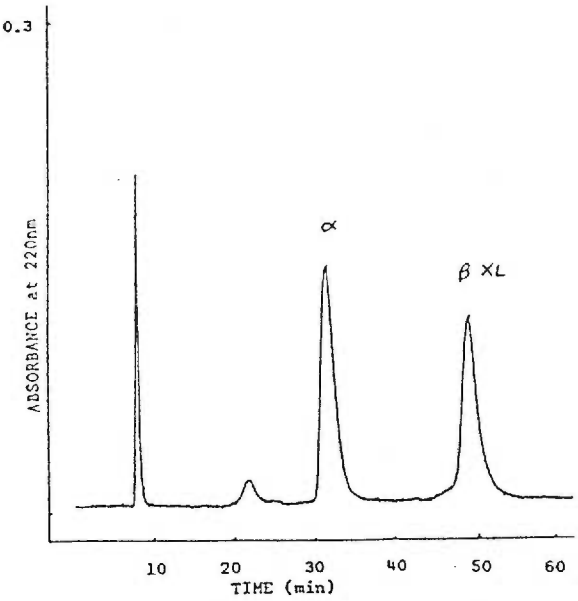
Increasing the length of time for the reaction resulted in a substantial decrease in overall reaction yield although the ratio of crosslinked to di-ATP Hb was somewhat increased (figure 26). Similar results were obtained upon increasing the temperature of the reaction bath to 25° (figure 26). An alternative strategy for increasing the yield of crosslinked product was devised utilizing sodium cyanoborohydride as a reducing agent rather than sodium borohydride. Cyanoborohydride was chosen because it is a milder and more specific reducing agent than borohydride. In particular, it will reduce Schiff base imine bonds to stable secondary amines but it will not reduce free aldehydes to alcohols (121). Cyanoborohydride should therefore convert the initial imine formed between hemoglobin and o-ATP to a stable 2° amine but it should not reduce the second free aldehyde from o-ATP until it has formed an imine with another amino residue on the protein. Figure 27 shows the cation-exchange HPLC of cyanoborohydride-reduced o-ATP-hemoglobin complex. It can be seen that the major products in the reaction mixture consist of the crosslinked fractions 2 and 3, indicating that cyanoborohydride had specifically favored the formation of crosslink. When 2 mM IHP was present in the reaction mixture, modification was blocked, providing evidence that crosslink formation was occurring in the $\beta_1\beta_2$ cleft.

Reversed-phase C_4 HPLC of fractions 2 and 3 was performed to separate constituent globins (figure 22). Both fractions displayed a globin peak which eluted in the position of native α globin. The crosslinked β globins, however, eluted in altered positions, running substantially slower than native β globin on the C_4 column. Fraction 2 in addition had two distinct β globin peaks, designated a and b, indicating that it was composed of at least 2 hemoglobins. Fraction 3

Figure 22. Reversed-phase C_4 HPLC globin separation of Hb A (top), o-ATP fraction 2 (middle), and o-ATP fraction 3 (bottom).


0.3
ABSORBANCE at 220nm
β
α
10
20
30
40
50
60
TIME (min)


0.3
ABSORBANCE at 220nm
α
β XL
a
b
10
20
30
40
50
60
TIME (min)


0.3
ABSORBANCE at 220nm
α
β XL
10
20
30
40
50
60
TIME (min)

had a single modified β globin peak.

Attempts to isolate modified peptides from enzyme and chemical partial hydrolysates of fractions 2 and 3 or to identify alterations in peptide maps from these fractions were unsuccessful. Tryptic and chymotryptic digests of fractions 2 and 3 as well as digests of globin derived from these fractions showed no differences in peptide patterns from native hemoglobin or globin (figure 23). However, by utilizing a second in-line variable wavelength flow detector set at 259 nm (λ_{max}o-ATP) for monitoring the enzyme hydrolysate HPLC, a non peptide-associated peak which eluted early in the gradient was detected (figure 24). This peak had a UV absorption profile characteristic of adenosine (figure 25). These results implied that that the crosslink adducts were labile and breaking down upon hydrolysis of the protein. The mechanism for this is not clear, but evidence for unusual reaction products such as β-elimination products found after affinity labeling with periodate-oxidized nucleotides has been reported (54,55).

Functional studies of fractions 2 and 3 were undertaken to investigate the effect of crosslinking on oxygen affinity of the two species. Figure 28 shows the oxygen equilibrium curves in 50 mM bis Tris pH 7.4, 0.1 M Cl^-, at 20°. It can be seen that both species have greatly increased oxygen affinities. Each fraction has a P_{50} equal to 1mm Hg, approximately a six fold increase in affinity compared with native hemoglobin. Both fractions still retain a significant degree of subunit cooperativity although it is greatly diminished, with fraction 3 having a somewhat higher n_{max} value than fraction 2 (1.8 v. 1.2).

2. Periodate-oxidized pyridine nucleotides (o-NAD and o-NADP)

Periodate-oxidized NADP (o-NADP) has been used as an affinity label for modification of 6-phosphogluconate dehydrogenase (53). Since both NAD and NADP contain negatively charged phosphate groups, site-specific modification of hemoglobin with these reagents was

Figure 23. HPLC of tryptic peptides from o-ATP crosslinked β globins from fractions 2 and 3. Eluant monitored at 214 nm.

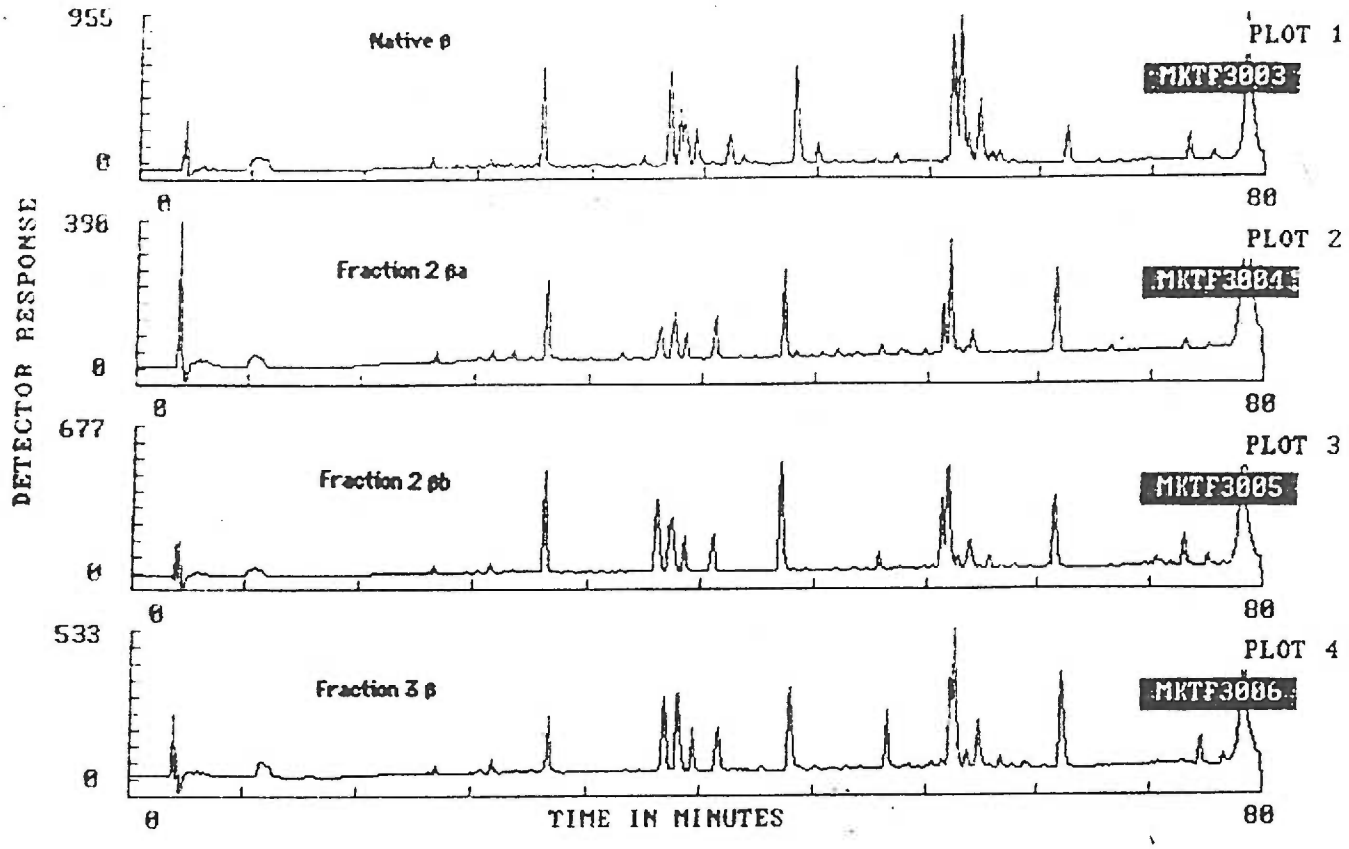

955
Native β
PLOT 1
MKTF3003
0
0
80
DETECTOR RESPONSE
398
Fraction 2 βa
PLOT 2
MKTF3004
0
0
80
677
Fraction 2 βb
PLOT 3
MKTF3005
0
0
80
533
Fraction 3 β
PLOT 4
MKTF3006
0
0
TIME IN MINUTES
80

Figure 24. HPLC of tryptic peptides from o-ATP crosslinked β globins from fractions 2 and 3, with detection at 259 nm. Arrows indicate position of 259 nm absorbing early peak.

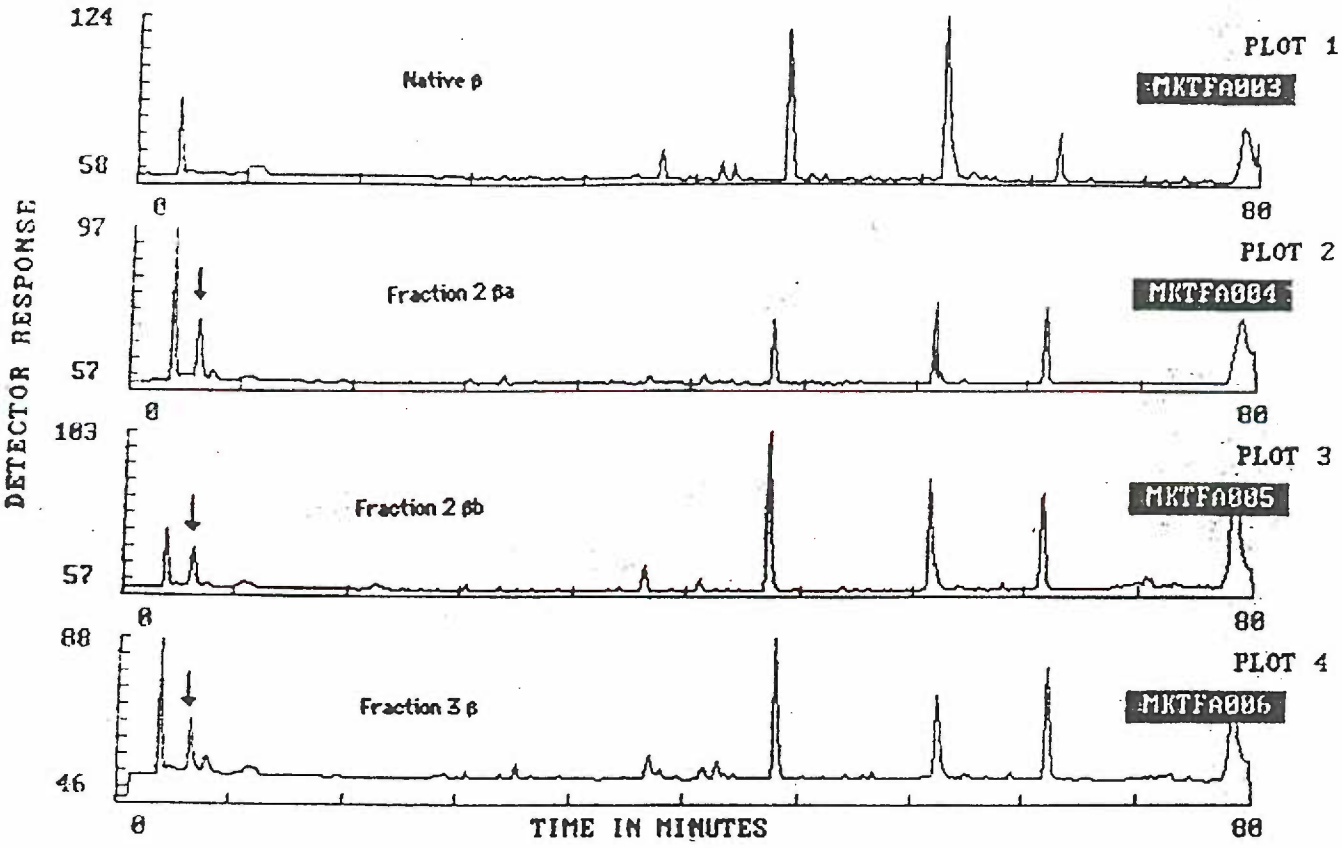

124
58
Native β
PLOT 1
MKTFA003
0
80
97
57
Fraction 2 βa
PLOT 2
MKTFA004
0
80
103
57
Fraction 2 βb
PLOT 3
MKTFA005
0
80
88
46
Fraction 3 β
PLOT 4
MKTFA006
0
80
DETECTOR RESPONSE
TIME IN MINUTES

Figure 25. UV spectrum of 259 nm-absorbing peaks indicated in figure 24 (solid line). Dashed line is spectrum of authentic ATP.

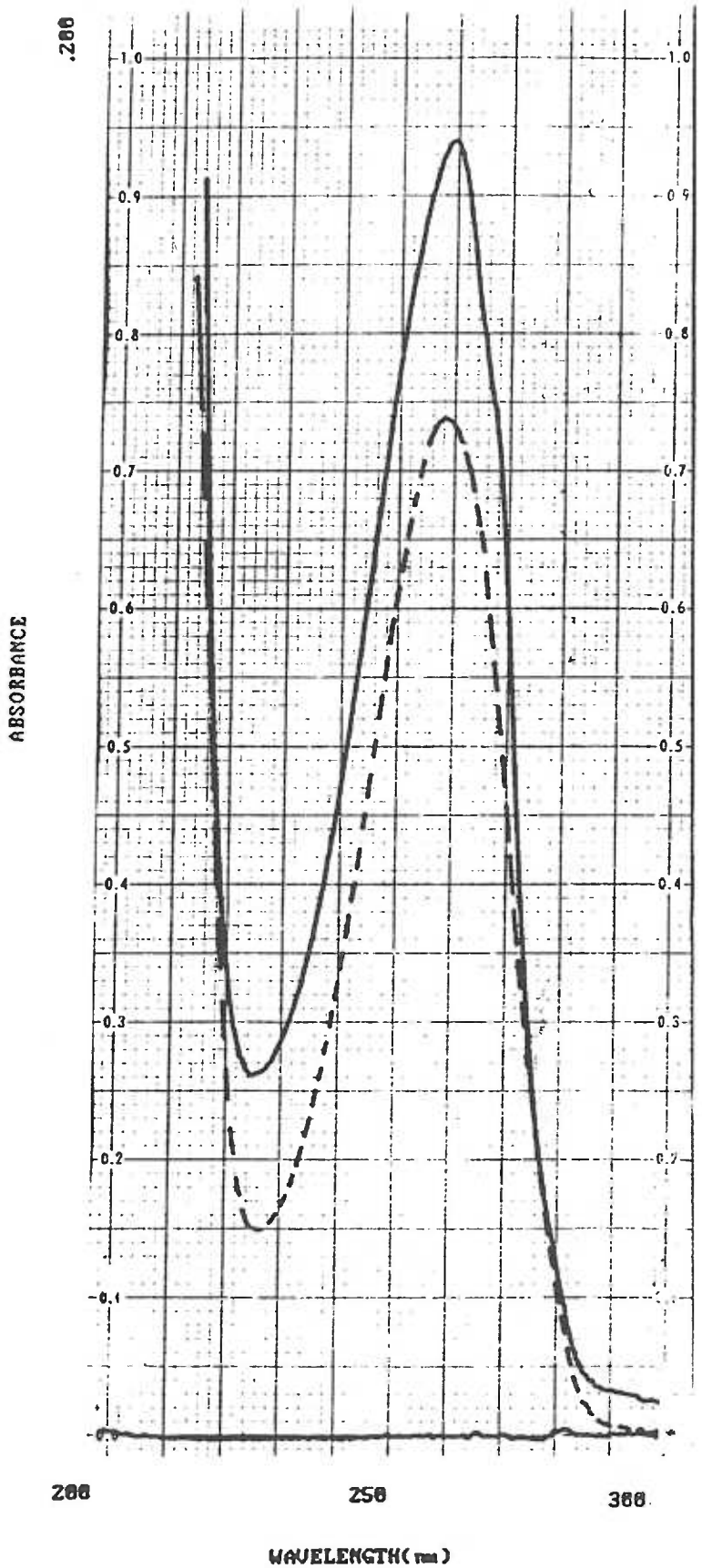
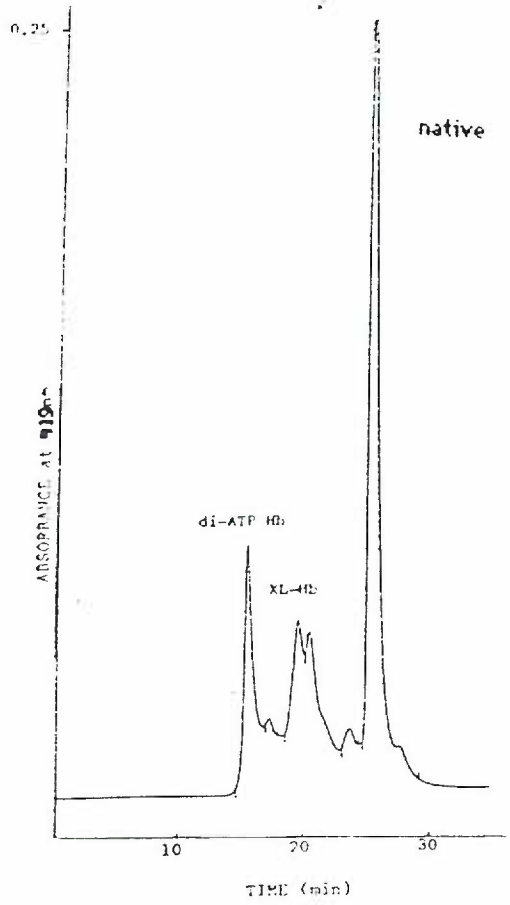

.200
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
200
250
300
WAVELENGTH(nm)

Figure 26. Cation-exchange HPLC of reaction mixture of 0.27 mM o-ATP with 0.25 mM hemoglobin. Bottom tracing represents reaction at 25° for 45 minutes followed by 90 minute reduction with 10 mM sodium borohydride. Top tracing represents reaction of Hb with o-ATP for 6 hours followed by reduction with 10 mM sodium borohydride for 90 minutes. Eluant monitored at 419 nm.


native
di-ATP Hb
XL-Hb
10
20
30
TIME (min)

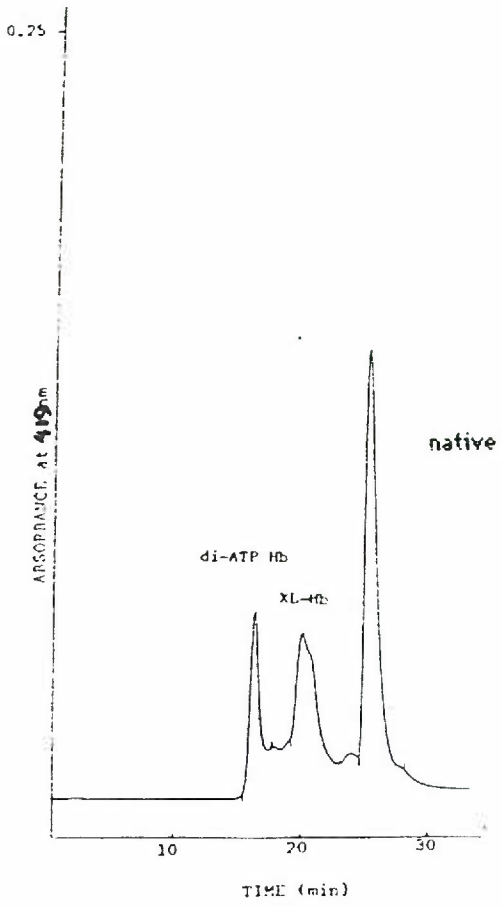

native
di-ATP Hb
XL-Hb
10
20
30
TIME (min)

Figure 27. Cation-exchange HPLC of reaction of 0.27 mM o-ATP plus 0.25 mM Hb reduced with 10 mM sodium cyanoborohydride for 60 minutes. Reaction buffer was 50 mM Tris-Cl pH 7.8, 0°.

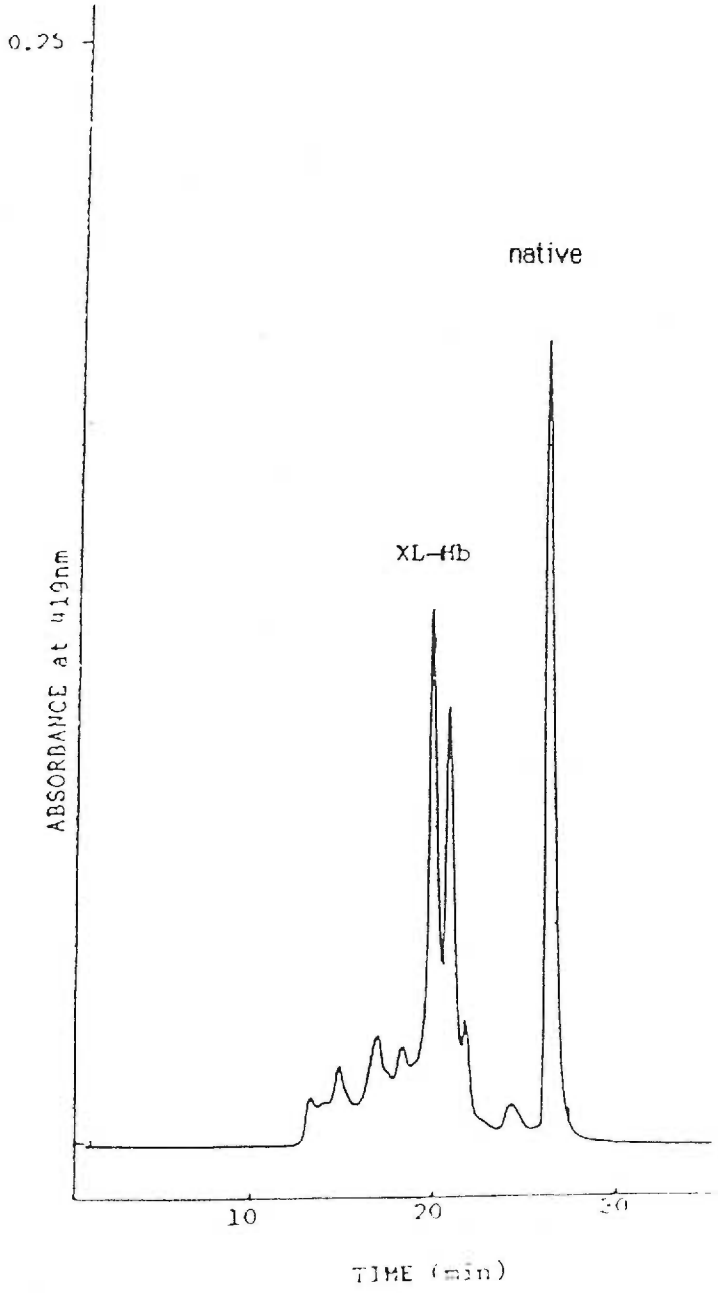
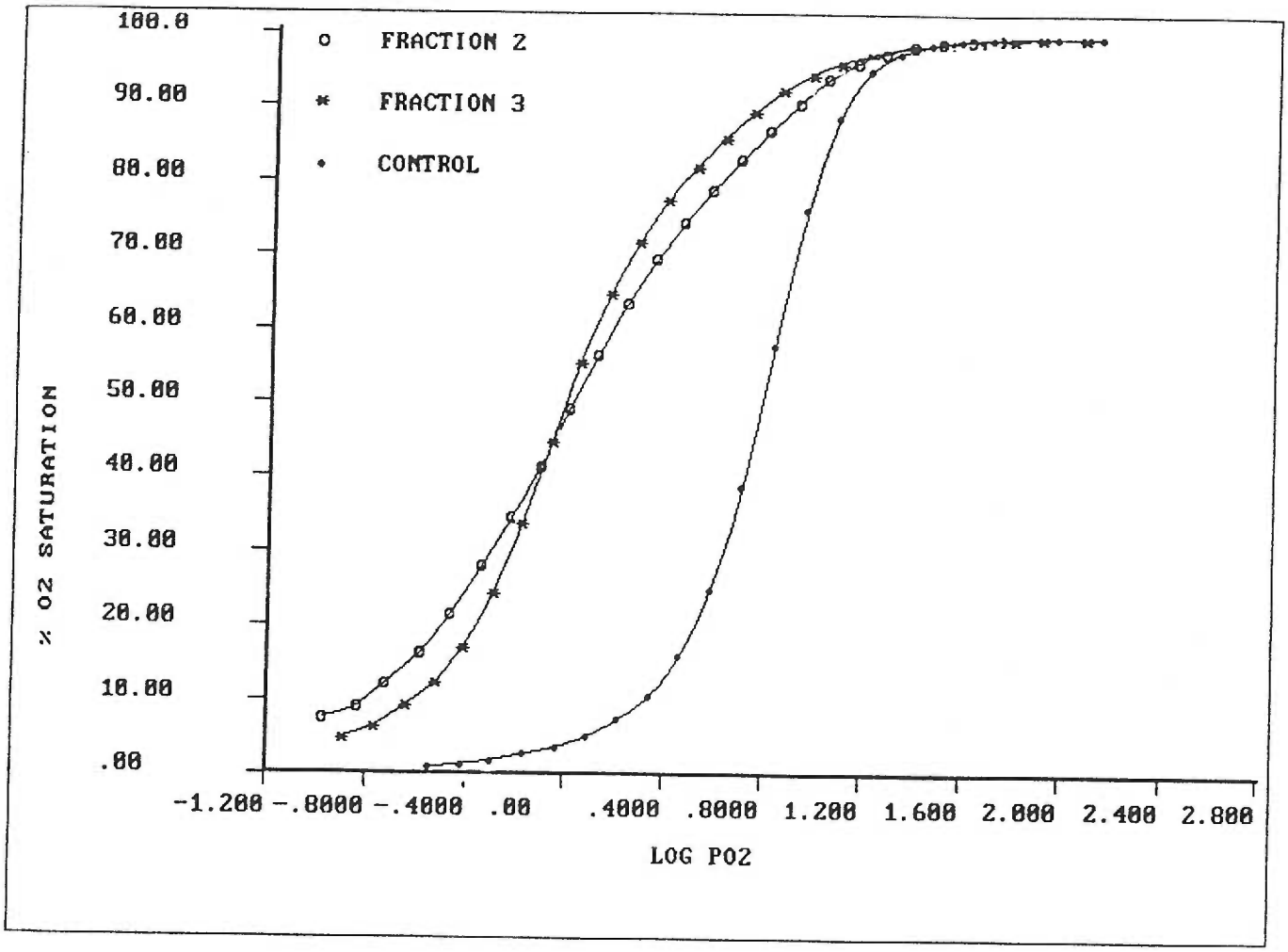

0.25
native
XL-Hb
ABSORBANCE at 419nm
10
20
30
TIME (min)

Figure 28. Oxygen equilibrium curves of o-ATP crosslinked fractions 2 and 3 (60 μM) in 50 mM bis-Tris pH 7.4, 0.1 M Cl, 20°.

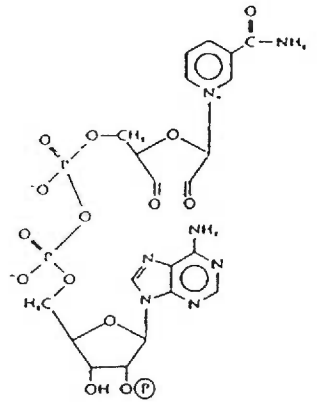

100.0
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
.00
% O2 SATURATION
FRACTION 2
FRACTION 3
CONTROL
-1.200
-.8000
-.4000
.00
.4000
.8000
1.200
1.600
2.000
2.400
2.800
LOG PO2

attempted. o-NAD and o-NADP were synthesized as described in Materials and Methods. The theoretical structures of the two oxidized pyridine nucleotides are shown in figure 29. Each reagent was allowed to react with 0.25 mM deoxyhemoglobin in a stoichiometric ratio in a buffer consisting of 50 mM Tris Cl^- pH 7.8, 0°, and reduced with sodium cyanoborohydride as described in Materials and Methods. The yield of modified product with o-NAD was significantly higher than for o-NADP as the cation-exchange HPLC profiles (figures 30,31) and time course of the reaction (figure 32) show. After addition of an equimolar amount of reagent, o-NAD led to modification yields over 80% whereas o-NADP led to less than 10% modification by 60 minutes (figure 32). Although there is an extra phosphate group in o-NADP not present in o-NAD, which would be expected to increase the binding affinity, there is an extra ring-opened dialdehyde derived from the adenosine ring in o-NAD not present in o-NADP (figure 29). This suggests that the presence of these adenosine-derived ring-opened dialdehyde groups is critical for the modification reaction with amino residues on the protein.

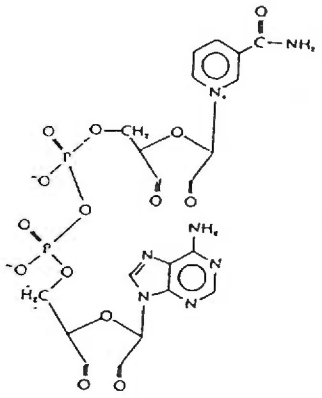
The o-NAD modified fraction was purified by chromatography on DEAE-Sephacel equilibrated with Tris-Cl pH 7.8 with a 0 to 200mM NaCl gradient. Determination of the $\Delta\varepsilon_{260}$ for this fraction indicated that there were 0.72 molecules of NAD incorporated per tetramer. The yield of modified product (>80%) and the stoichiometric ratio of o-NAD to tetramer in the reaction mixture led to the conclusion that one molecule of o-NAD had been incorporated per tetramer. SDS-PAGE of the o-NAD modified hemoglobin indicated that it contained crosslinked globin (figure 33). Reversed-phase C_4 HPLC of o-NAD Hb revealed the presence of material which co-eluted with native α globin (figure 34). In addition, a second peak identified as β globin by tryptic mapping was present and eluted in a position significantly slower than native β globin. SDS-PAGE of the modified β globin showed that it was crosslinked while the α globin showed normal mobility. It was therefore concluded that the β chains had been crosslinked by a single molecule of o-NAD. As was the case with

Figure 29. Postulated structures of periodate-oxidized affinity labels.

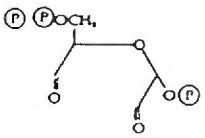
o-NADP



o-NAD



o-PRPP



o-G-1-P

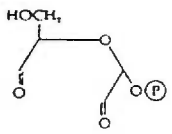


Figure 30. Cation-exchange HPLC (SynChrom CM-Silica) of reaction mixture consisting of 0.27 mM o-NADP 0.25 mM Hb, 10 mM cyanoborohydride, in 50 mM Tris-Cl buffer pH 7.8, 0°.

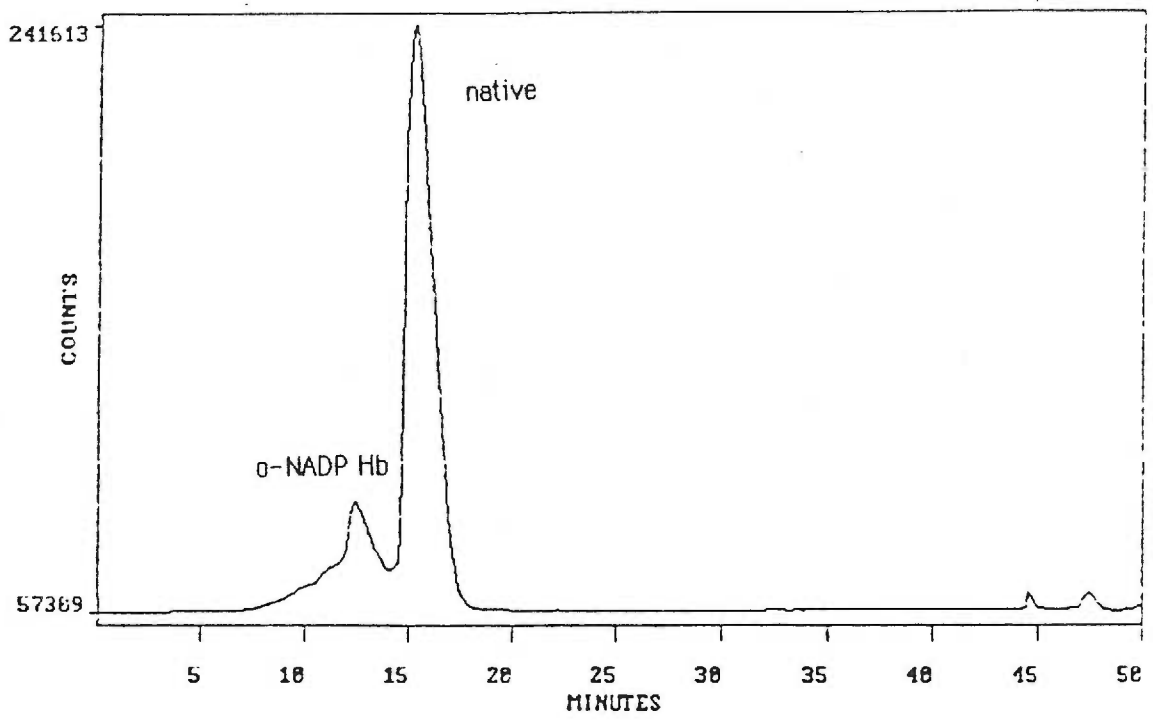

241613
57369
COUNTS
native
o-NADP Hb
5
10
15
20
25
30
35
40
45
50
MINUTES

Figure 31. Cation-exchange HPLC (SynChrom CM-Silica) of reaction mixture consisting of 0.27 mM o-NAD 0.25 mM Hb, 10 mM cyanoborohydride, in 50 mM Tris-Cl buffer pH 7.8, 0°.

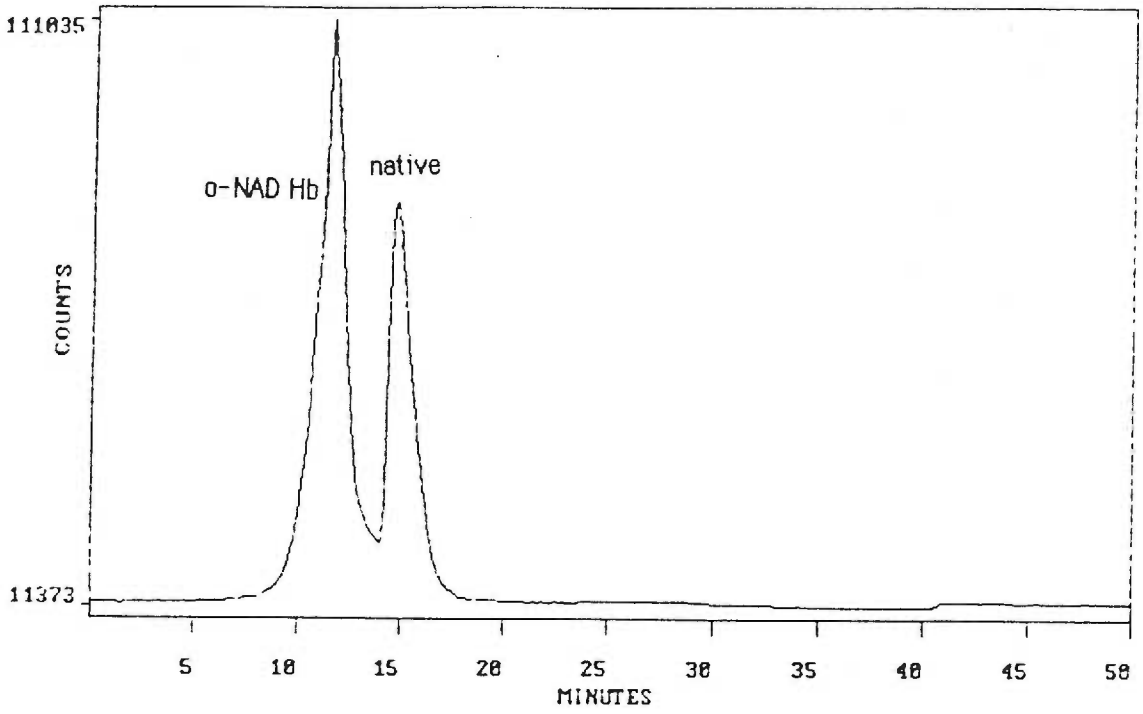

111035
11373
COUNTS
o-NAD Hb
native
5
10
15
20
25
30
35
40
45
50
MINUTES

Figure 32. Time course of modification of 0.25 mM hemoglobin by 0.27 mM o-NADP and o-NAD plus 10 mM cyanoborohydride in 50 mM Tris-Cl buffer pH 7.8, 0°.

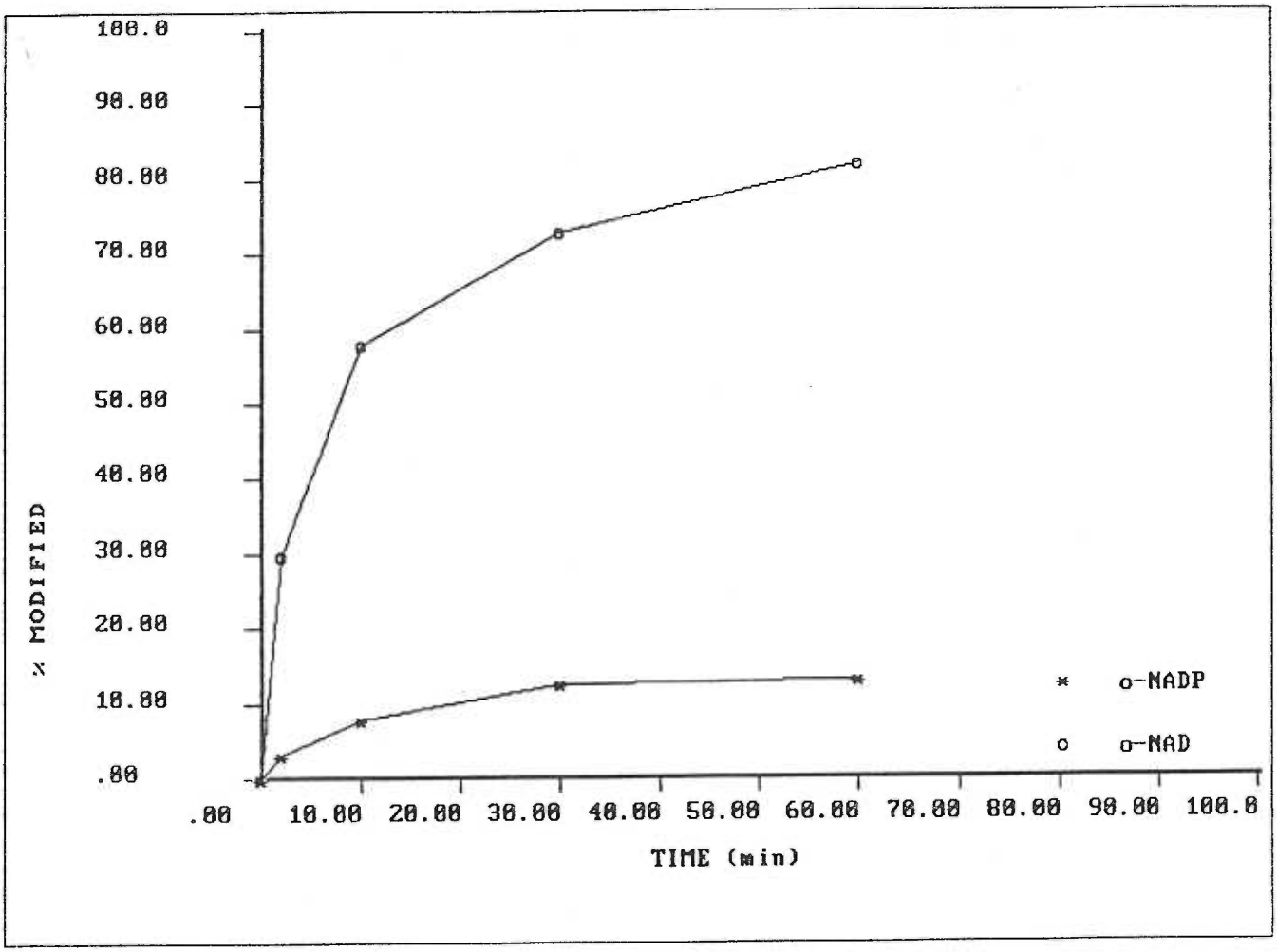

100.0
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
.00
% MODIFIED
.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.0
TIME (min)
*
o-NADP
o
o-NAD

Figure 33. SDS-PAGE of o-NAD crosslinked Hb. Lane 1: Lane 1: standard crosslinked bovine hemoglobin mixture (17K,34K,41K,58K,75K)(Sigma); Lane 2: o-NAD crosslinked Hb; Lane 3: native Hb

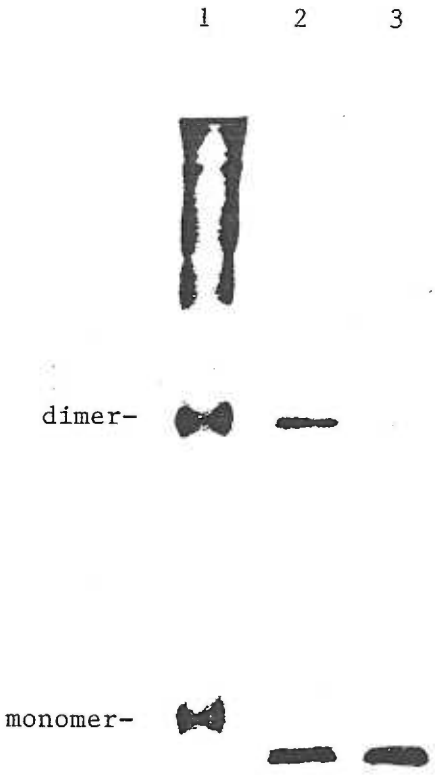
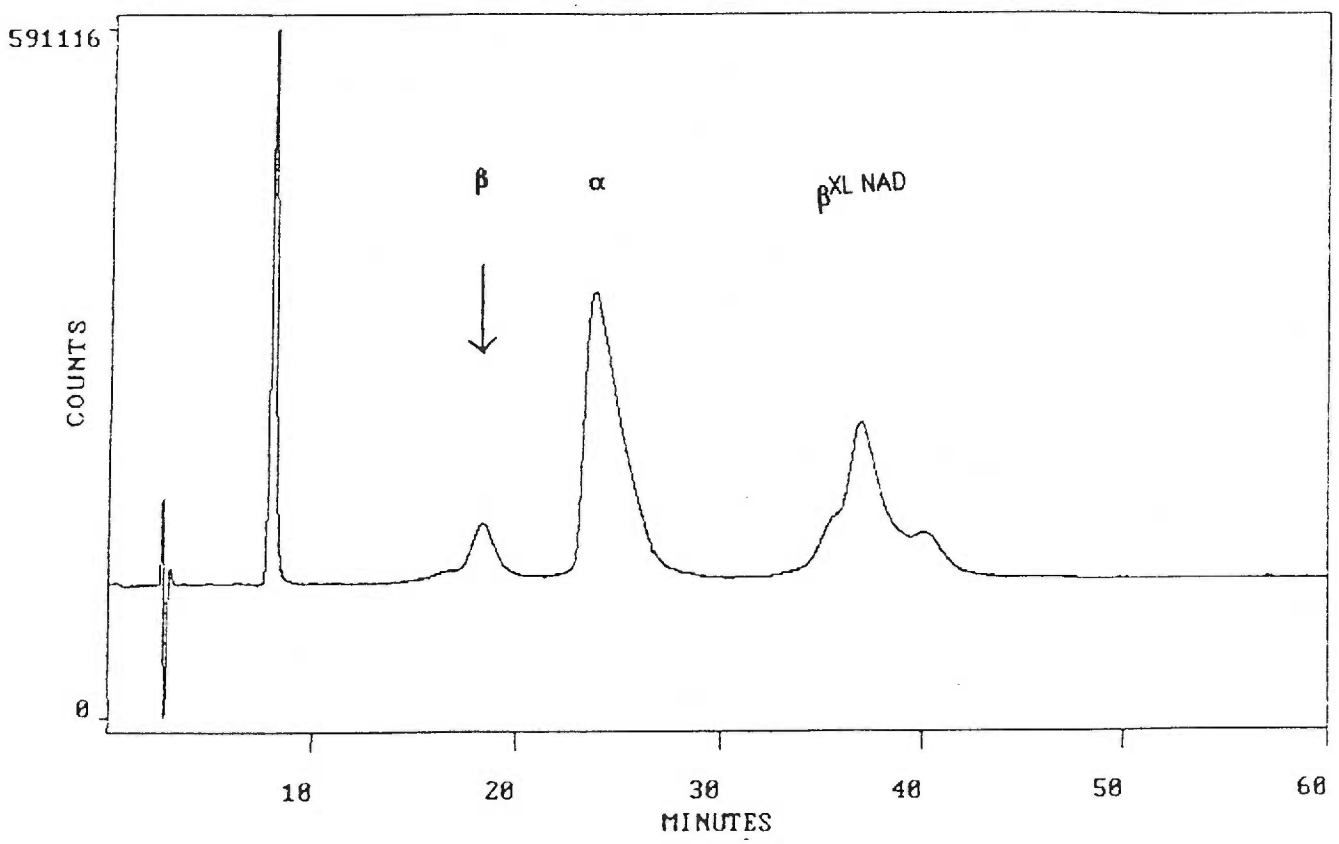

1
2
3
dimer-
monomer-

Figure 34. Reversed-phase C_4 HPLC globin separation of o-NAD modified Hb.


591116
COUNTS
0
β
α
β^XL NAD
10
20
30
40
50
60
MINUTES

hemoglobin crosslinked with o-ATP, partial hydrolysis of the o-NAD modified hemoglobin by chymotrypsin, trypsin, and 0.25 M acetic acid revealed no alterations in peptide patterns compared to native hemoglobin, presumably due to lability of the crosslink adducts. The presence of 2 mM IHP in the reaction mixture completely blocked modification of deoxyhemoglobin by o-NAD, suggesting that the modification was occurring in the organic phosphate binding site.

Functional analysis of o-NAD modified hemoglobin was performed in 50 mM bis-Tris buffer, pH 7.4, 0.1 M Cl^-, at 20° (figure 35). The oxygen affinity of the modified hemoglobin was greatly increased (P_{50} = 0.75 mm Hg). In addition, the subunit cooperativity was significantly decreased (n_{max} = 1.4).

3. Periodate oxidized sugars (o-Glc-1-P and o-PRPP)

Ring-opened glucose-1-phosphate (o-glc-1-P) and phosphoribosyl pyrophosphate (o-PRPP) were synthesized as described in Materials and Methods and allowed to react in a stoichiometric ratio with 0.25 mM deoxyhemoglobin in 50 mM Tris Cl^-, pH 7.8, at 0°, using sodium cyanoborohydride for reduction. Figure 36 shows the results of cation-exchange HPLC of the reaction mixtures for each reagent. Reaction with stoichiometric amounts of o-glc-1-P led to modification yields of approximately 20%, while reaction with o-PRPP gave higher yields, approaching 75%. Time courses for the two reactions are displayed in figure 37. The main fraction from the o-PRPP modified hemoglobin was selected for further study and purified by chromatography on carboxymethyl-Sephadex using a pH gradient from 6.6 to 7.0 in 50 mM Tris-maleate buffer. Analysis of SDS-PAGE of this fraction showed it to contain crosslinked globin (figure 38). Reversed-phase C_4 chromatography of this fraction indicated the presence of a peak which co-eluted with native α globin whereas the β globin, which was found to be crosslinked by SDS-PAGE, had decreased mobility on the C_4 column (figure 39). Tryptic peptide maps of the o-PRPP modified hemoglobin revealed no

Figure 35. Oxygen equilibrium curve for 60 μM o-NAD modified Hb in 50 mM bis-Tris pH 7.4 0.1 M Cl, 20°.

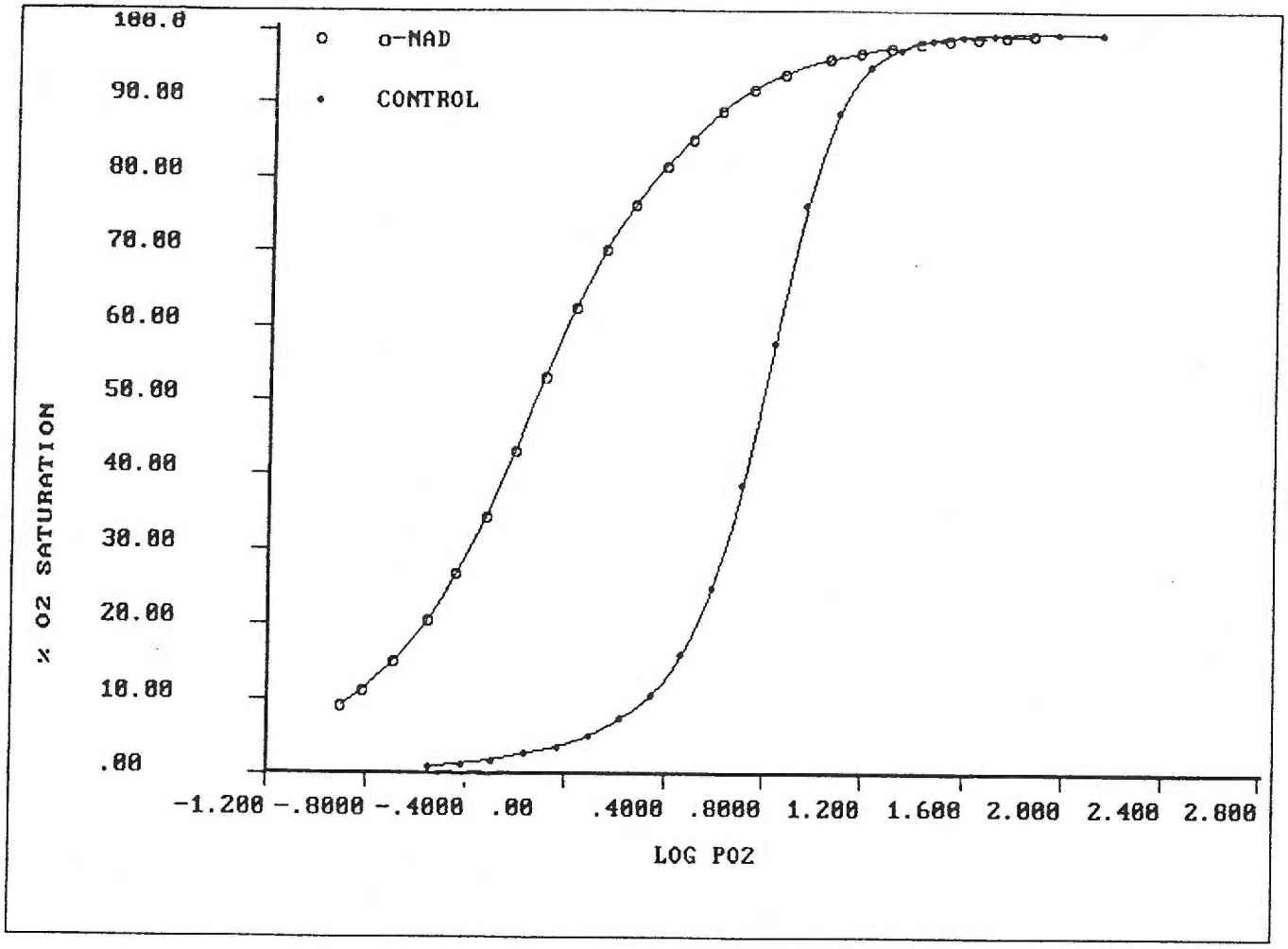
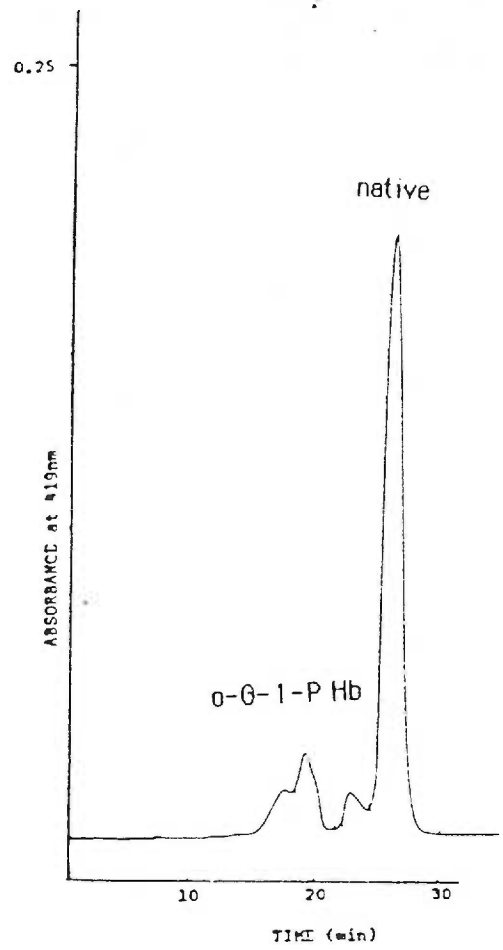

100.0
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
.00
% O2 SATURATION
o-NAD
CONTROL
-1.200
-.8000
-.4000
.00
.4000
.8000
1.200
1.600
2.000
2.400
2.800
LOG PO2

Figure 36. Cation-exchange HPLC of reaction of 0.25 mM Hb plus 0.27 mM o-G-1-P (top) or o-PRPP (bottom) reduced with 10 mM sodium cyanoborohydride for 60 minutes. Reaction buffer was 50 mM Tris-Cl pH 7.8, 0°.


0.25
native
ABSORBANCE at 419nm
o-G-1-P Hb
10
20
30
TIME (min)

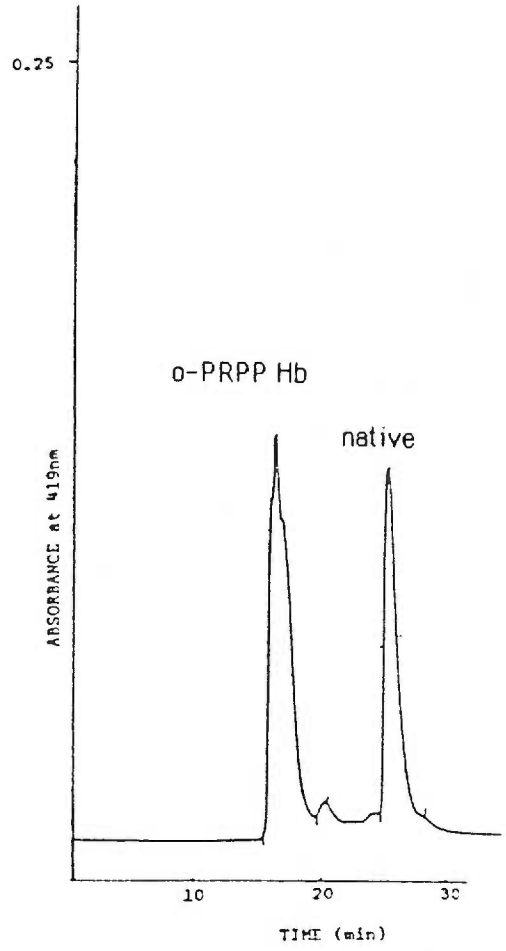

0.25
o-PRPP Hb
native
ABSORBANCE at 419nm
10
20
30
TIME (min)

Figure 37. Time course for modification of Hb with o-G-1-P and o-PRPP. Reaction of 0.25 mM Hb plus 0.27 mM o-G-1-P or o-PRPP reduced with 10 mM sodium cyanoborohydride. Reaction buffer was 50 mM Tris-Cl pH 7.8, 0°.

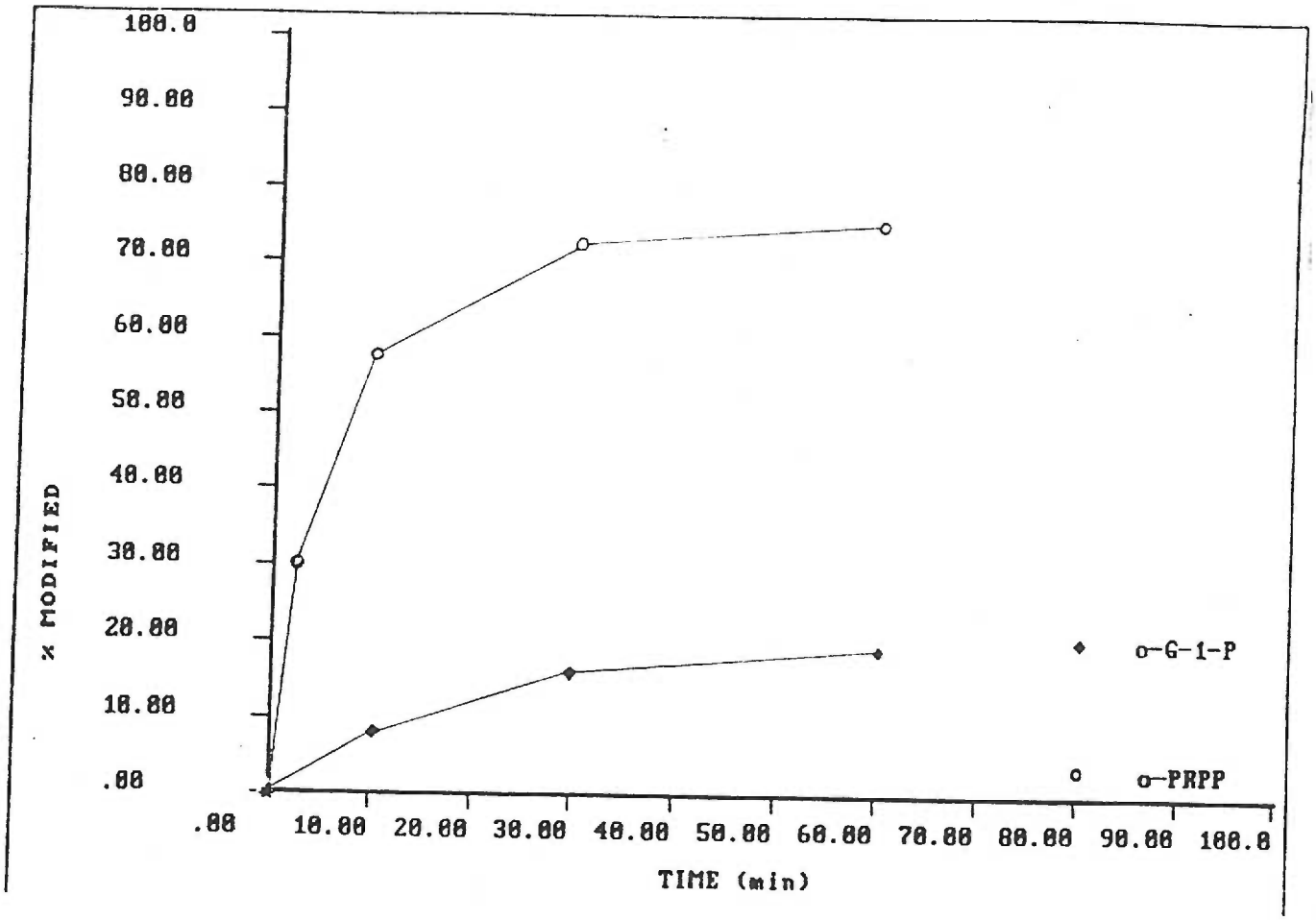

100.0
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
.00
% MODIFIED
.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.0
TIME (min)
o-G-1-P
o-PRPP

Figure 38. SDS-PAGE of o-PRPP modified Hb. Lane 1: o-PRPP-crosslinked Hb; Lane 2: native Hb; Lane 3: standard crosslinked bovine hemoglobin mixture (17K,34K,41K,58K,75K)(Sigma).

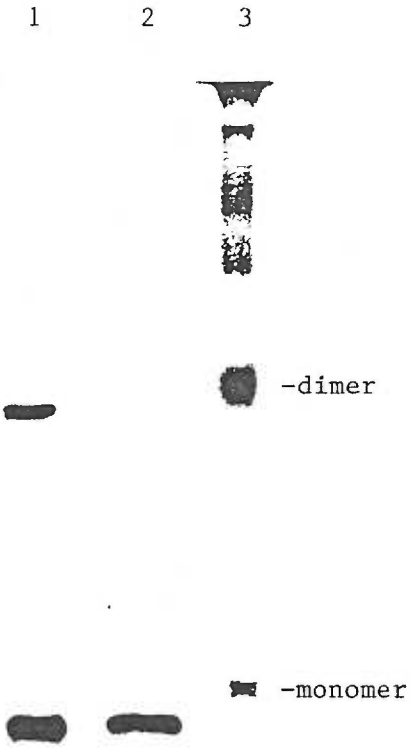

1
2
3
-dimer
-monomer

Figure 39. Reversed-phase C_4 HPLC globin separation of o-PRPP modified Hb.

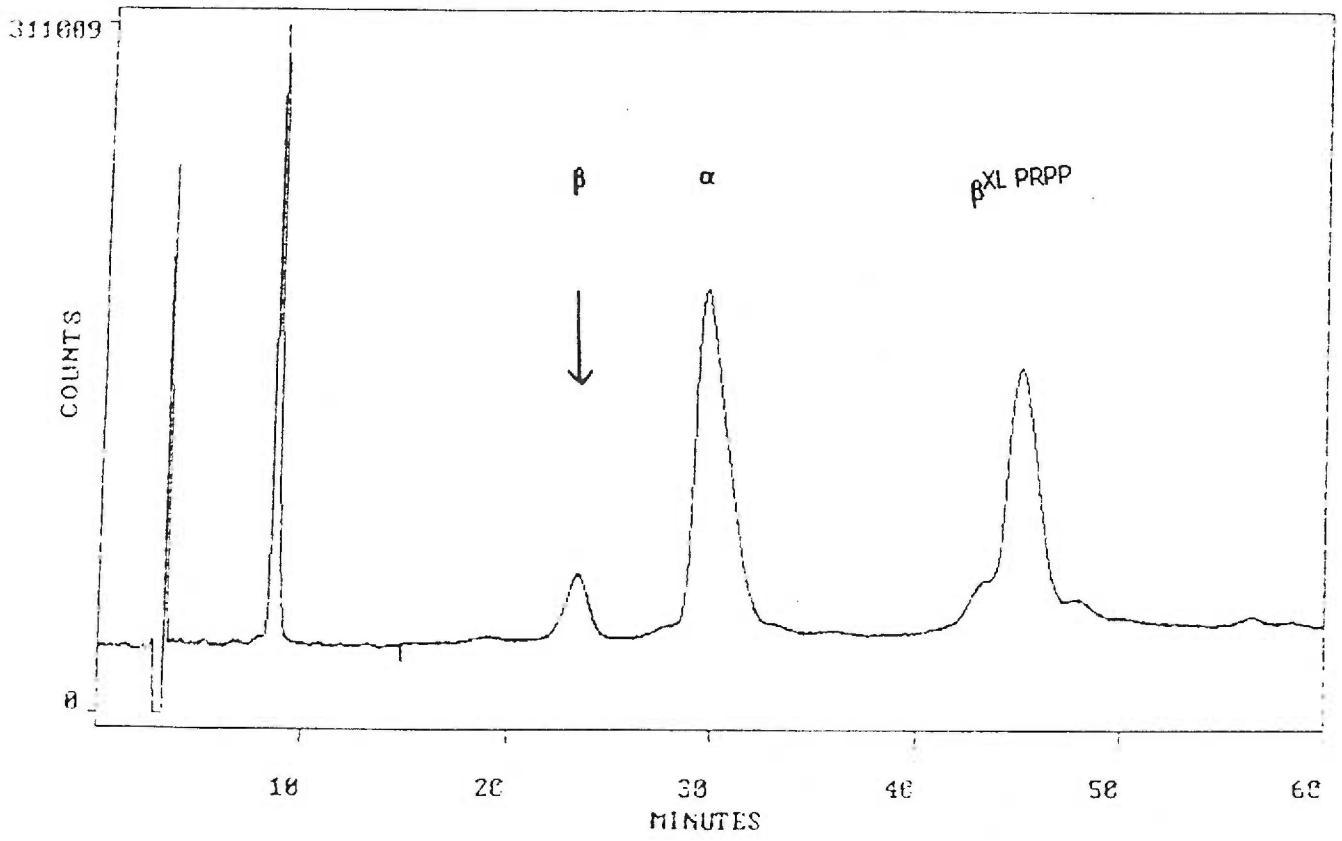
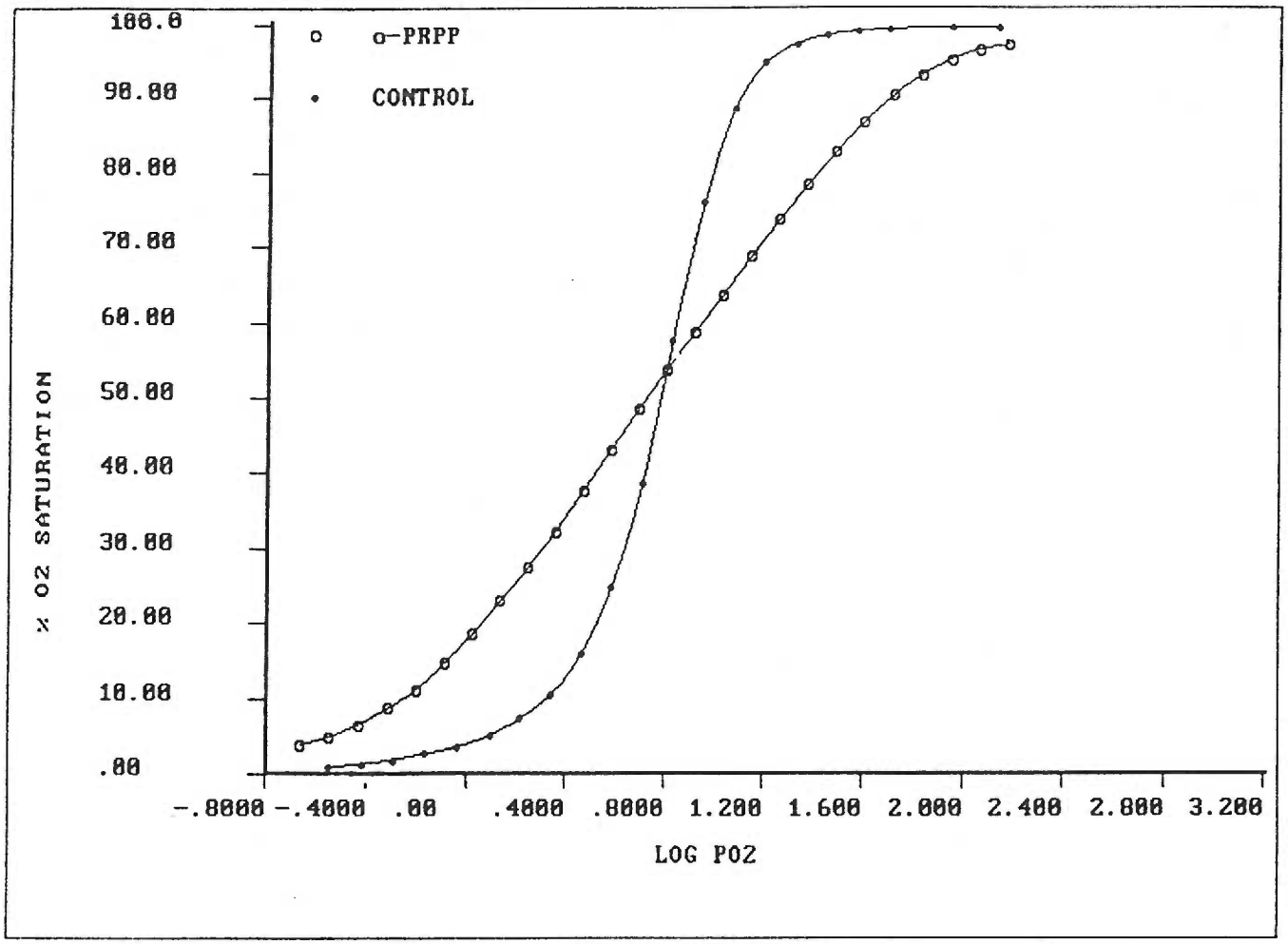

311609
β
α
βXL PRPP
COUNTS
0
10
20
30
40
50
60
MINUTES

Figure 40. Oxygen equilibrium curve for 60 μM o-PRPP modified Hb in 50 mM Tris pH 7.4, 0.1 M Cl^-, 20°.


100.0
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
.00
% O2 SATURATION
o—PRPP
• CONTROL
-.8000 -.4000 .00 .4000 .8000 1.200 1.600 2.000 2.400 2.800 3.200
LOG PO2

significant differences in peptide patterns for either the α or β globins.

Functional studies were performed with o-PRPP modified hemoglobin in 50 mM bi-Tris pH 7.4, 0.1 M Cl^- at 20° (figure 40). The P_{50} of the crosslinked hemoglobin is close to that of unmodified hemoglobin under these conditions, but the subunit cooperativity is significantly reduced (n_{max} = 1.3).

Reaction of hemoglobin with aromatic dialdehydes

Functional studies revealed that all of the hemoglobins crosslinked with periodate-oxidized ligands derived from a basic ring-opened ribose structure exhibited high oxygen affinity and reduced subunit cooperativity. This ring opened dialdehyde structure, which has a bridging span of 7.5 Å in the extended conformation, would be expected to have a degree of flexibility and could be therefore expected to constrain residues which it had crosslinked to an intermolecular distance of 7.5 Å or less, depending on the free energy of the various conformations. If crosslinking occurs in the $\beta_1\beta_2$ cleft between two residues which move normally closer together during the T to R structural transition, then a net force pulling them together would result in a shift in the allosteric equilibrium towards the high affinity R state. In order to investigate the effect of crosslinking hemoglobin in the organic phosphate binding site with a rigid bridging structure which would impose a well defined structural constraint, modification with the aromatic dialdehydes 5-formyl salicylaldehyde and 2,6-pyridine dicarboxylic acid chloride was attempted. Each of these bifunctional compounds has an intermolecular bridging distance of approximately 7.5 Å. However, neither of the compounds led to significant modification of hemoglobin as determined by cation-exchange HPLC and SDS-PAGE. A possible explanation for this lack of reactivity is the absence of anionic charge in the compounds to confer specificity for binding in the $\beta_1\beta_2$ cleft.

Reaction of hemoglobin with DIDS

In order to further investigate the effects of covalent modification in the organic phosphate binding site with a rigid crosslinking agent, trans-4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) was used as a modification reagent. DIDS is a stilbene analog (figure 41) which has been characterized as an erythrocyte membrane anion transport inhibitor (122). The molecule has a rigid biphenyl geometry with two isothiocyanate functional groups spaced approximately 15 Å apart. The functional group spacing in DIDS is close to the distance between the β amino termini in deoxyhemoglobin (16 Å) as determined by x-ray crystallography (123). In addition there are two negatively charged sulfonate moieties in the molecule which should impart a degree of specificity for binding in the polyanion binding site of hemoglobin.

1. Reaction of DIDS with deoxyhemoglobin

The time course for the reaction of 0.5 mM deoxyhemoglobin with a stoichiometric amount of DIDS in Tris-Cl buffer pH 7.8 is shown in figure 42. The reaction proceeds very rapidly above pH 7, with high yields of modified product. When a stoichiometric amount of DIDS is added to a deoxyhemoglobin solution, a yield of approximately 75% modified hemoglobin results as determined by integration of peaks from cation-exchange HPLC. Using a two-fold molar excess of DIDS, essentially 100% of the deoxyhemoglobin in the reaction mixture becomes modified. Cation-exchange HPLC of the reaction mixture shows the appearance of a number of products (figure 43). Components from the deoxyhemoglobin reaction mixture were purified by ion-exchange chromatography on carboxymethyl-Sepadex using a pH gradient from 6.5 to 7.4 in Tris-maleic acid. Three major components (labeled $Hb(DIDS)^T$, $Hb(DIDS)_2{}^R$, and $Hb(DIDS)_2{}^T$, figure 43) from the deoxyhemoglobin reaction mixture were further purified by preparative anion-exchange rechromatography on DEAE-Sephacel

Figure 41. Structure of 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS).

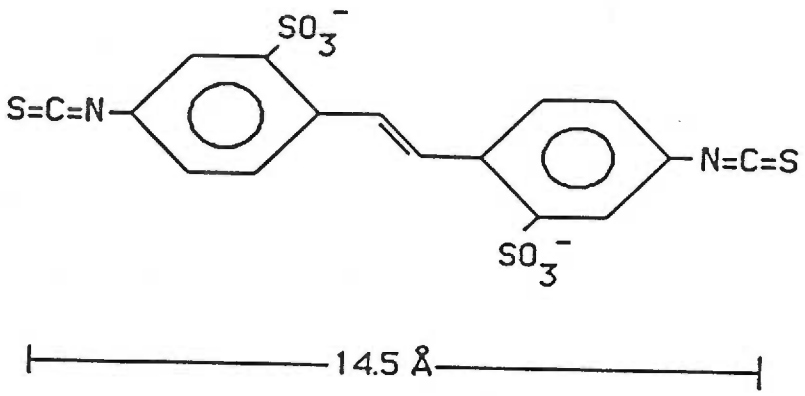
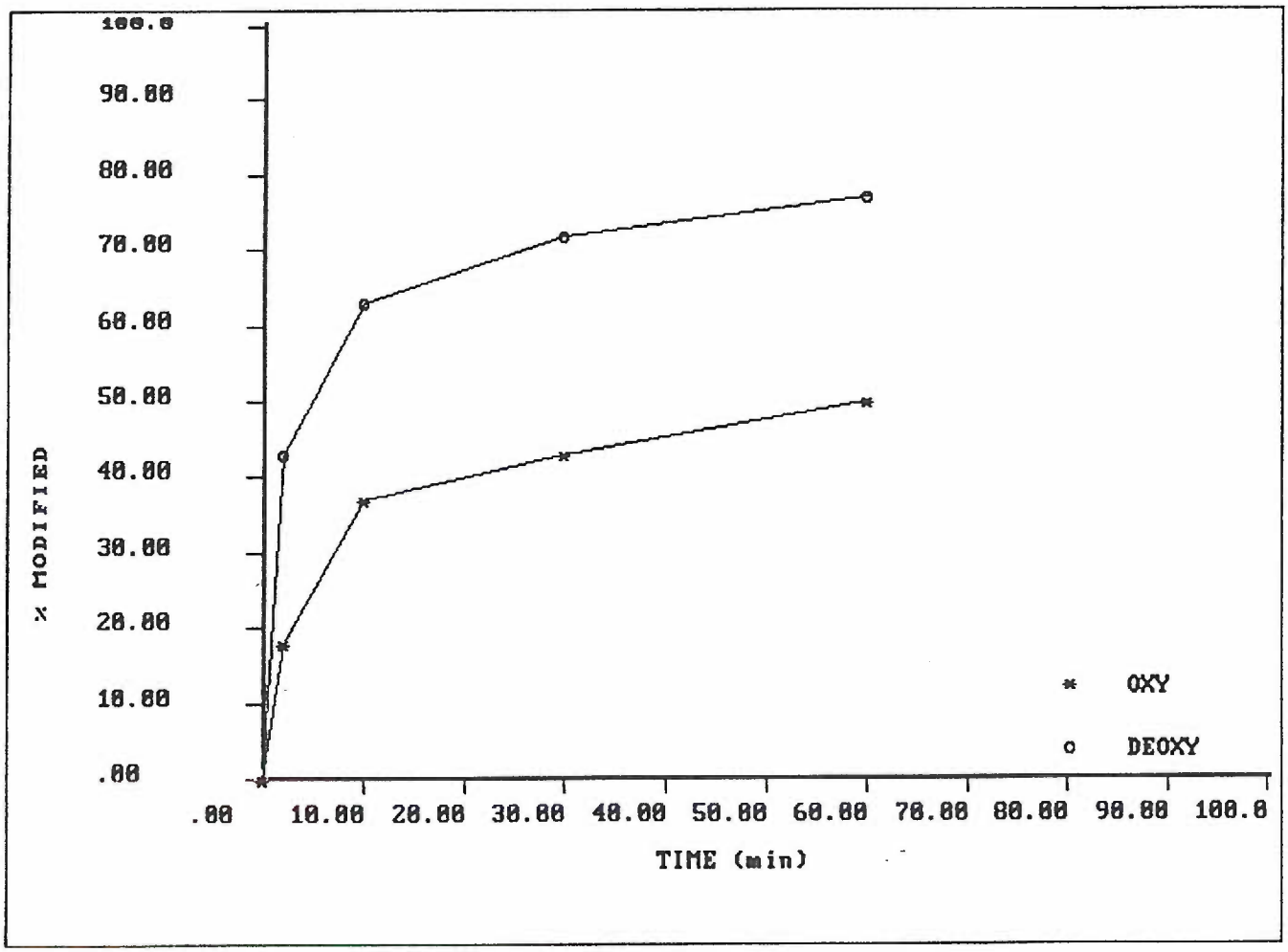

SO3-
S=C=N
N=C=S
SO3-
14.5 Å

Figure 42. Time course for modification of 0.25 mM oxy and deoxy hemoglobin with 0.27 mM DIDS in 50 mM Tris-Cl pH 7.8, 0°.


100.0
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
.00
% MODIFIED
.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.0
TIME (min)
* OXY
o DEOXY

equilibrated with 50 mM Tris-Cl pH 7.8 using an NaCl gradient from 0 to 100 mM. The purity of the components was checked by cation exchange HPLC (figure 45).

2. Reaction of DIDS with oxyhemoglobin

In contrast to deoxyhemoglobin, the reaction of oxyhemoglobin with DIDS appeared to be more specific, leading to the formation of only one major component with smaller amounts of other products (figure 44). Reaction of oxyhemoglobin with a stoichiometric amount of DIDS leads to approximately a 50% yield of modified product. With a two-fold molar excess of DIDS, close to 100% modification occcurs. The major modified product from the oxyhemoglobin reaction was purified by cation-exchange on carboxymethyl-Sephadex with a pH gradient in 50mM Tris-maleate from 6.5 to 7.2. Cation-exchange HPLC was used to check the purity of the modified component (figure 45).

3. Characterization of DIDS-modified hemoglobins

A. $Hb(DIDS)^T$

$Hb(DIDS)^T$ ran on cation-exchange HPLC in a position intermediate between unmodified hemoglobin and $Hb(DIDS)_2{}^R$ and $Hb(DIDS)_2{}^T$ (figure 43). SDS-PAGE of $Hb(DIDS)^T$ (figure 46) demonstrated that it contained a crosslinked band migrating with an M_r approximately double that of native globin monomer in addition to a band running in a position corresponding to native globin. Because of the large absorption by DIDS at 344 nm (ε_{344} = 30.5) (figure 51), it was possible to obtain an estimate of the number of molecules incorporated into $Hb(DIDS)^T$. Table 2 shows the ε_{344} values for native hemoglobin and $Hb(DIDS)^T$, allowing the calculation of $\Delta\varepsilon_{344}$ for $Hb(DIDS)^T$. From this determination the number of molecules of

Figure 43. Cation-exchange HPLC of 0.5 mM deoxyhemoglobin plus 0.55 mM DIDS reaction mixture. Reaction in 50 mM Tris-Cl buffer pH 7.8, 0° for 60 minutes.

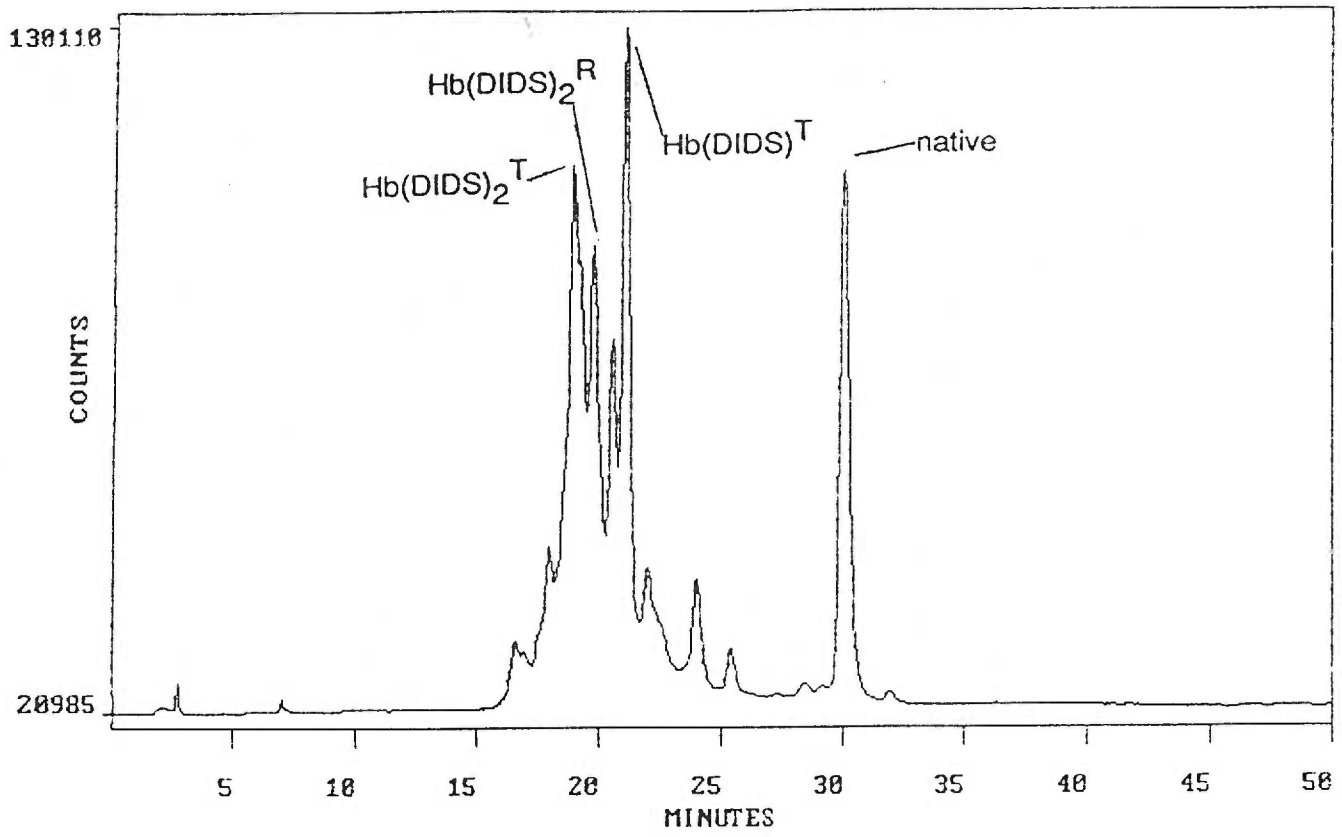

130110
20985
COUNTS
Hb(DIDS)2R
Hb(DIDS)2T
Hb(DIDS)T
native
5
10
15
20
25
30
35
40
45
50
MINUTES

TABLE 2. Determination of ε_{344} for native and DIDS-modified hemoglobins. Hemoglobin concentration was determined in the cyanmet form on the basis of $\varepsilon_{540}^{CNMetHb} = 44\ mM^{-1}cm^{-1}$ (94). $\varepsilon_{344}^{DIDS} = 30.5 mM^{-1}cm^{-1}$.

Hemoglobin	Abs_{540}(conc.)	Abs_{344}	$\varepsilon_{344} mM^{-1}$	$\Delta\varepsilon_{344} mM^{-1}$
native	0.199 (4.53 μM)	0.484	106.8	(0)
$Hb(DIDS)^T$	0.202 (4.59 μM)	0.635	138.3	31.5
$Hb(DIDS)_2{}^T$	0.209 (4.75 μM)	0.800	168.8	62.0
$Hb(DIDS)_2{}^R$	0.256 (5.82 μM)	0.905	155.5	48.7

Figure 44. Cation-exchange HPLC of 0.5 mM oxyhemoglobin plus 0.75 mM DIDS reaction mixture. Reaction took place in 50 mM Tris-Cl buffer pH 7.8, 0° for 60 minutes.

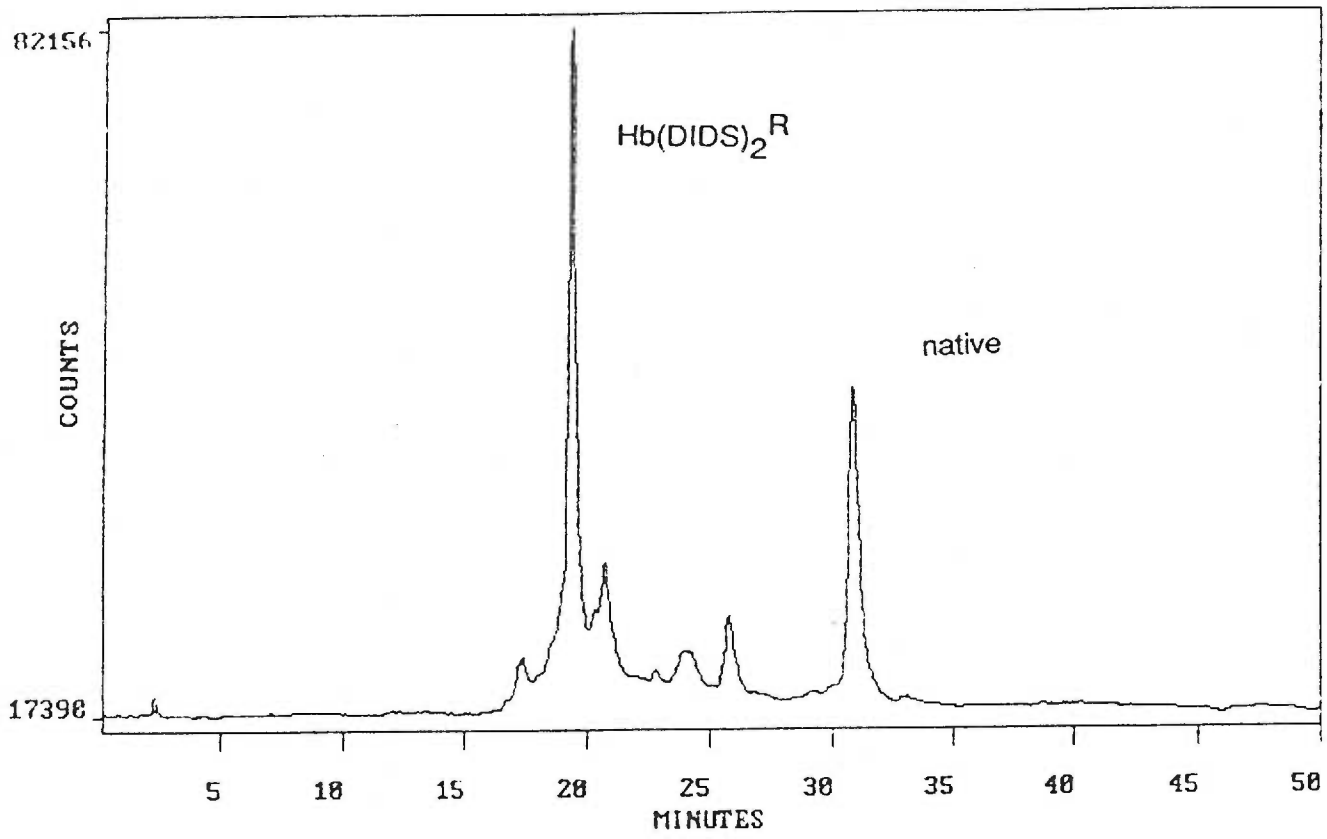
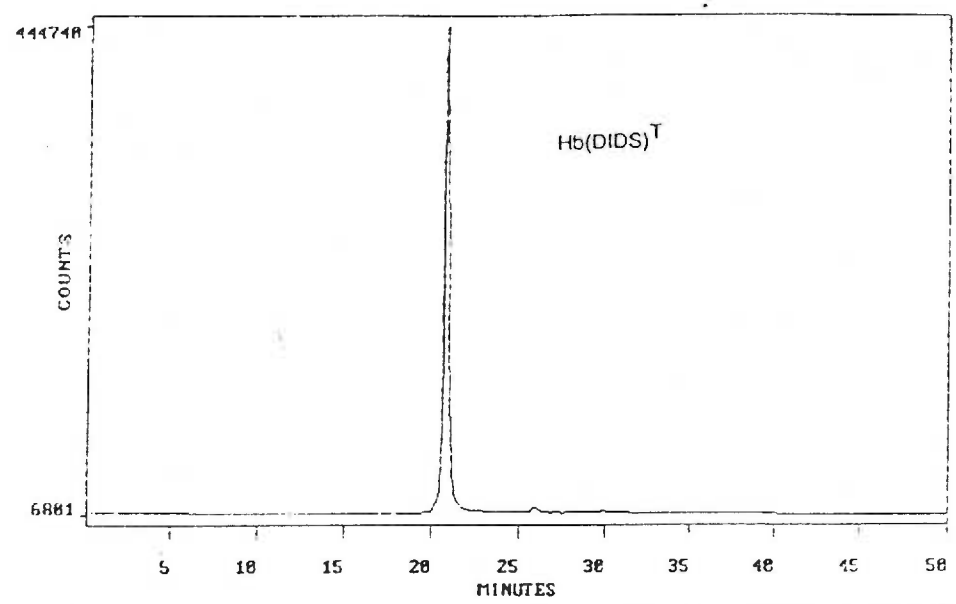
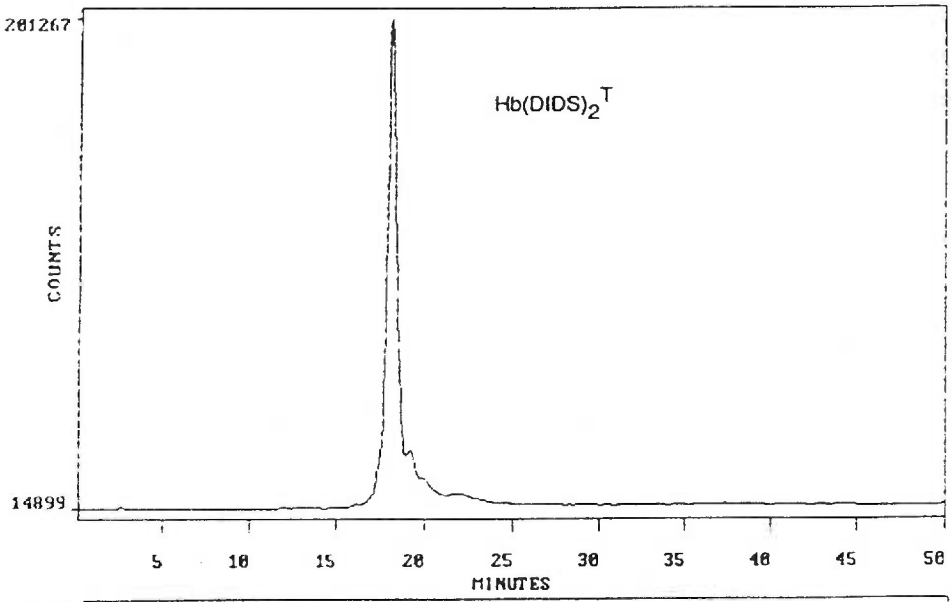

82156
17398
COUNTS
Hb(DIDS)2R
native
5
10
15
20
25
30
35
40
45
50
MINUTES

Figure 45. Cation-exchange HPLC of chromatographically purified DIDS-modified hemoglobins.


444748
Hb(DIDS)T
COUNTS
6881
5
10
15
20
25
30
35
40
45
50
MINUTES


201267
Hb(DIDS)2T
COUNTS
14899
5
10
15
20
25
30
35
40
45
50
MINUTES

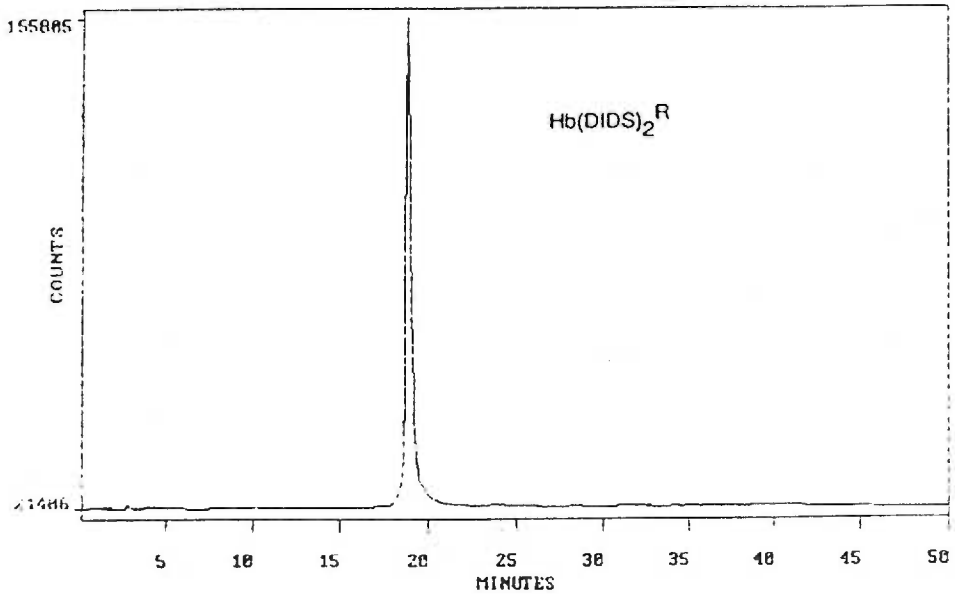
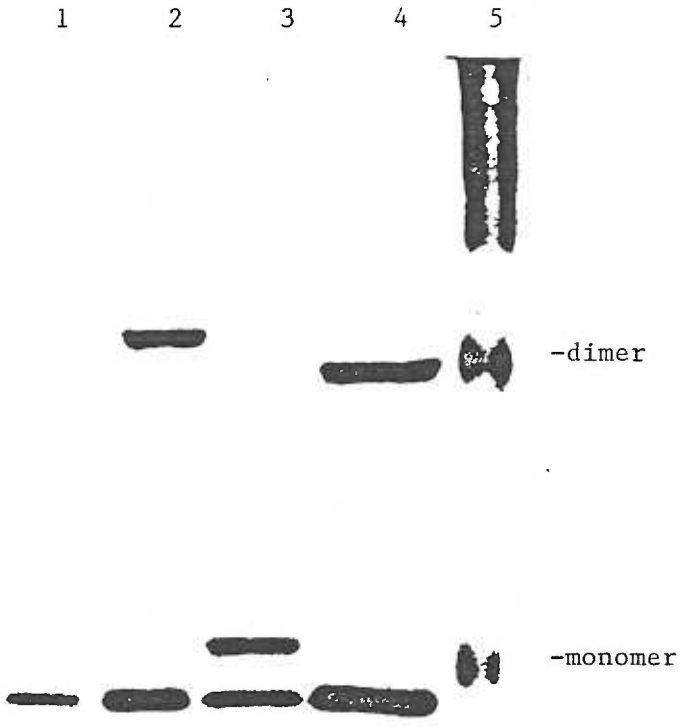

155885
Hb(DIDS)2R
COUNTS
5
10
15
20
25
30
35
40
45
50
MINUTES

Figure 46. SDS-PAGE of DIDS-modified hemoglobins. Lane 1: native Hb; Lane 2: $Hb(DIDS)^{T}$; Lane 3: $Hb(DIDS)_2^{R}$; Lane 4: $Hb(DIDS)_2^{T}$; Lane 5: standard crosslinked bovine hemoglobin mixture (17K,34K,41K,58K,75K)(Sigma)


1
2
3
4
5
-dimer
-monomer

DIDS incorporated per tetramer was estimated to be 1.03. The absorbance increase at 344 nnm was associated with the β-globin from $Hb(DIDS)^T$ but not the α-globin (figure 55). Analytical reversed-phase C_4 chromatography of $Hb(DIDS)^T$ indicated that the material had one globin peak which coeluted with native α globin while the β globin eluted much later than native β globin (figure 47). Partial hydrolysis of the α and β globins from $Hb(DIDS)^T$ was accomplished with trypsin, and the peptides were chromatographed by reversed-phase C_{18} HPLC as described in Materials and Methods. The eluant was monitored at 214 nm and 344 nm by two in-line detectors. Figure 48 demonstrates that the peptide pattern resulting from hydrolysis of $Hb(DIDS)^T$ α globin is indistinguishable from that of native α globin, whereas that for $Hb(DIDS)^T$ β globin differs from native β globin by the loss of βt-1, the amino-terminal tryptic peptide (residues 1-8) (figure 49). In addition, a new peptide appears in the hydrolysate as indicated in figure 49. This peptide was detected at 214 nm and also absorbed strongly at 344 nm, a wavelength at which an absorption maximum by DIDS occurs. The peptide was further purified by C_{18} HPL rechromatography using an acetate buffer-acetonitrile system described in Materials and Methods. A spectral scan of the peptide appears in figure 51 in comparison with authentic DIDS. The UV spectrum of the peptide shows that it has a profile very similar to that of DIDS with an identical λ_{max} at 344 nm, a wavelength at which no significant absorption by any amino acid occurs.

Results of amino acid analysis of the peptide are displayed in table 3. The amino acid composition of the peptide is identical to that of the amino terminal tryptic peptide βt-1 with the exception of the N-terminal valine residue, which is missing. By using the previously determined ε_{344} of DIDS it was possible to independently estimate the molar quantity of the DIDS-peptide both on the basis of absorption at 344 nm as well as by amino acid analysis (Table 3). These data indicated that there were approximately 1.7 moles of βt-1 per mole of DIDS in the modified peptide. The tryptic peptide map (figure 49)

TABLE 3. Amino acid analysis of modified peptides from $Hb(DIDS)^T$ and $Hb(DIDS)_2^R$. Analyses were done on 500-1000 pmol peptide as described in Materials and Methods. *Molar ratios of DIDS were determined from peptide absorbance at 344 nm prior to hydrolysis assuming an ε_{344} of 30.5 mM^{-1}.

	$\beta t\text{-}1^{native}$	$\beta t\text{-}X^{Hb(DIDS)T}$	$\beta t\text{-}X^{Hb(DIDS)_2R}$
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	(0)	0.60*	1.28*

Figure 47. Reversed-phase C_4 HPLC globin separation of Hb(DIDS)^T.

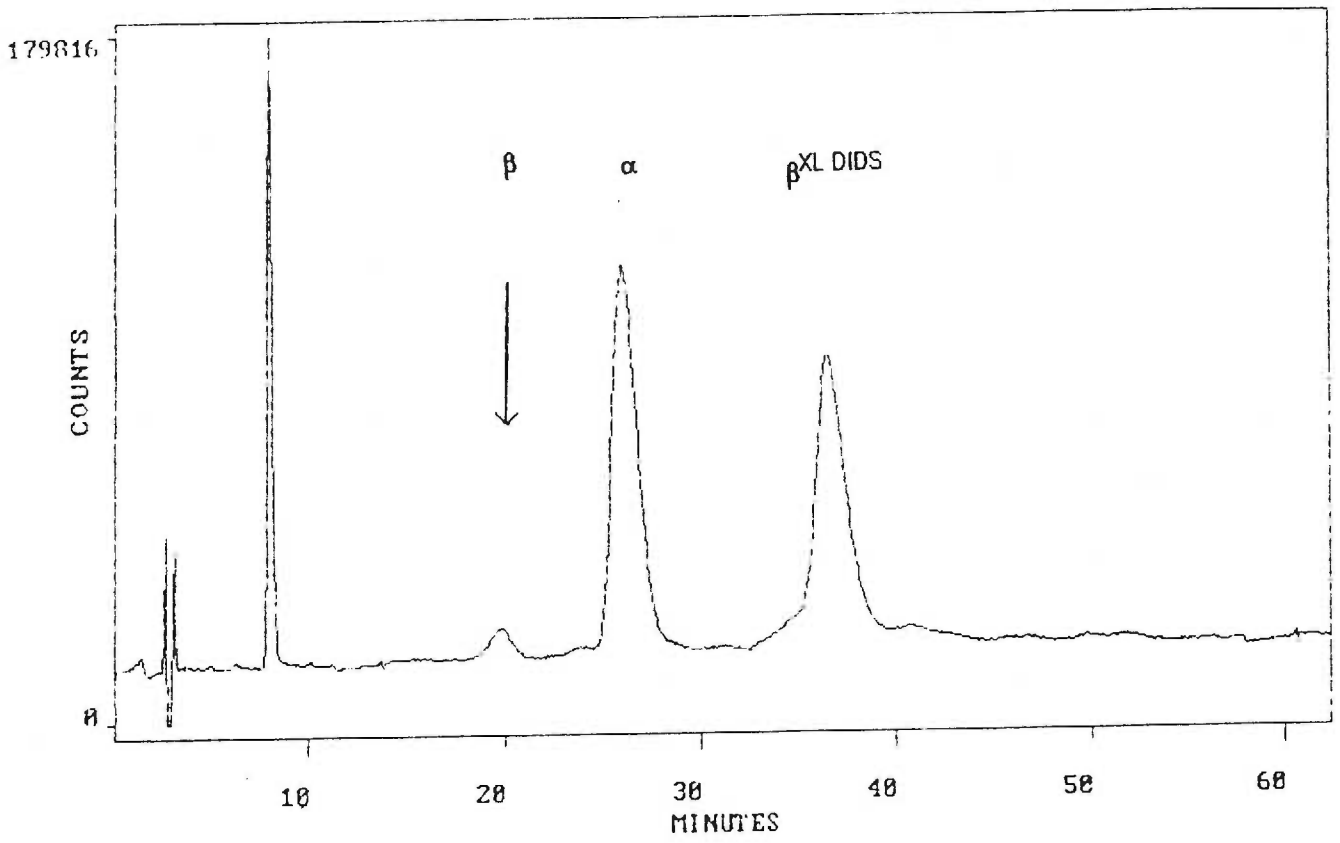
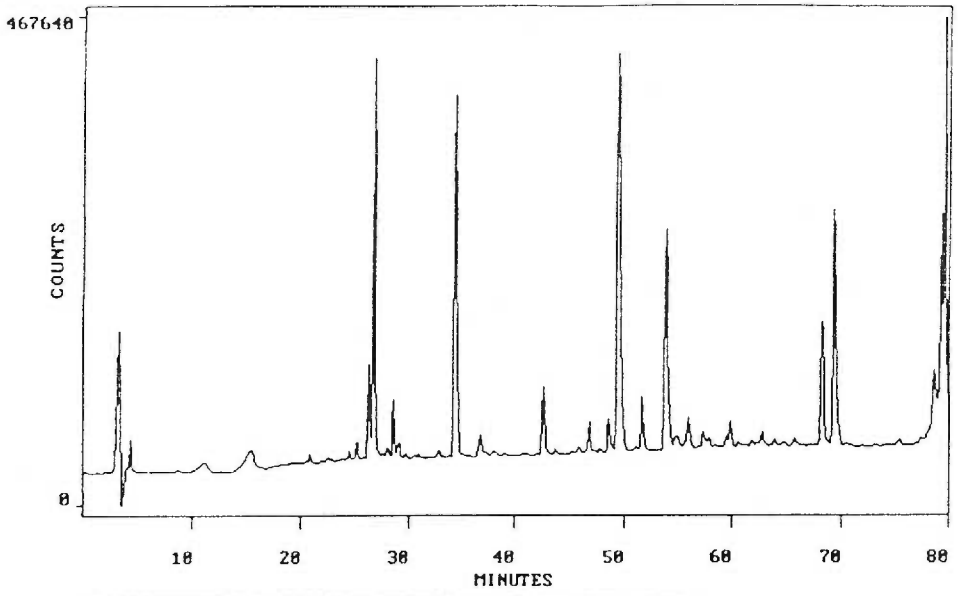

179816
β
α
βXL DIDS
COUNTS
0
10
20
30
40
50
60
MINUTES

Figure 48. HPLC of tryptic peptides from native α globin (bottom) and Hb(DIDS)^{T} α globin (top).


467648
COUNTS
0
10
20
30
40
50
60
70
80
MINUTES

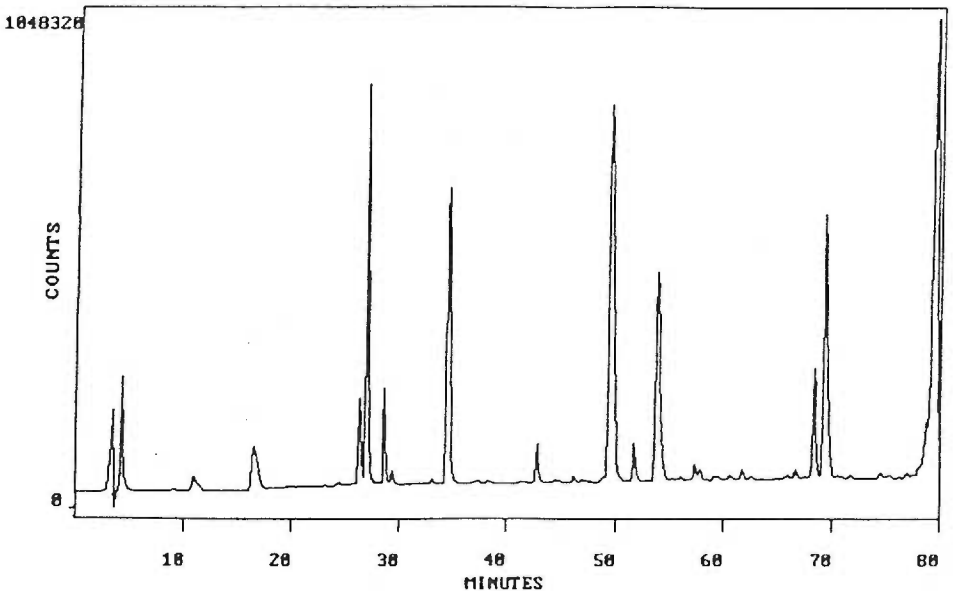

1048320
COUNTS
0
10
20
30
40
50
60
70
80
MINUTES

Figure 49. HPLC of tryptic peptides from Hb(DIDS)^{T} β globin. Effluent was simultaneously monitored at 214 nm (top) and 344 nm (bottom).

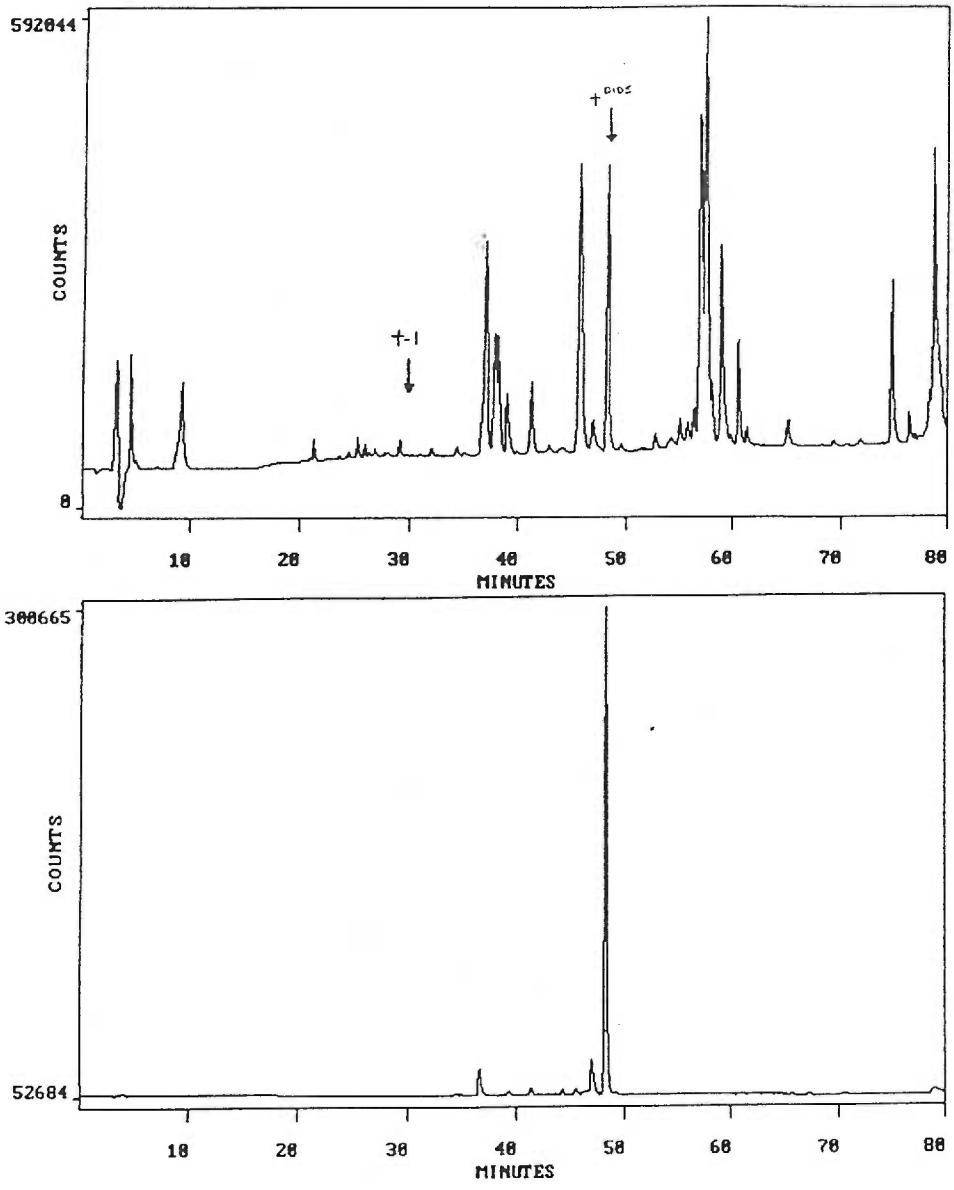

592044
COUNTS
0
+-I
+ DIDS
10
20
30
40
50
60
70
80
MINUTES
308665
COUNTS
52684
10
20
30
40
50
60
70
80
MINUTES

Figure 50. HPLC of tryptic peptides from native β globin. Effluent was simultaneously monitored at 214 nm (top) and 344 nm (bottom).

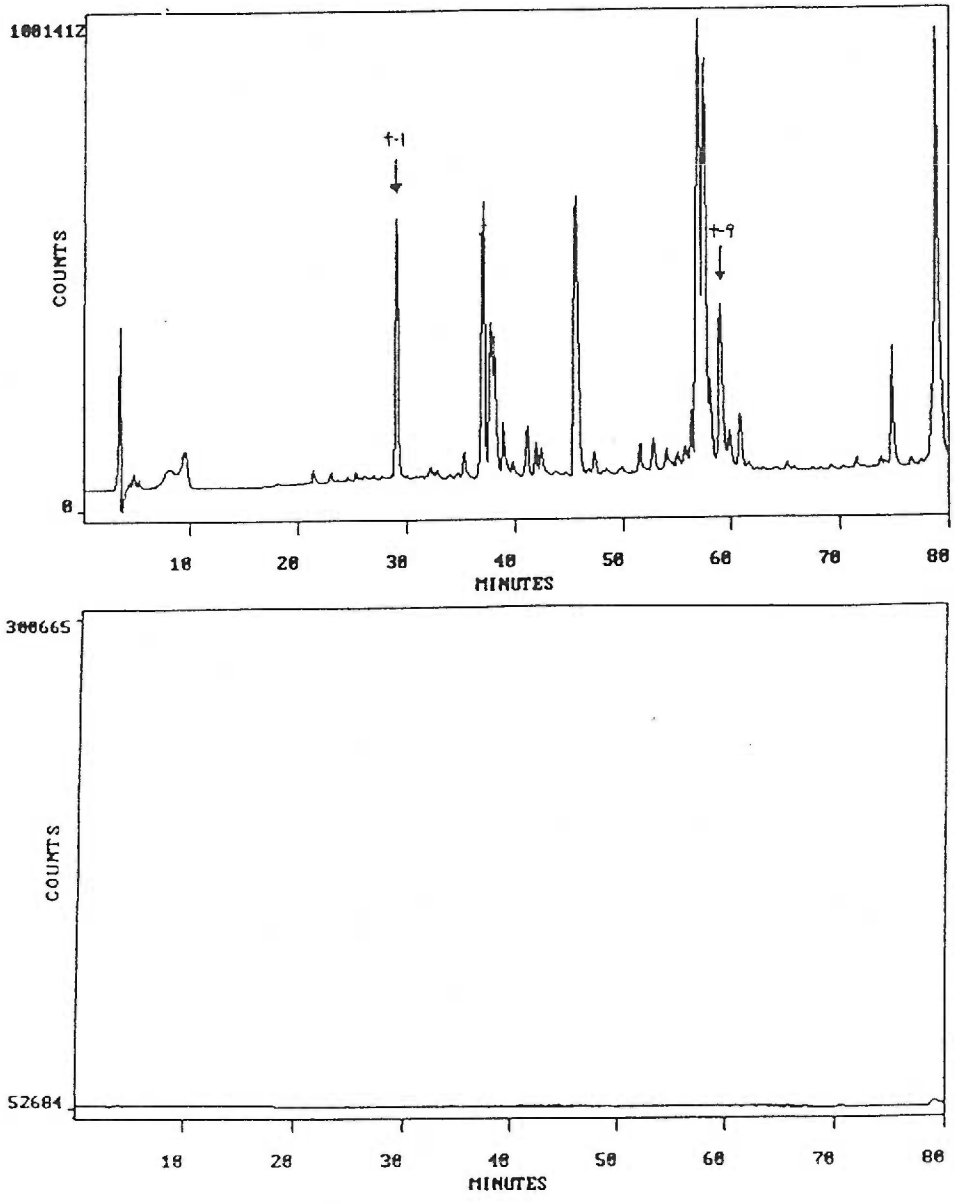
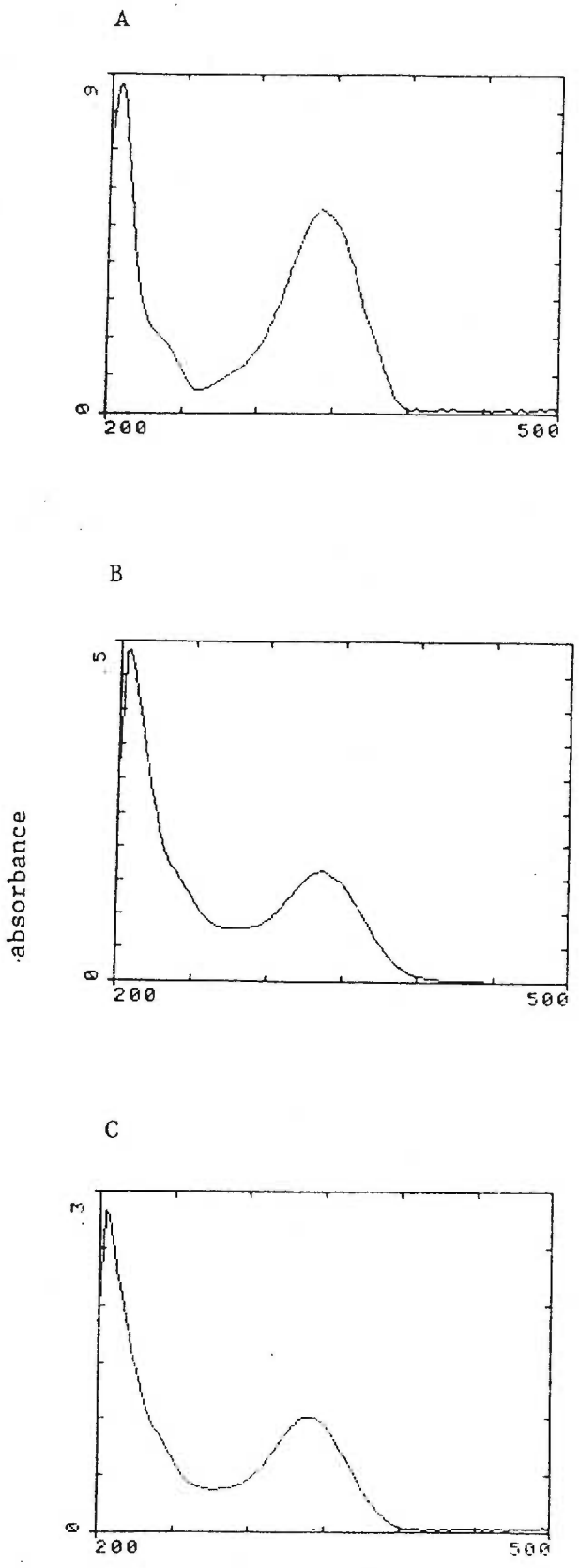

108141Z
COUNTS
0
t-1
t-9
10
20
30
40
50
60
70
80
MINUTES
300665
COUNTS
52684
10
20
30
40
50
60
70
80
MINUTES

Figure 51. Spectra of DIDS (top) and modified peptides from $Hb(DIDS)^T$ (middle) and $Hb(DIDS)_2^R$ (bottom).


A
.9
0
200
500
B
.5
0
200
500
C
.3
0
200
500
absorbance
wavelength (nm)

of the modified β globin indicated that there was no material running in the position of βt-1. To verify that all of the amino terminal peptide had been converted and was present in this modified form, the molar yield of the modified peptide was compared to that of βt-1 in a tryptic hydrolysate of native β-globin. By normalizing the molar yields from amino acid analysis to areas under five other tryptic peptide peaks, it was determined that the yield of the modified peptide was approximately 95% of that of native βt-1. A model for $Hb(DIDS)^T$ was constructed based on these data in which the β chain amino termini are crosslinked by a molecule of DIDS in the organic phosphate binding site (figure 52).

Functional studies of $Hb(DIDS)^T$ were performed in 50 mM bis-Tris buffer, pH 7.4, 0.1 M Cl^-, at 20° (figure 53). $Hb(DIDS)^T$ had a significantly decreased oxygen affinity, with a P_{50} of 28 mm Hg, approximately equal to that of whole blood. The subunit cooperativity was significantly decreased (n_{50}=1.4, n_{max}=2.5).

B. $\underline{Hb(DIDS)_2^T}$

$Hb(DIDS)_2^T$ eluted on cation-exchange HPLC before $Hb(DIDS)^T$, indicating that it had an even greater increased negative charge (figure 43). SDS-PAGE of $Hb(DIDS)_2^T$ demonstrates that a crosslink has occured in the molecule (figure 46). However, the mobility of the crosslinked globin was slightly greater than that of the crosslinked band in $Hb(DIDS)^T$, implying that the dimers had different conformations in the SDS gel. Spectroscopic studies of $Hb(DIDS)_2^T$ revealed that the molecule had an increased absorbance at 344 nm consistent with the incorporation of 2.03 molecules of DIDS per tetramer (figure 55, Table 2). Reversed-phase C_4 HPLC of $Hb(DIDS)_2^T$ demonstrated a globin peak eluting in the position of native α-globin while the β-globin eluted more slowly than native β globin (figure 54). The absorbance increase at 344 nnm was associated with the β-globin from $Hb(DIDS)_2^T$ but not the α-globin (figure 55). Tryptic peptide mapping of globins from $Hb(DIDS)_2^T$ showed that the α-globin

had a pattern identical to native α-globin (figure 56). The tryptic map of the β-globin however, revealed some differences from that of native β-globin. βt-1, the amino terminal tryptic peptide, was missing from the hydrolysate (figure 57). In addition, βt-9 (residues 67 to 82) is present in one-half yield, as determined by integration and comparison of the normalized area under the peak. A small peak was identified in the $Hb(DIDS)_2^T$ tryptic peptide HPL chromatogram which absorbed at 344 nm and which eluted at the same position as crosslinked βt-1 from $Hb(DIDS)^T$. Amino acid analysis and quantitation of DIDS in this peptide were also identical to that for crosslinked βt-1 from $Hb(DIDS)^T$. It was therefore concluded that the two modified peptides were identical. However, the yield of crosslinked βt-1 in the $Hb(DIDS)_2^T$ hydrolysate was less than 10% of the theoretical yield. This suggested that the presence of crosslinked βt-1 in the tryptic hydrolysate of $Hb(DIDS)_2^T$ was due to contamination of $Hb(DIDS)_2^T$ with a small amount of material crosslinked between the β amino termini. The reduction in yield of βt-9 by one-half suggests the possibility that there may be a crosslink formed between Val1 β_1 and Lys 82 β_2. This possibility is also supported by the difference in mobility of the crosslinked β globins from $Hb(DIDS)_2^T$ and $Hb(DIDS)^T$ during SDS-PAGE. β globin crosslinked between the amino terminus of one chain and the middle of the other (i.e., Val 1 and Lys 82) would be expected to be in a less extended conformation in an SDS gel than β globin crosslinked between the amino termini, and therefore have a higher mobility.

Functional studies of $Hb(DIDS)_2^T$ in 50 mM bis Tris, pH 7.4, 0.1 M Cl, 25° revealed it to have a significantly lowered oxygen affinity (figure 58). The P_{50} of $Hb(DIDS)_2^T$ is 26 mm Hg, close to that of $Hb(DIDS)^T$, but the subunit cooperativity is somewhat more reduced (n_{50}=1.2, n_{max}=2.0).

C. $\underline{Hb(DIDS)_2^R}$

When oxyhemoglobin is allowed to react with DIDS, the predominant modified species elutes on cation-exchange HPLC in a position close

to $Hb(DIDS)_2^T$. SDS-PAGE of this fraction reveals that it has an extra band which runs slightly slower than native globin, similar to di-ATP Hb, but no bands running in the position of crosslinked globin (figure 46). Analytical reversed-phase C_4 HPLC of $Hb(DIDS)_2^R$ indicates that it has a peak which elutes in the position of native α-globin, and a slower moving β globin peak corresponding to the slow running band on SDS-PAGE (figure 59). Spectrophotometric studies of this component revealed a $\Delta\varepsilon_{344}$ of 48.7 mM^{-1}, reflecting a theoretical incorporation of 1.6 molecules of DIDS per tetramer (Table 2). The uv spectrum of the globins from $Hb(DIDS)_2^R$ reveal that the increased absorbance at 344 nm is associated solely with the β globin (figure 55). Tryptic peptide mapping of $Hb(DIDS)_2^R$ revealed an unchanged pattern for the α-globin (figure 60) and a normal pattern for the β-globin except for the complete absence of the amino-terminal tryptic peptide βt-1 (figure 61). In addition a new peptide appeared in the hydrolysate which eluted later than βt-1 and absorbed at 344 nm. This peptide had a uv spectrum like that of DIDS (figure 51). When the peptide was subjected to amino acid analysis, the composition was identical to βt-1 except for the loss of the amino terminal valine residue (Table 3). The molar ratio of DIDS to peptide was 1.28, approximately double that of the crosslinked βt-1 from $Hb(DIDS)^T$ (Table 3). These data suggested a structure for $Hb(DIDS)_2^R$ in which two molecules of DIDS are incorporated per tetramer, with each molecule involved in a monofunctional adduct with a β chain amino terminus (figure 62). In contrast to the reaction of deoxyhemoglobin with o-ATP, which also resulted in the formation of monoadducts, two molecules of DIDS must be able to react sequentially with a single tetramer. The limit for specific modification of hemoglobin with o-ATP was found to be 50% disubstituted tetramer after subunit dissociation and reformation during ion-exchange chromatography. With DIDS, however, close to 100% yields of disubstituted tetramer were obtained when a two-fold excess of reagent was used.

Functional studies of $Hb(DIDS)_2^R$ in 50 mM bis-Tris pH 7.4, 0.1 M Cl,

at 20° were performed. The molecule exhibited unusual behavior during the oxygen equilibria determinations (figure 63). Upon initial deoxygenation, $Hb(DIDS)_2{}^R$ exhibited an increased oxygen affinity relative to native hemoglobin. Upon reoxygenation followed by a second deoxygenatiion, however, the oxygen affinity curve became markedly biphasic, reflecting the appearance of a lower affinity component. This behavior suggested the possibility that $Hb(DIDS)_2{}^R$ had undergone a structural change during the equilibrium experiment. Cation-exchange HPLC of $Hb(DIDS)_2{}^R$ after the equilibrium experiment revealed the appearance of a new peak which eluted in the same position as $Hb(DIDS)_2{}^T$. This experiment was later repeated by simply deoxygenating a sample of $Hb(DIDS)_2{}^R$ followed by cation-exchange HPLC (figure 64). The new peak appearing in the position of $Hb(DIDS)_2{}^T$ suggested that the structural transition of the protein from the R to the T state allowed a further covalent modification to occur involving a free unreacted isothiocyanate group (figure 62). This hypothesis was further supported by the appearence of a 34 kD band after SDS-PAGE of $Hb(DIDS)_2{}^R$ following deoxygenation.

An explanation for the difference in structures of the R and T modified forms is suggested by the structural changes in the $\beta_1\beta_2$ cleft which occur during the T to R transition. Oxygenation (a shift to the R state) causes the β chain amino termini to move apart several angstroms and assume a geometry unfavorable for crosslinking by DIDS. (The intermolecular distance between funtional groups in DIDS is approximately 15Å, while the N-termini are 20 Å apart in the R state (123)). In this case, two monofunctional adducts are formed leading to the formation of $Hb(DIDS)_2{}^R$ (figure 62). $Hb(DIDS)_2{}^R$ was also isolated from the reaction mixture of deoxyhemoglobin with DIDS (figure 43) and was concluded to be the same as $Hb(DIDS)_2{}^R$ isolated from the oxyhemoglobin reaction mixture based on cation-exchange HPLC elution, tryptic mapping, and functional studies. The formation of $Hb(DIDS)_2{}^R$ in the deoxyhemoglobin reaction mixture could be due to a residual amount of liganded hemoglobin or alternatively could be

formed at the conclusion of the reaction when the reaction mixture is reoxygenated and rapidly chromatographed on a Sephadex G-25 column. A third possibility is that some monofunctional adduct can be formed even when the protein is in the T state.

Figure 52. Model of Hb(DIDS)^{T} showing crosslink formation between β chain amino termini.

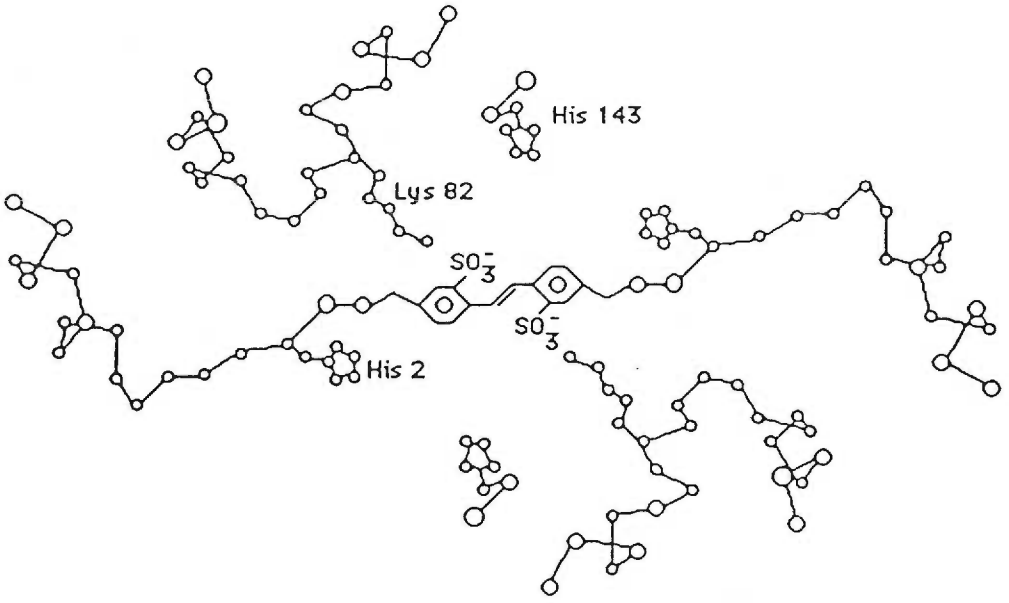

His 143
Lys 82
SO_3^-
SO_3^-
His 2

Figure 53. Oxygen equilibrium curve for 60 μM Hb(DIDS)^{T} in 50 mM bis-Tris pH 7.4 0.1 M Cl, 20°.

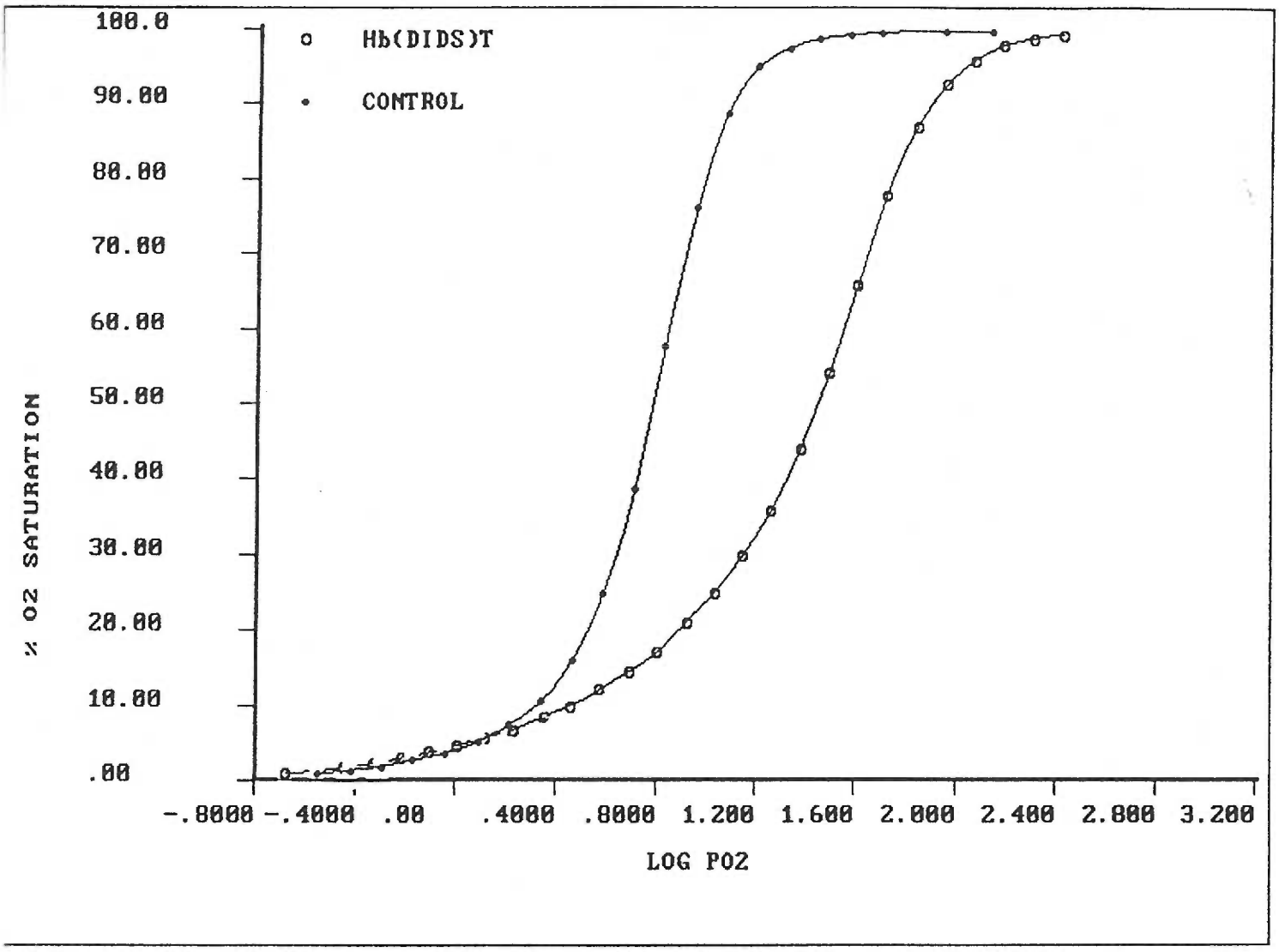

Hb(DIDS)T
CONTROL
100.0
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
.00
% O2 SATURATION
-.8000 -.4000 .00 .4000 .8000 1.200 1.600 2.000 2.400 2.800 3.200
LOG PO2

Figure 54. Reversed-phase C_4 HPLC globin separation of $Hb(DIDS)_2^T$.

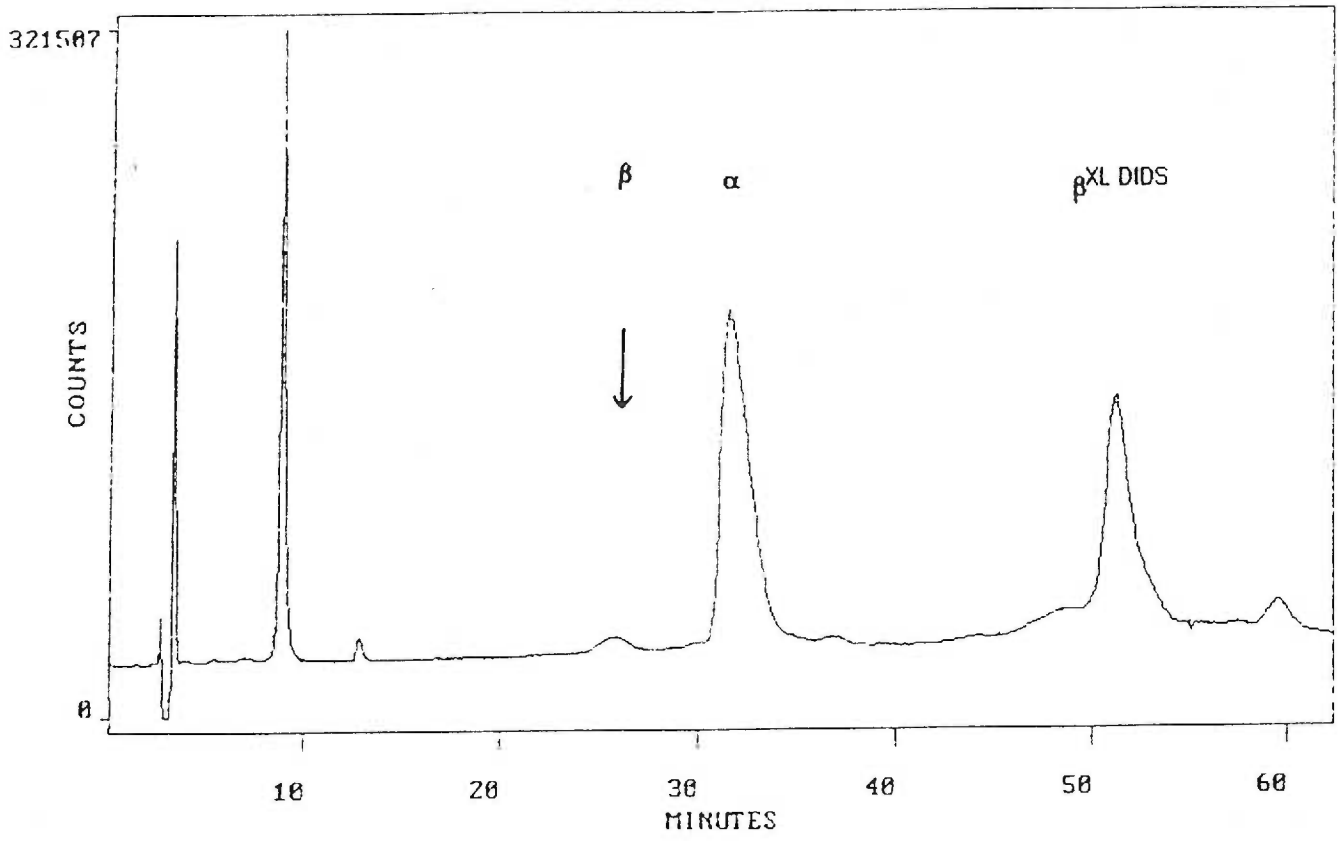

321507
COUNTS
0
β
α
βXL DIDS
10
20
30
40
50
60
MINUTES

Figure 55. UV spectra of globins from native and DIDS-modified hemoglobins.

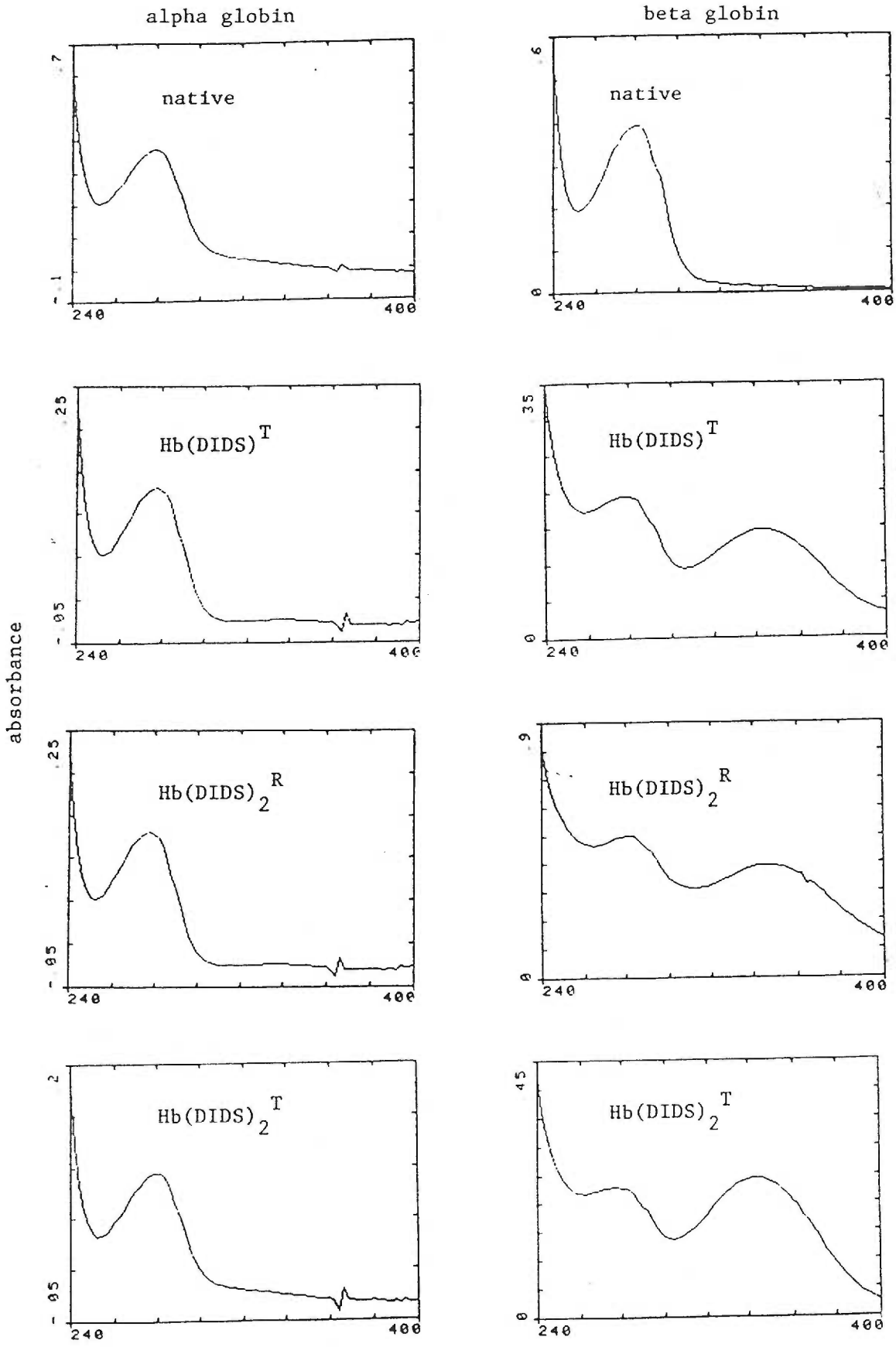
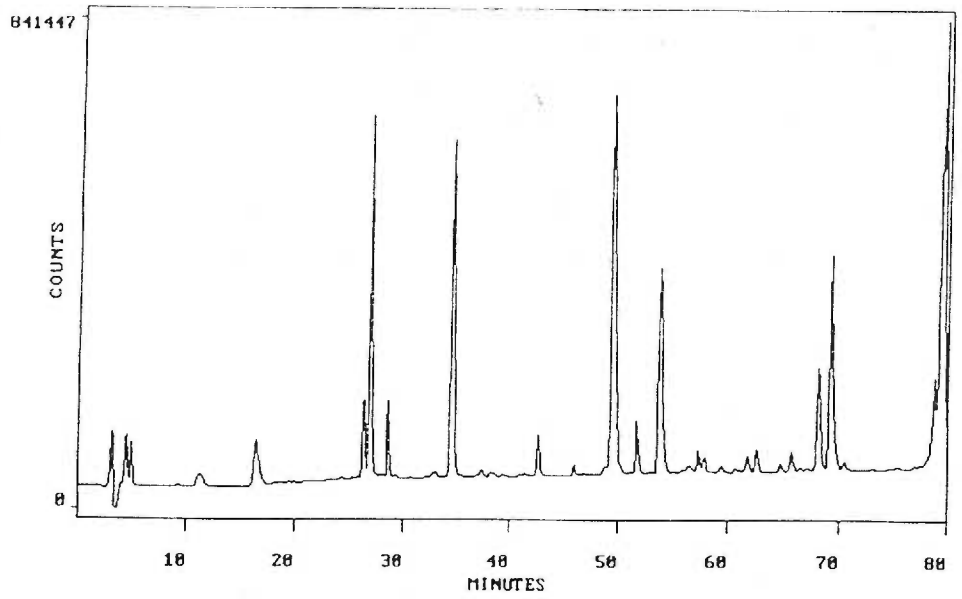

alpha globin
beta globin
native
native
Hb(DIDS)T
Hb(DIDS)T
Hb(DIDS)2R
Hb(DIDS)2R
Hb(DIDS)2T
Hb(DIDS)2T
absorbance
wavelength (nm)
240
400

Figure 56. HPLC of tryptic peptides monitored at 214 nm from native α globin (bottom) and $Hb(DIDS)_2^T$ α globin (top).


841447
COUNTS
0
10
20
30
40
50
60
70
80
MINUTES

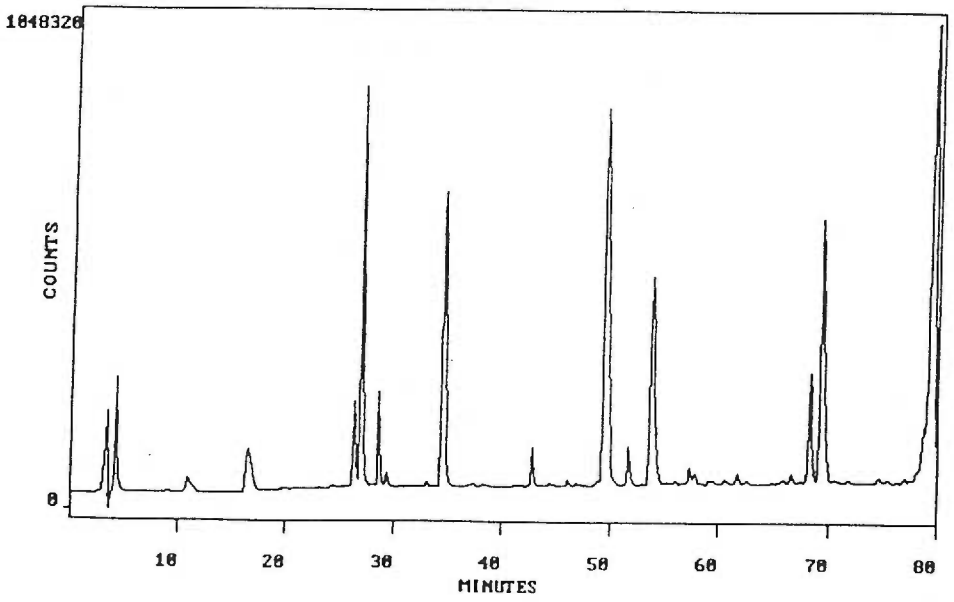

1048320
COUNTS
0
10
20
30
40
50
60
70
80
MINUTES

Figure 57. HPLC of tryptic peptides from $Hb(DIDS)_2^T$ β globin simultaneously monitored at 214 nm (top) and 344 nm (bottom).

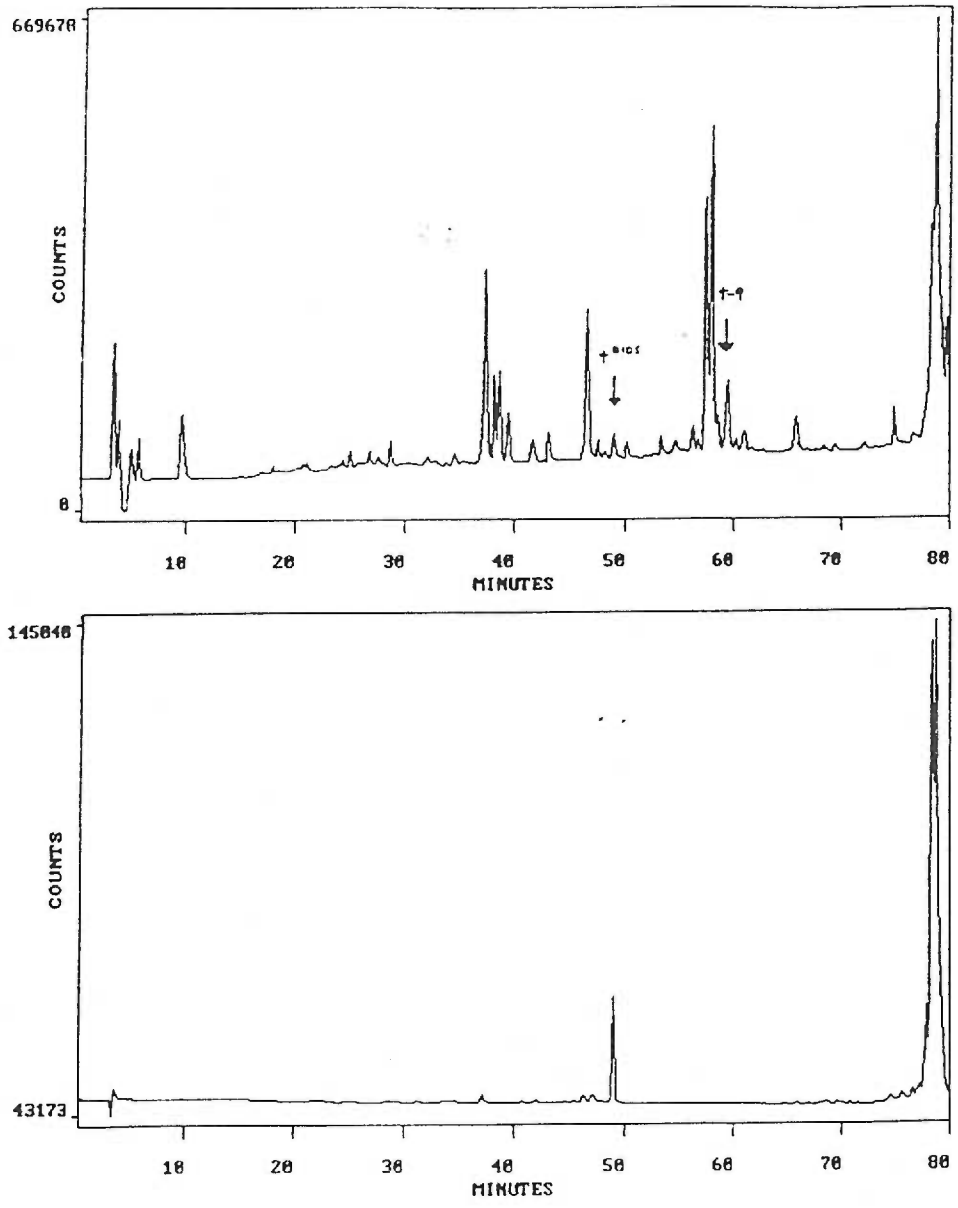

669670
COUNTS
0
10
20
30
40
50
60
70
80
MINUTES
145040
COUNTS
43173
10
20
30
40
50
60
70
80
MINUTES

Figure 58. Oxygen equilibrium curve for 60 μM $Hb(DIDS)_2^T$ in 50 mM bis-Tris pH 7.4 0.1 M Cl, 20°.

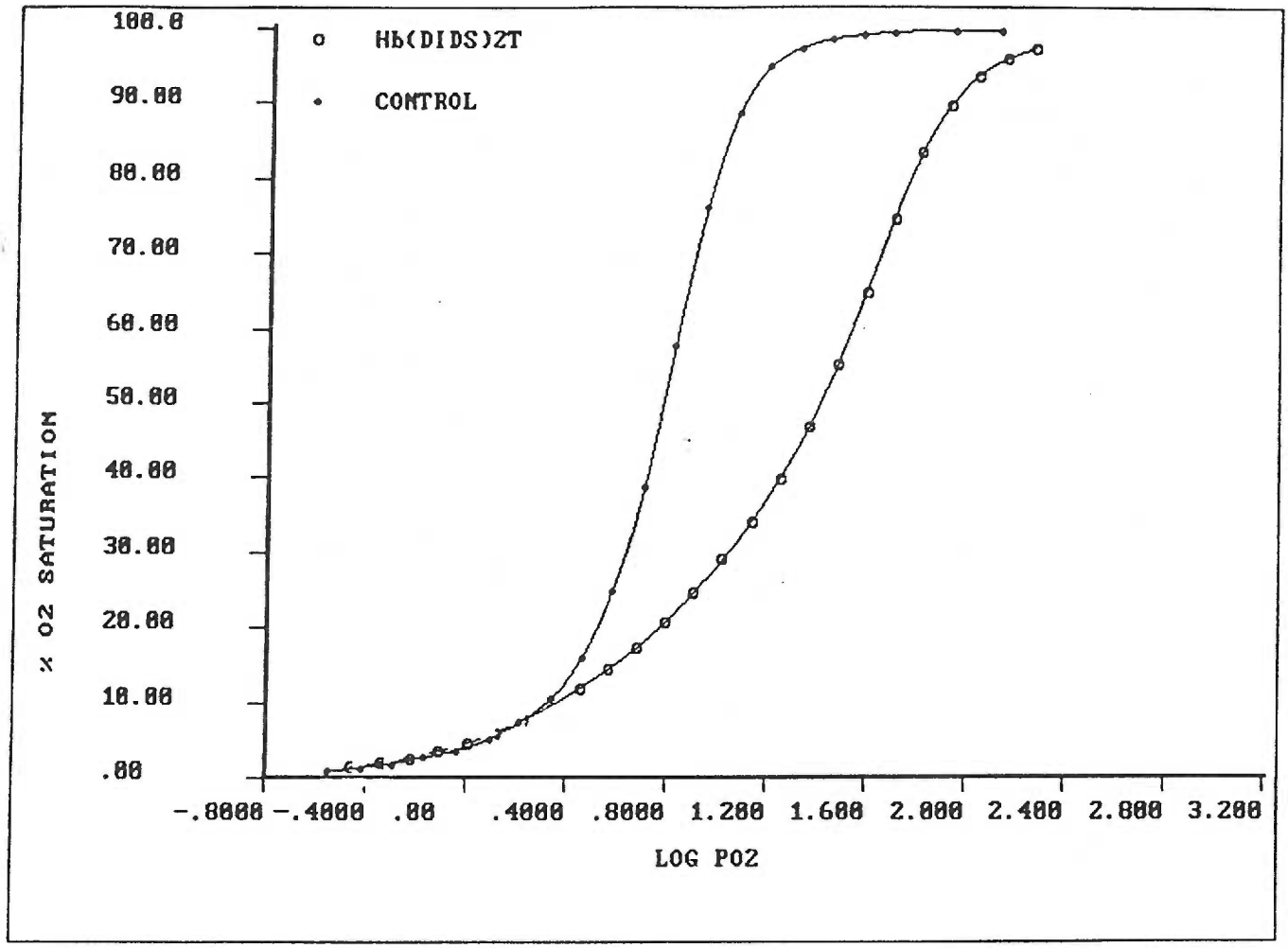

100.0
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
.00
% O2 SATURATION
o Hb(DIDS)2T
• CONTROL
-.8000 -.4000 .00 .4000 .8000 1.200 1.600 2.000 2.400 2.800 3.200
LOG PO2

Figure 59. Reversed-phase C_4 HPLC globin separation of $Hb(DIDS)_2^R$.

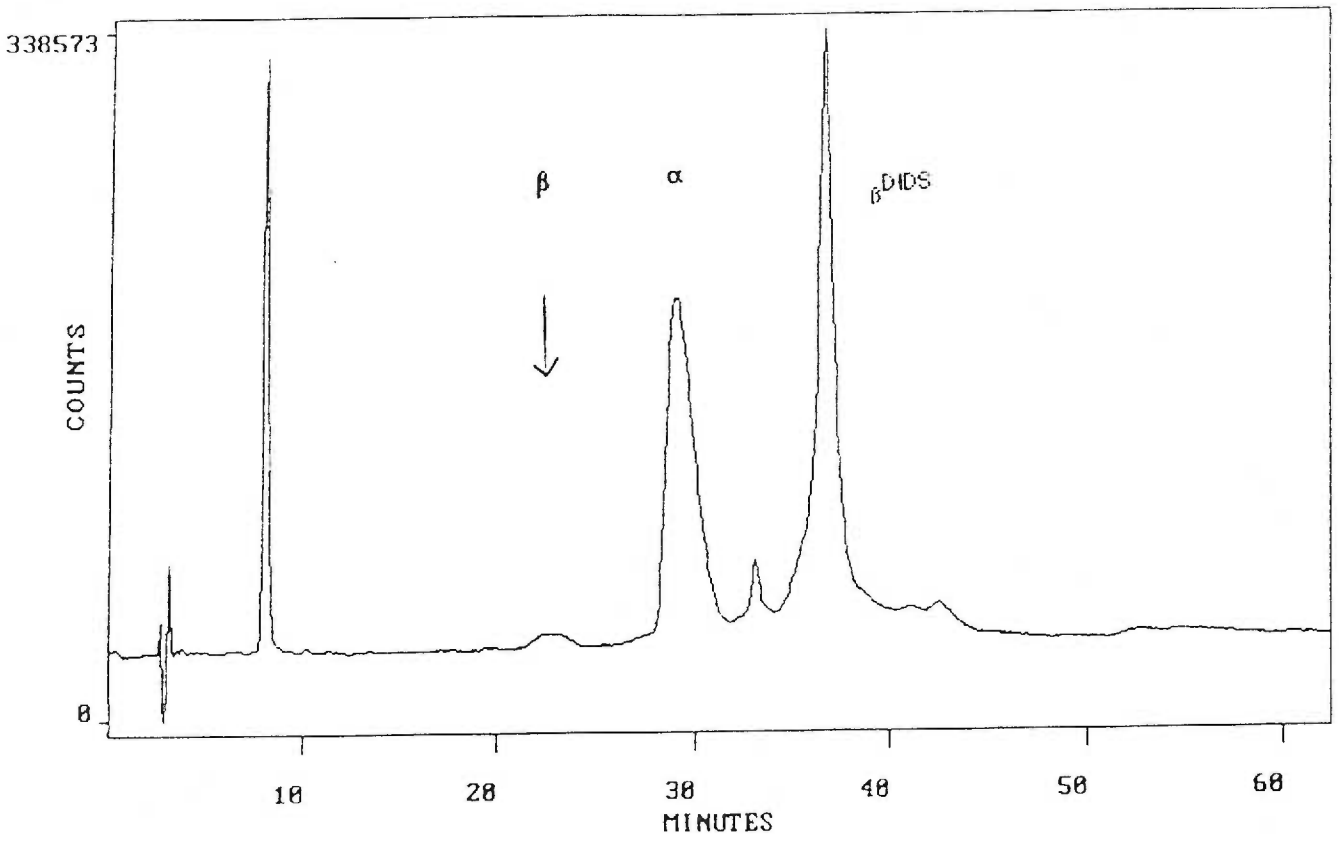

338573
0
COUNTS
β
α
βDIDS
10
20
30
40
50
60
MINUTES

Figure 60. HPLC of tryptic peptides monitored at 214 nm from native α globin (bottom) and $Hb(DIDS)_2^R$ α globin (top).

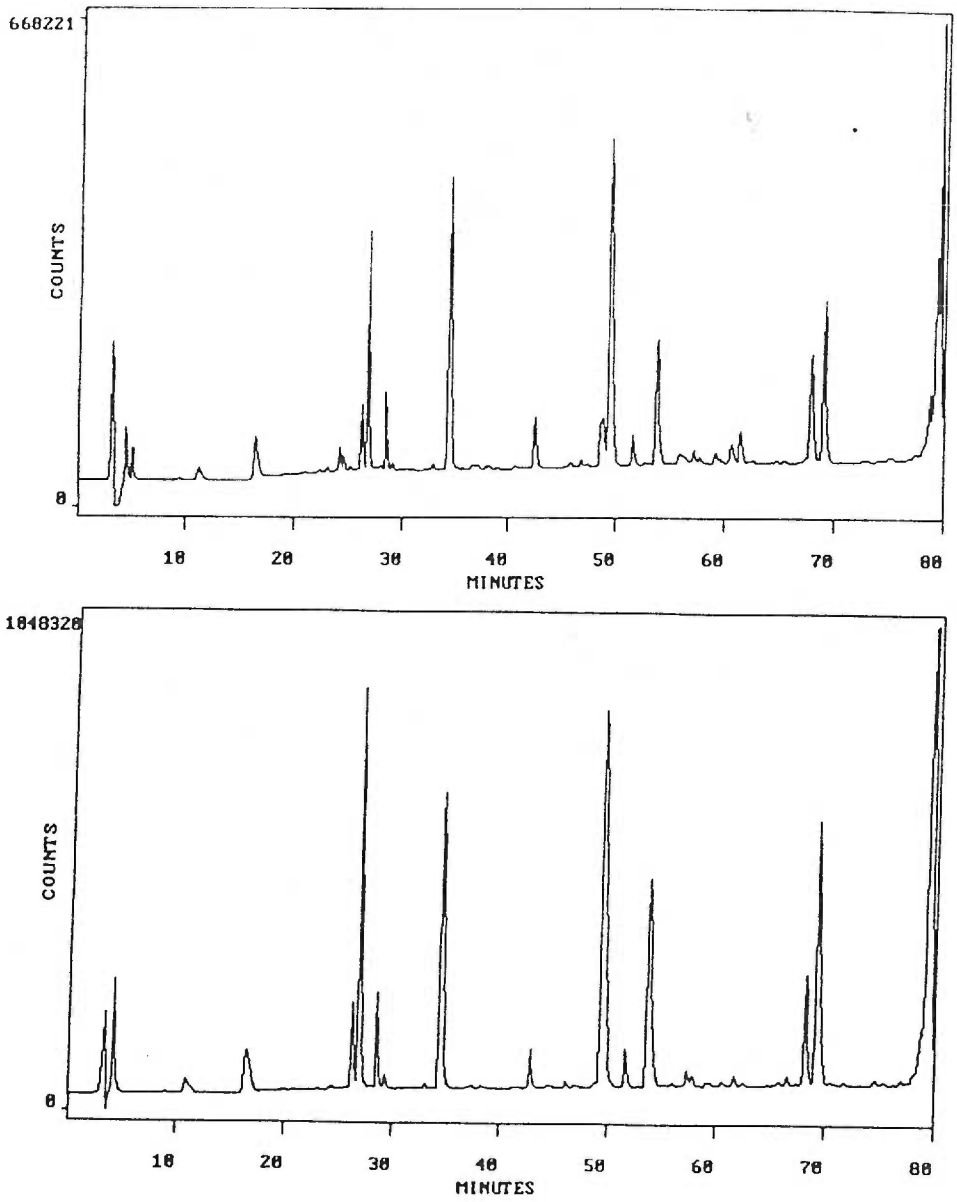
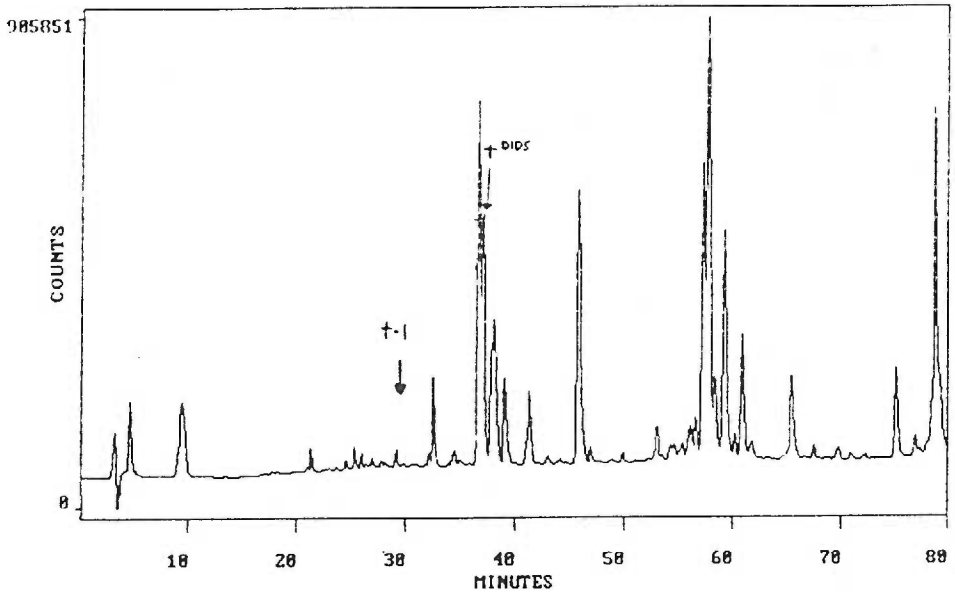

668221
COUNTS
0
10
20
30
40
50
60
70
80
MINUTES
1848328
COUNTS
0
10
20
30
40
50
60
70
80
MINUTES

Figure 61. HPLC of tryptic peptides from $Hb(DIDS)_2^R$ β globin simultaneously monitored at 214 nm (top) and 344 nm (bottom).


985851
COUNTS
0
DIDS
10
20
30
40
50
60
70
80
MINUTES

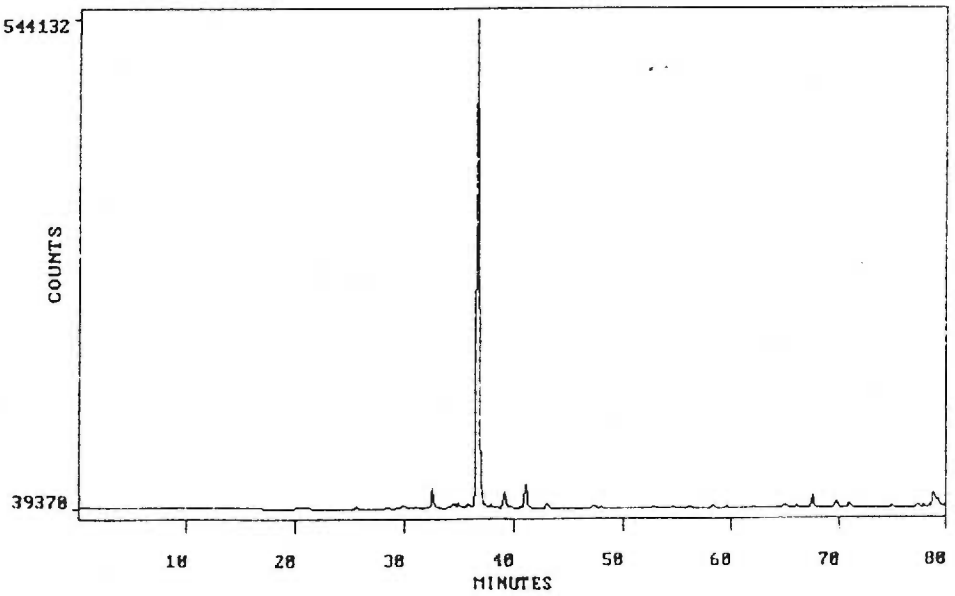

544132
COUNTS
39370
10
20
30
40
50
60
70
80
MINUTES

Figure 62. Molecular model of $Hb(DIDS)_2^R$.

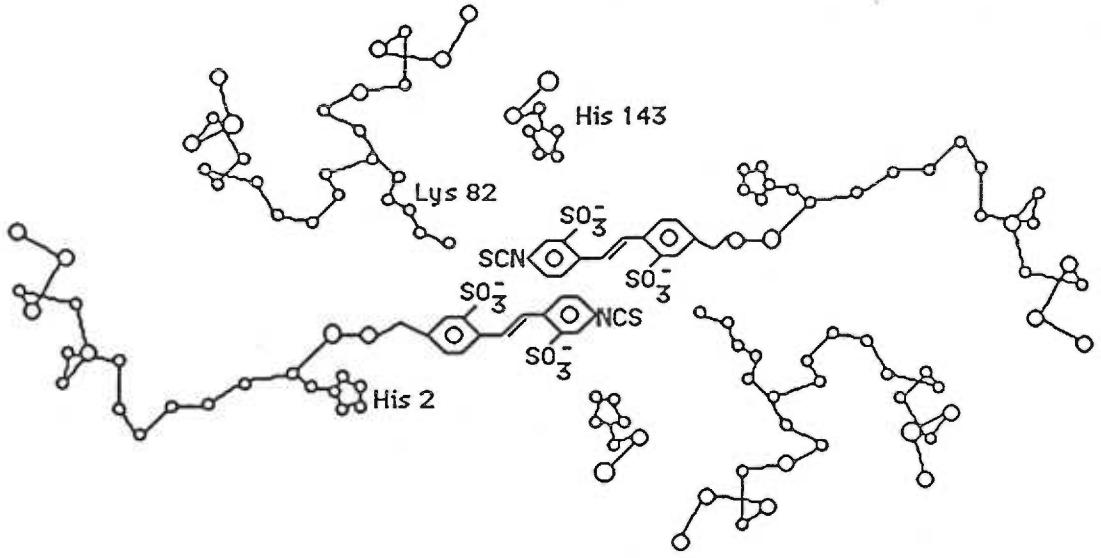

His 143
Lys 82
SO3-
SCN
SO3-
SO3-
NCS
SO3-
His 2

Figure 63. Oxygen binding curve for 60 μM $Hb(DIDS)_2^R$ in 50 mM bis-Tris pH 7.4 0.1 M Cl, 20°. Curve 1(°) is initial deoxygenation, curve 2 (*) is second deoxygenation following reoxygenation.

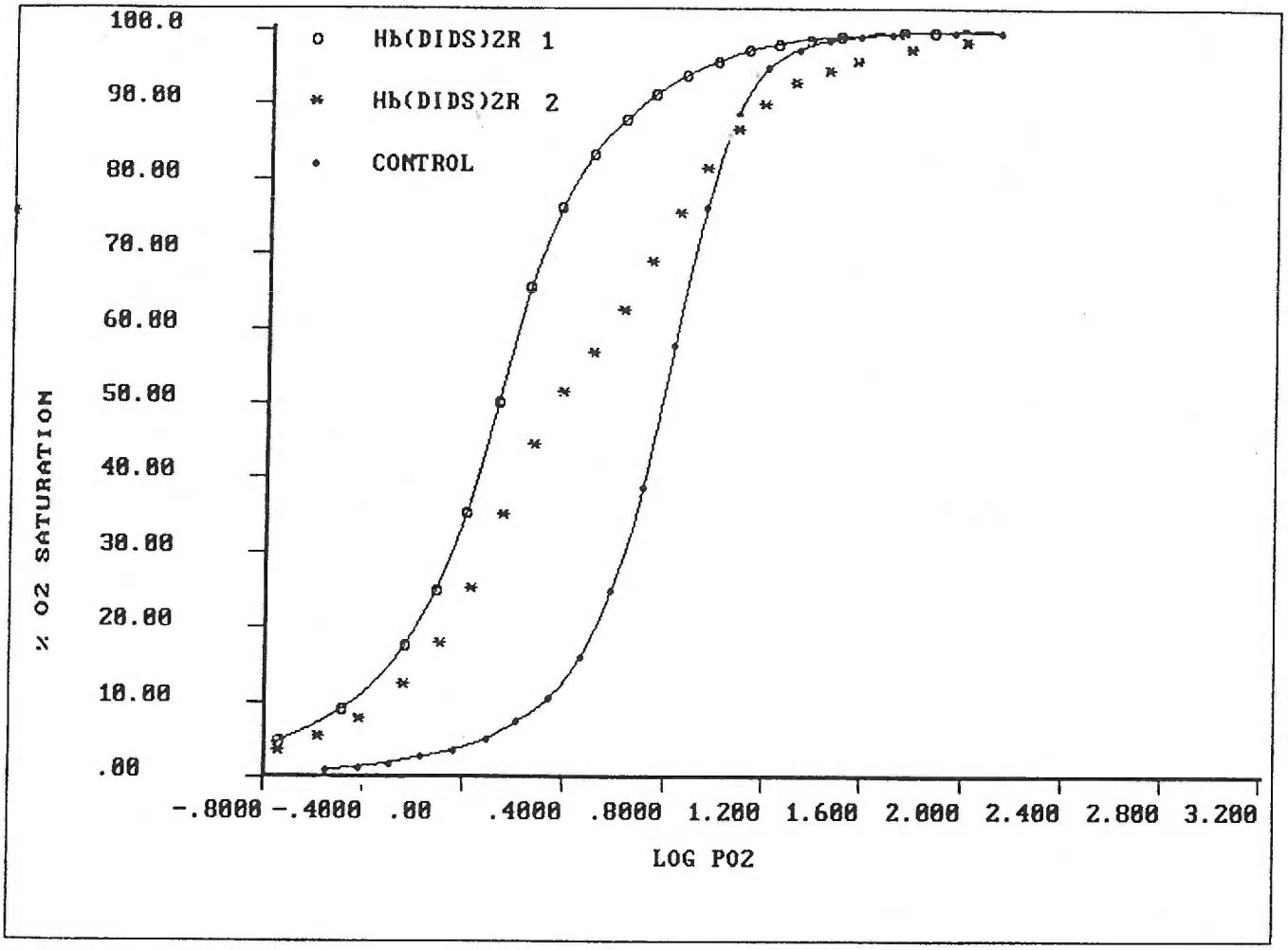
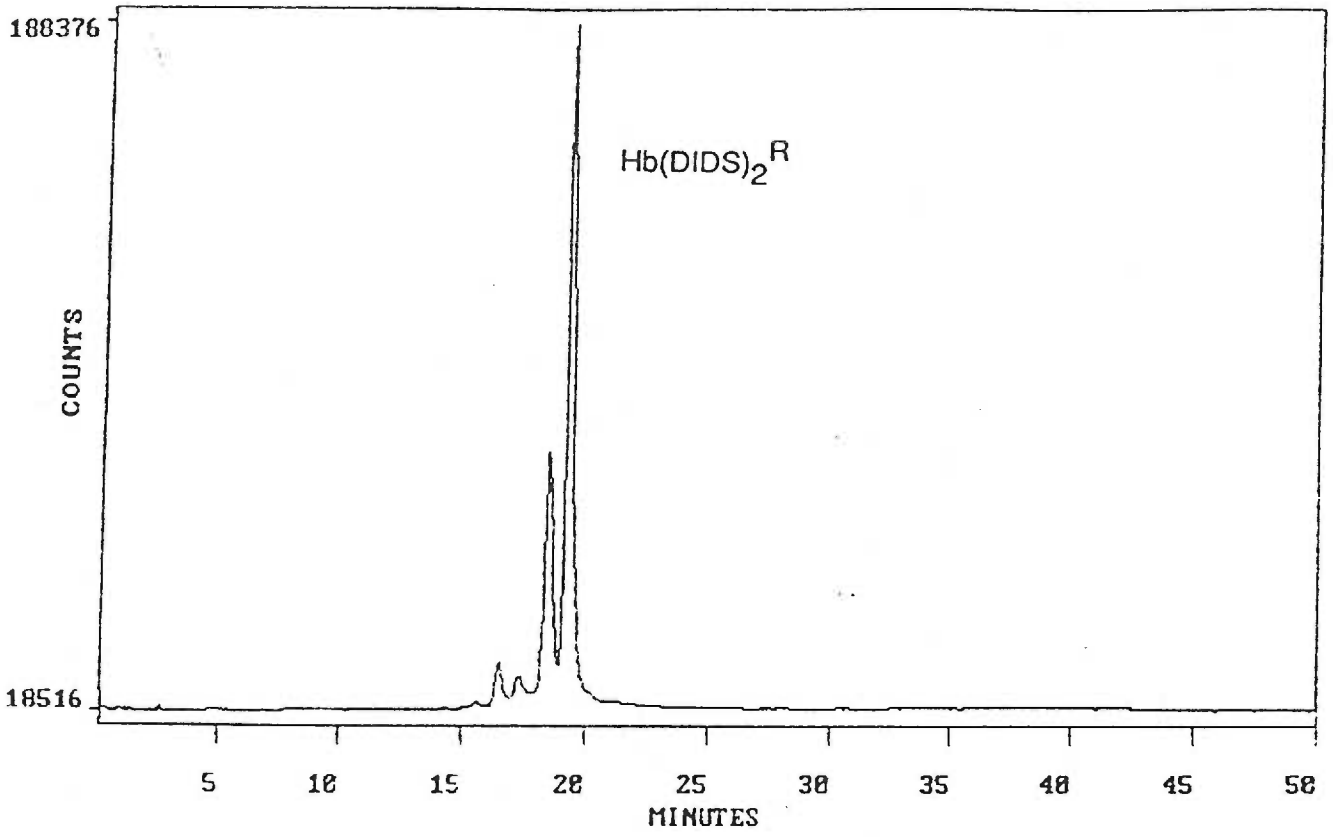

100.0
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
.00
% O2 SATURATION
o Hb(DIDS)2R 1
* Hb(DIDS)2R 2
• CONTROL
-.8000 -.4000 .00 .4000 .8000 1.200 1.600 2.000 2.400 2.800 3.200
LOG PO2

Figure 64. Cation-exchange HPLC of $Hb(DIDS)_2^R$ following deoxygenation for 3 hours at 0° showing appearance of new peak eluting in position of $Hb(DIDS)_2^T$.


188376
18516
COUNTS
Hb(DIDS)2R
5
10
15
20
25
30
35
40
45
50
MINUTES

CONCLUSIONS

The synthesis of a homogeneous crosslinked hemoglobin with low oxygen affinity has been the focus of much effort over the past several years. It has been suggested that periodate-oxidized adenosine triphosphate (o-ATP) may be a useful reagent for the production of a crosslinked low affinity hemoglobin suitable for use as a blood substitute (60, 61). The results presented in this dissertation, however, indicate that the properties of the various o-ATP modified hemoglobins are incompatible with this use. It was found that the hemoglobin fractions which had been crosslinked by o-ATP (fractions 2 and 3) had significantly increased oxygen affinity. Conversely, a fraction with low oxygen affinity which was also isolated was found to be disubstituted and not crosslinked (fraction 1). Attempts to produce a crosslinked molecule with low oxygen affinity using o-ATP were unsuccessful, it is hypothesized, because of the geometry and conformational flexibility of the ribose ring-opened dialdehyde bridging structure.

During the transition in hemoglobin from the R state to the T state, the EF regions in which Lys 82 β_1 and β_2 are found to move apart several angstroms and the opening to the central cavity enlarges (123). Crosslinking between residues in the EF region, e.g. Lys 82 β, may hinder this movement depending on the geometry and length of the bridging group. For example, when the reagent bis (3,5-dibromosalicyl) fumarate is used for crosslinking Lys 82 β_1 to Lys 82 β_2 with a 6.8 Å four-carbon spacer group, the resulting molecule has an increased oxygen affinity (42). Although the β globin residues involved in crosslink formation by o-ATP have not been definitively identified, circumstantial evidence points to involvement of Lys 82 and/or the amino termini. These groups are the only primary amines in the organic phosphate binding site and would thus

be the most likely candidates for reaction with the ring-opened dialdehyde. The intermolecular distance between the functional groups in o-ATP is approximately 7.5 Å in the theoretical extended antiperiplanar conformation, although the actual conformation is likely not to be extended (56,76). The existence of three distinct o-ATP-crosslinked β globins has been demonstrated, indicating that various bridging combinations may be occurring in this reaction. There are three possible permutations of bridging by o-ATP in the central cavity: Lys 82 β_1- Lys 82 β_2, and two possible Lys 82 β_1-Val 1 β_2 structures which can exist since the aldehyde groups in o-ATP are not symmetric. Crosslink formation between Lys 82 β_1 and Lys 82 β_2 by o-ATP could result in a high affinity molecule by a mechanism analogous to bis (3,5-dibromosalicyl) fumarate crosslinking, where the EF regions are constrained by the bridge between them. In the event of crosslink formation between Lys 82 β_1 and Val 1 β_2 by o-ATP, the mechanism for an increase in oxygen affinity is not as clear. Crosslinking by 2-nor-2-formyl pyridoxal phosphate, which has a rigid conformation and a 7.5 Å intermolecular functional group distance, has been shown by x-ray crystallography to involve a bridge between Lys 82 β_1 and Val 1 β_2. This modification results in lowered oxygen affinity (63). However, the only major structural perturbation seen in nfPLP-crosslinked hemoglobin is a movement of the N-terminal helix 3 Å toward the central cavity (67) (figure 9). The EF region in nfPLP-crosslinked hemoglobin is relatively unperturbed in the deoxy state, and in its normal T state position shifted away from the central cavity. It is possible that crosslinking between Lys 82 β_1 and Val 1 β_2 by o-ATP with its flexible bridging structure could impose additional constraints and hinder motion of the EF region away from the cavity. X-ray crystallographic studies of the o-ATP crosslinked hemoglobins will be crucial in interpreting the functional data and distinguishing among these possibilities.

The major product obtained after ion-exchange chromatography of the borohydride-reduced o-ATP reaction mixture has been shown to be di-ATP hemoglobin. Biochemical and x-ray crystallographic evidence have indicated that the modification in this product is an adduct involving Lys 82 β. This is in contrast to the major product obtained after the reaction of hemoglobin with pyridoxal phosphate, di-PLP hemoglobin, which has been shown to involve an adduct with the β amino termini (67). Because of the greater nucleophilicity (lower pK) of the β chain amino termini compared with the ε-amino group of Lys 82 β, they are more reactive toward carbonyl groups and susceptible to modification by general amino group reagents (69,71). Modification of Lys 82 β by o-ATP therefore implies a highly specific binding conformation in the central cavity which would cause a near-neighbor enhancement of the rate of reaction with the ε-amino group.

In contrast to the o-ATP crosslinked species, the di-ATP product had a significantly lowered oxygen affinity (P_{50} = 20.8 mm Hg vs 1.0 mm Hg for crosslinked). This is the first report of a hemoglobin modified at this residue with a lowered oxygen affinity. The structure of di-ATP Hb determined by x-ray crystallography (figure 20) suggests a possible mechanism for the reduction in oxygen affinity of this molecule. Both molecules of o-ATP are protruding into the $\beta_1\beta_2$ cleft with the planes of the adenine rings 3.4 Å apart and parallel to each other. The subunit motion involved in switching from the T to the R state would tend to push the adenine rings closer together resulting in steric repulsion which would inhibit the T -> R shift. The allosteric equilibrium would therefore be shifted toward the T state and a low oxygen affinity would result. This mechanism would also be consistent with the reduction in subunit cooperativity observed in di-ATP Hb.

A novel set of site-specific molecular modifications of hemoglobin

with 4,4'-diisothiocyanato-2,2'-stilbene disulfonate (DIDS) has also been described in this dissertation. It was found that DIDS had a high affinity for the organic phosphate binding site, reflected in the specificity of modification of residues in this region. When deoxyhemoglobin was reacted with DIDS, a number of modified products resulted, three of which were characterized structurally and functionally. The major product after addition of a stoichiometric amount of DIDS to deoxyhemoglobin was Hb(DIDS)^T, which was shown to crosslink the β chains via phenylthiocarbamyl adducts with the α-amino termini. This hemoglobin had a dramatically lowered oxygen affinity, comparable to that of whole blood. It also exhibited reduced subunit cooperativity. Until x-ray crystallographic studies are done with this molecule, any proposed mechanism for its reduced oxygen affinity must be based on theoretical considerations of its atomic structure. It would seem reasonable to speculate, however, that on the basis of the geometry and intramolecular bifunctional distance of DIDS, the amino terminal helices of Hb(DIDS)^T are constrained in a T-like conformation. The distance between the isothiocyanate groups in DIDS is approximately 15 Å, while the distance between the α-amino termini of the β chains is 16 Å in the deoxy state (123). When the native protein shifts to the R state, the termini move apart 4 Å (123). The formation of thiocarbamyl linkages between the amino termini and the isothiocyanate groups thus would prevent movement of the helices away from the cleft and result in significant structural perturbation of the R state. This would in turn bring about a shift in the allosteric equilibrium toward the T state, resulting in a decreased oxygen affinity. Further functional studies on Hb(DIDS)^T will be required to investigate its detailed allosteric properties and to determine the relative changes in the allosteric constants c (K^T / K^R) and L ($[Hb^T]/[Hb^R]$). If there is a decrease in K^T of Hb(DIDS)^T, this would be direct evidence that movement of the amino helices toward the central cavity like that seen in the 2,3-DPG-hemoglobin complex results in a lowering of the T state oxygen affinity.

A second modified hemoglobin isolated from the deoxygenated reaction mixture was $Hb(DIDS)_2^T$. This hemoglobin was shown to have incorporated two molecules of DIDS by forming adducts with both β chain amino termini and, by indirect evidence, a crosslink between one of the termini and Lys 82 on the opposite β chain. $Hb(DIDS)_2^T$ was found to have an increase in P_{50} similar to that seen in $Hb(DIDS)^T$, but the subunit cooperativity was significantly lower, indicating differences in the mechanism of this effect. X-ray structural data on this hemoglobin will be required to draw definitive conclusions on the structural basis of the functional changes in $Hb(DIDS)_2^T$. It seems likely that the mechanism would be different than that seen in hemoglobin crosslinked between Lys 82 β_1 and Val 1 β_2 with 2-nor-2-formyl pyridoxal phosphate, since the bridging lengths of DIDS and nfPLP are 15 Å and 7.5 Å respectively. In nfPLP-crosslinked hemoglobin, the N-terminus is drawn into the central cavity while the EF region surrounding Lys 82 is relatively undisturbed (67). The bridging length of DIDS precludes this structure but may instead involve a perturbation of the EF region so that it is repelled outwards in a T-like conformation.

When oxyhemoglobin was reacted with DIDS, the predominant product was shown to be hemoglobin disubstituted at the β amino termini ($Hb(DIDS)_2^R$). This hemoglobin was also present in the mixture of products seen in the deoxy reaction. $Hb(DIDS)_2^R$ had a significantly increased oxygen affinity. Each α-amino group has apparently formed a thiocarbamyl derivative with one isothiocyanate moiety, leaving the second isothiocyanate unreacted (figure 62). Supporting evidence for the second functional group being unreacted comes from data on $Hb(DIDS)_2^R$ which reveals a change in functional properties after deoxygenation. This change apparently reflected formation of a new structural derivative which behaved like $Hb(DIDS)_2^T$ on cation exchange HPLC and SDS-PAGE. Apparently the conformational shift

undergone by the molecule in switching from the R to the T state allowed formation of a new adduct, presumably with Lys 82 on the opposite β chain. Additionally, incubation of $Hb(DIDS)_2^T$ in 1.0 M glycine at pH 9.0 for 24 h at 20° results in quantitative conversion of $Hb(DIDS)_2^T$ to a component that elutes earlier on cation-exchange HPLC (data not shown). This result suggests that glycine has reacted with the extra isothiocyanate group to form a phenylthiocarbamyl-carboxymethyl derivative with a greater negative charge.

The structural basis for the high oxygen affinity of $Hb(DIDS)_2^R$ is at this time unknown and awaits elucidation by x-ray crystallography. One possible mechanism would involve a steric repulsion analogous to that seen in di-ATP Hb. Rather than repulsion of the EF region resulting in a T-shifted structure like that of di-ATP Hb, however, $Hb(DIDS)_2^R$ may have its amino termini forced apart resulting in a high affinity R-like conformation.

In summary, the feasibility of rationally designing a molecular engineering scheme for specifically altering protein structure and function using a site-directed bifunctional heterotropic ligand analog has been demonstrated. With a knowledge of the x-ray crystallographic atomic coordinates of hemoglobin, the chemistry and geometry of the functional groups in the heterotropic ligand analog, and the structural changes occurring in the $\beta_1\beta_2$ cleft during the R to T state transition, a novel crosslinked low affinity hemoglobin, $Hb(DIDS)^T$, was designed and synthesized. The physical chemical properties of this modified hemoglobin match those which have been described as desirable in a hemoglobin-based blood substitute.

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