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LOYOLA UNIVERSITY CHICAGO

MOLECULAR MODELING STUDIES OF HEMOGLOBIN CROSSLINKING REACTIONS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY

BY
PATRICIA A. PAVLIK

CHICAGO, ILLINOIS
JANUARY 1995

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ACKNOWLEDGMENTS

I would like to thank Yvonne Martin, Tetsuro Oie, Mark Bures and Cele Abad-Zapatero for all their help and support. I would like to express my appreciation to Abbott Laboratories for allowing my research to be completed using their facilities. Special thanks and gratitude for their advice and guidance is for my advisors, Dr. Mary Boyd and Dr. Kenneth Olsen, and committee member, Dr. Leslie Fung. Lastly, but not least, I would also like to thank Charles Hutchins for his patience, guidance and encouragement to persevere.

DEDICATION

To my family and friends, both living and those who have passed on, who believed in me.

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LIST OF ABBREVIATIONS

ACD	Available Chemical Database
ASCII	American Standards Code for Information Interchange
BPG	Bisphosphoglycerate
BZF	Bezafibrate
CFA	Clofibric Acid
DBSF	Bis-(3,5-Dibromosalicyl)Fumarate
NMR	Nuclear Magnetic Resonance
PDB	Protein Data Bank
RMS	Root Mean Square

CHAPTER 1

INTRODUCTION

The Need for a Blood Substitute

The search for a blood substitute has been an ongoing research area for at least the last twenty-five years due to problems within the blood supply.[1] A blood substitute would eliminate worries about the spread of infectious diseases such as AIDS and hepatitis from the blood supply, blood shortages would not be as prevalent, there would be no need to specify blood type, and the blood substitute would hopefully have a longer shelf life than fresh blood. However, a blood substitute has to possess many of the properties of normal blood. The blood substitute would have to be able to transport oxygen to various tissues as needed, and be a large-sized molecule so as not to be removed by the kidneys.[2] It would also have to be stable, easily purified from any contaminants, and non-toxic.

Hemoglobin, which is contained in red blood cells, would seem to be a natural substitute for blood because it is already present in the human body. When crosslinked, hemoglobin can also be heated to eliminate any viral contaminants.[2] Hemoglobin has two main functions within the human body: a) to deliver oxygen to tissues via the binding of oxygen to the heme iron atoms, and b) to transport the carbon dioxide byproducts and hydrogen ions from the tissues to the lungs in order to eliminate the carbon dioxide as waste.[3] Therefore, hemoglobin already functions as an oxygen transporter.

There are various types of hemoglobin which contain combinations of different subunits. Hemoglobin A discussed in this thesis, is 97.5% of the total hemoglobin in adult primates.[3] Human hemoglobin A is a tetramer of two alpha and two beta subunits. Each of the alpha subunits contains 141 amino acid residues and each beta subunit has 146 amino residues.[4] Each subunit of hemoglobin has one heme which consists of a porphyrin ring with one iron atom, which is the oxygen binding site.

One alpha and one beta subunit interact to form a dimer. An alpha subunit has few interactions with the other alpha subunit of a tetramer, and the same is true for the beta subunits. The major subunit interfaces are $\alpha1\beta1$, $\alpha2\beta2$, $\alpha1\beta2$ and $\alpha2\beta1$.

Hemoglobin exists in two conformations: the R (relaxed) state and the T (tense) state. In the T state, the hemoglobin is the deoxyhemoglobin or decreased oxygen binding state. Hemoglobin is a very taut or rigid molecule in this state due to the formation of eight salt links (electrostatic interactions) as well as other hydrogen bonds maintaining the deoxyhemoglobin conformation.[3]

In order for the transition to the R or oxyhemoglobin state to occur, some of the salt links and hydrogen bonds must be disrupted.[3] This allows the hemoglobin molecule to move and the oxygen to bind more easily to the iron binding site. The binding of oxygen to deoxyhemoglobin is cooperative.[3] After the first oxygen binds, it then becomes easier for the next oxygen to bind and so forth. As stated previously, in the transition from the deoxy to oxy state, the hemoglobin molecule has to change conformation. One dimer, $\alpha_1\beta_1$, shifts by 15 degrees relative to the other dimer, $\alpha_2\beta_2$, and the β chains move closer together.[3]

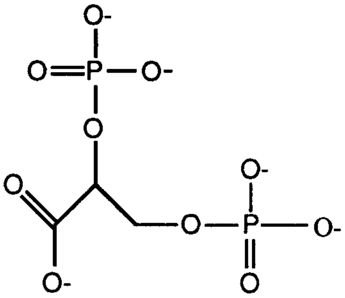
One problem with the use of hemoglobin as a blood substitute is that in the oxyhemoglobin form it will dissociate into dimers and be filtered through the kidneys. One way to solve this problem is to use a crosslinker to stabilize the tetramers. This crosslinked hemoglobin must then be able to carry out similiar functions as normal blood, such as transporting oxygen. Thus the search for the best crosslinker of hemoglobin continues.

History of Hemoglobin Crosslinkers and Allosteric Modifiers

The majority of the published work with crosslinkers has been due to attempts to solve the problem of sickling caused by Hemoglobin S.[5-11] However, these potential crosslinkers have been tested in both Hemoglobin A and S in order to determine the sites of reaction.

There are many known types of crosslinkers. Schiff base adducts,[12] aspirin analogues[5, 6, 11, 13-18] and bis-pyridoxal polyphosphates[19] are only some of the choices. A crosslinker has many possible interaction sites available on hemoglobin. The areas of specific interest to us are the bisphosphoglycerate (BPG) binding site and the α99 site in oxyhemoglobin and in deoxyhemoglobin.

Upon binding to the deoxyhemoglobin (T state), BPG **1** bridges the β chains, which stabilizes the tetramer in the T state and decreases the oxygen affinity. In oxyhemoglobin (R state), the proximity of the β subunits forces the BPG out of the binding pocket. Thus, BPG is acting as an allosteric regulator of both the oxygen binding and the conformation (T or R) of hemoglobin.



BPG

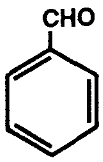
1

Schiff base adducts are one type of monofunctional crosslinker. Zaugg and coworkers examined 31 carbonyl compounds (Figure 1) which react with an amino group on the hemoglobin A and S proteins to form reversible Schiff base compounds.[12] Aromatic aldehydes produced the greatest changes in intracellular hemoglobin in binding of oxygen compared to aliphatic aldehydes (less reactive) and ketones (none were reactive). The hemoglobin modified by aromatic aldehydes resulted in an increase in oxygen affinity compared to the hemoglobin modified by aliphatic aldehydes and ketones which exhibited no increase in oxygen affinity. Aromatic aldehydes were suggested to form relatively stable bonds in hemoglobin through reaction with an amine of a lysine residue. Additionally, the addition of a second functional group on the aromatic ring significantly affected the oxygen binding. The rate of the Schiff base formation reaction increased when an electron-withdrawing substituent was present. Bulky substituents next to the carbonyl prohibited this reaction from occuring. Compounds were most potent when there was a hydroxyl group in the ortho position. This was suggested to help guide the ligand to a specific site on hemoglobin.

Beddell[20, 21] and coworkers used molecular modeling to propose three possible compounds that could interact in the BPG binding site of

FIGURE 1

31 Schiff Base Adducts Tested as Monofunctional Crosslinkers.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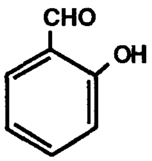
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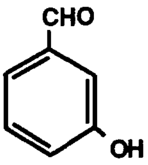
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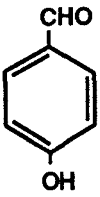
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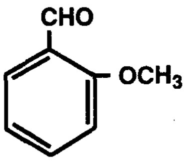
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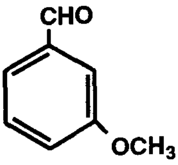
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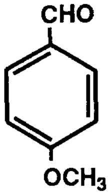
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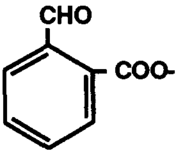
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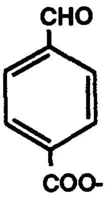
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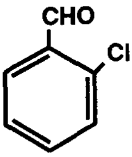
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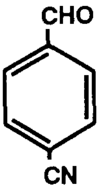
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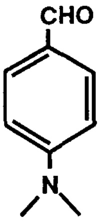
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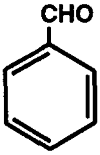
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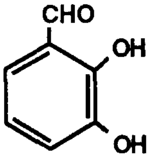
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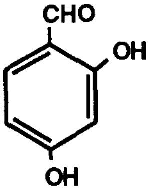
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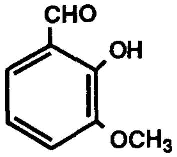
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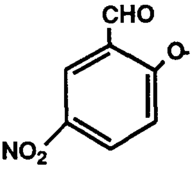
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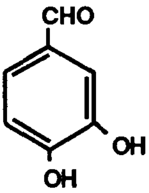
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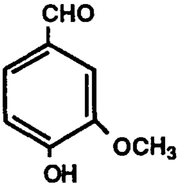
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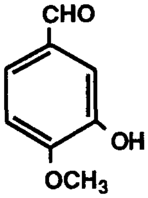
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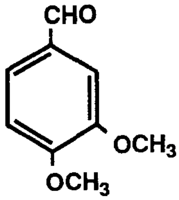
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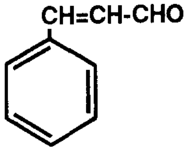
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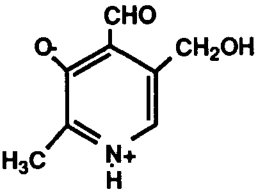
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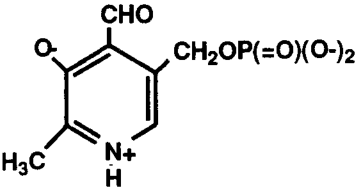
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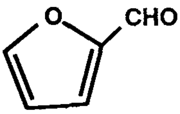
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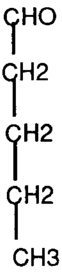
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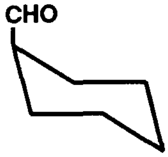
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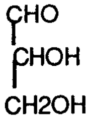
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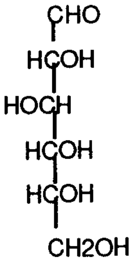
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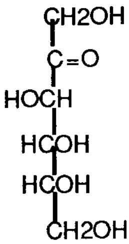
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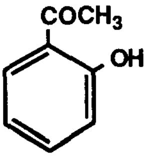
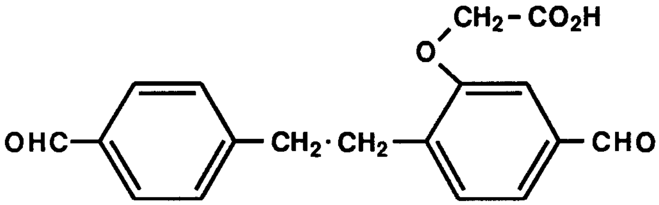
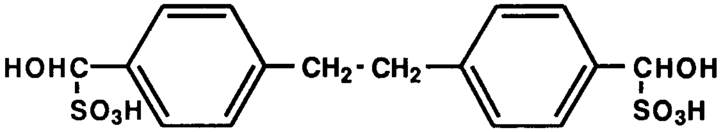

COCH3
OH

deoxyhemoglobin. Beddell examined the BPG binding in the X-ray crystal structure of deoxyhemoglobin.[22] Eight nitrogens in the BPG site were further examined: two from the terminal amino groups of the β chains, and from His β2, Lys β82, and His β143 from each β subunit. A desired characteristic of a ligand was a lower polarity than BPG, allowing the compound to cross the red cell membrane. Other desired characteristics were that the molecule be novel, conformationally limited and easy to synthesize. This molecule was designed to react at the terminal amino groups of the β subunits. The starting ligand was one proposed by Robertson[23] - a bibenzyl structure. Aldehyde groups were added in the para positions to enable the molecule to form Schiff bases with the deoxyhemoglobin. Unfortunately, this molecule was not soluble in water, so the structure was modified to increase solubility resulting in ligand **2**.



2

Other compounds that were suggested included a bisulphite derivative **3** and an analogue **4** which was a combination of compounds **2** and **3**.



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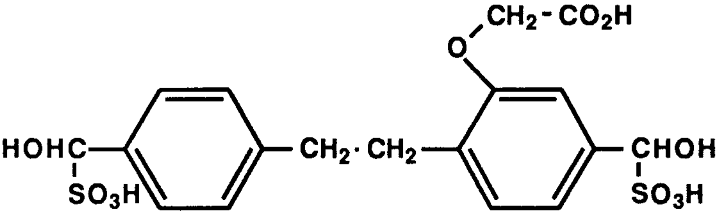
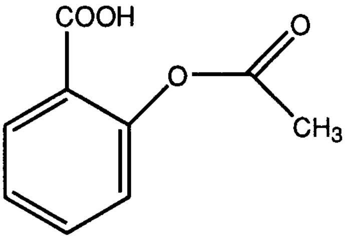


4

Synthesis and testing of these compounds showed the design was successful and all the compounds promoted liberation of oxygen from deoxyhemoglobin, similar to BPG. **3** was even comparable in potency to BPG as measured by the right shift in the oxygen affinity graph, indicating a decrease in oxygen affinity. NMR experiments[21] provided support for the proposed mode of interaction of **2** and **3** in the BPG binding site.

Aspirin and diaspirin analogues have long been known to act as potent acylating agents of the amine groups on hemoglobin.[5, 6, 11, 13-18] Aspirin **5** is a member of the acylsalicylates group and diaspirin, for example **6**, is a double-headed aspirin with various groups bridging the two aspirin portions.

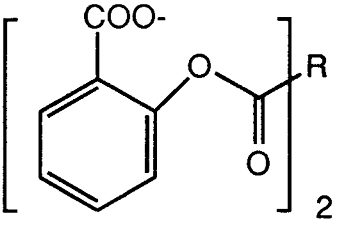
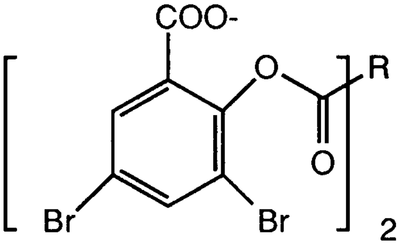


5

From these studies much can be learned about the crosslinking sites on oxyhemoglobin. Acyl groups with 2 or 3 carbons in the chain are more reactive than those with longer carbon chains of 4 to 10 methylene groups[11, 13] (see Table 1). However, the compounds with longer chains permeate the red cell membrane easier than those with shorter carbon chains.[13] Diaspirins, whether

TABLE 1

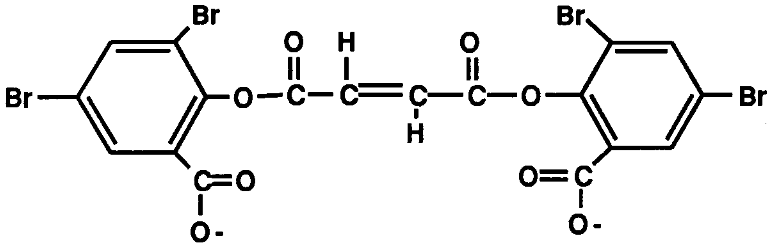
Percent Crosslinking for Acyl Groups with Varying Carbon Chain Lengths.

	R[b]	% Cross-linked[c]
	$-CH=CH-$	85
	$-CH_2CH_2-$	10
	$-(CH_2)_4-$	0
	$-(CH_2)_8-$	0
	$-(CH_2)_{10}-$	0
	$-CH=CH-$	70
	$-CH_2CH_2-$	40
	$-(CH_2)_8-$	0

Source:R.H. Zaugg, J.A. Walder, R.Y. Walder, J.M. Steele, and I.M. Klotz. Modification of Hemoglobin with Analogs of Aspirin. J. Biol. Chem. 255: 2816-2821 (1980).

b Bridge between aspirin groups

c % Cross-linked is the percentage of hemoglobin β chains that are crosslinked by various bis(salicyl) diesters



DBSF

6

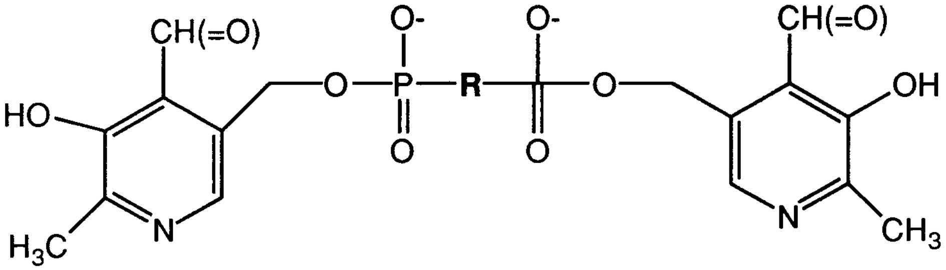
brominated or not, are more reactive than monoaspirins.[13] Bromination of the aromatic ring helps transport the ligand across the membrane.[13] In addition, since bromine is an electron-withdrawing substituent, nucleophilic attack by a hemoglobin amine group on the carbonyl group is facilitated.[13] Unsaturated chains have a higher percentage of crosslinking compared to saturated chains, which can be attributed to the inductive effect of the olefin which increases the lability of the ester.[15]

These data explain why bis-(3,5-dibromosalicyl)fumarate (DBSF) **6** is able to crosslink oxyhemoglobin. DBSF is a diaspirin with a two carbon chain length. The bromine substituents aid in crossing the cell membrane, and the electron-withdrawing nature of the substituents improves nucleophilic attack on the carbonyl group. Also, DBSF has an unsaturated chain which increases the rate of crosslinking.

Polyphosphates[19, 24-26] are another group of crosslinkers which have been used. The proposed crosslinking site of bis-pyridoxal polyphosphates (Table 2) is from the N-terminal amine on one beta subunit to the β82 lysine on the other β subunit.[19, 26] Benesch and coworkers varied the number of phosphate groups between two pyridoxal rings to determine the effect on crosslinking.[19] Surprisingly, the 4 phosphate groups between pyridoxal rings resulted in a

TABLE 2

Percent Crosslinking for Bis-pyridoxal Polyphosphate Compounds



R	% Tetramer[a]
Oxygen	16
Orthophosphate	18
Pyrophosphate	68
Methylene diphosphate	53
Fructose 1,6-diphosphate	41
2,3-diphosphoglycerate	70

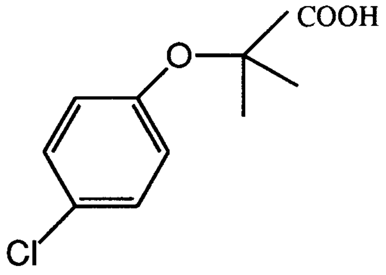
Source: R.E. Benesch and S. Kwong. Bis-Pyridoxal Polyphosphates: A New Class of Specific Intramolecular Crosslinking Agents for Hemoglobin. Biochem. Biophys. Res. Commun. **156**: 9-14 (1988).

[a] % Tetramer: Covalent Intramolecular Crosslinking of Deoxyhemoglobin by the Bis-Pyridoxal Polyphosphates

recovering of 68% of the crosslinked tetramer.[19] This ligand is clearly much longer than the 11Å distance observed in the X-ray structure between the N-terminal amine and β282 lysine. One possible explanation is that the pyridine rings of the ligand must interact with each other. Thus, hemoglobin crosslinked with this class of compounds could be considered as a blood substitute since there is a very high yield of crosslinked tetramer, and experimentally it was determined to have a low oxygen affinity.

The initial work of Abraham and coworkers was in antisickling agents[7, 8] with later efforts in the allosteric modification of hemoglobin.[9, 27, 28] Many of these allosteric modifiers are not crosslinking agents, however they do bind at specific sites in hemoglobin. Allosteric modifiers affect the preferred conformation of the hemoglobin molecule in the same way as the biological allosteric effector molecule, BPG.

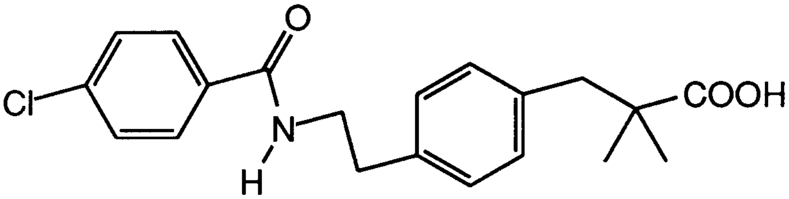
The first nonnatural allosteric inhibitor reported by Abraham was clofibric acid (CFA) **7**.[7] The binding sites of CFA were determined by both X-ray and solution-binding studies.[28] CFA binds in deoxyhemoglobin in two primary (high occupancy) sites, between the α subunits, and two secondary (low occupancy) sites. The secondary sites are located in the central cavity where the α and β subunits meet.



CFA

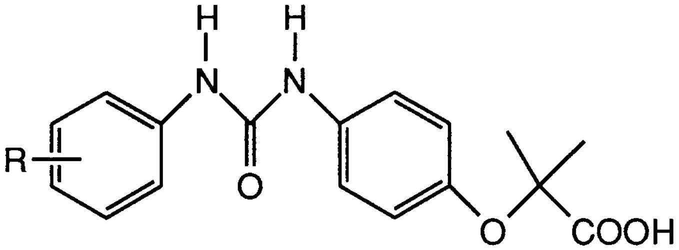
7

Perutz and Poyart discovered a more potent allosteric inhibitor, bezafibrate (BZF) **8**.[27] BZF occupied one of the primary sites, and also one of the secondary sites filled by CFA. Lalezari and coworkers reported that analogues of BZF, urea derivatives, **9**, were even more potent allosteric effectors than BZF itself.[28]



Bezafibrate (BZF)

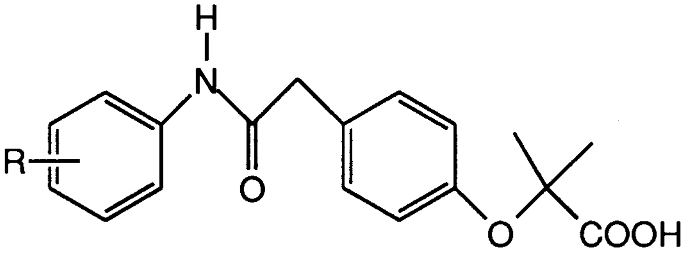
8



a = 3,4 - Cl
b = 3,5 - Cl
c = 3,4,5 - Cl

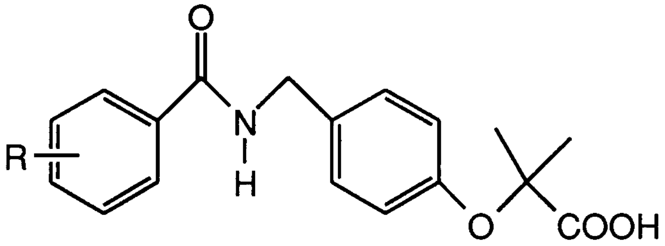
9

These derivatives were also found to bind in a similar manner as BZF. Abraham and coworkers then reported on three series of compounds, **10-12**, similar in structure to Perutz's compounds **8** and **9**, which also are allosteric effectors.[28] **10** and **11** occupy a binding site in the α subunits near lysine 99 of hemoglobin similar to BZF and the urea derivatives.



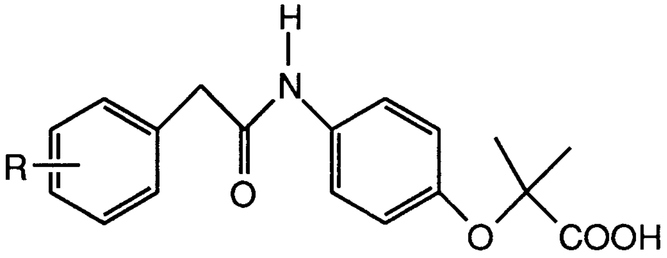
a = 4 - Cl
b = 3,5 - Cl
c = 3,4,5 - Cl

10



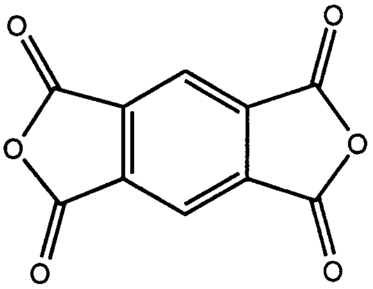
a = 4 - Cl
b = 3,5 - Cl
c = 3,4,5 - Cl

11



12

Even more recently, mellitic dianhydrides[29] **13** have been shown to crosslink deoxyhemoglobin. These compounds decrease oxygen affinity, maintain much of the cooperativity of binding and reduce the Bohr effect. Interestingly, these compounds are believed to crosslink deoxyhemoglobin in the α99 site because of the results of competition experiments with known crosslinkers.



13

Goal of This Study

The goal of this research was to use molecular modeling techniques to develop a molecule that would crosslink hemoglobin upon irradiation. In order to achieve this goal, the initial research effort was to examine hemoglobin, the sites where crosslinking is known to occur, and many literature references about crosslinking hemoglobin. The possible mechanism of crosslinking was explored using molecular dynamics and minimization experiments. After the simulation of binding of known crosslinkers, we then used various computer programs to develop a molecule which is suggested to crosslink deoxyhemoglobin between the α subunits.

CHAPTER 2

EXAMINATION OF HEMOGLOBIN

Introduction

Our initial goal was to gain familiarity and intuition into the binding and crosslinking of hemoglobin.

Conformational Search of Diaspirin

The research began with an examination of a known crosslinker in various sites of the deoxyhemoglobin[22] and oxyhemoglobin[30] X-ray crystal structures from the Brookhaven Protein Database,[31] 2HHB and 1HHO, respectively. The known crosslinker is bis-(3,5-dibromosalicyl)fumarate, or diaspirin (DBSF), **6,** which crosslinks oxyhemoglobin at the β82-β82 residues.[15]

The conformation which DBSF adopts to bind to hemoglobin is unknown, therefore a conformational search was performed. The molecule was constructed in Chem-X,[32] and its energy was minimized using molecular mechanics to remove any steric interactions. One hundred conformations were generated using a distance geometry program called Dgeom[33], with the Chem-X minimized structure as the initial starting structure.

Dgeom describes the structure of a molecule with a distance matrix for each pair of atoms in the molecule. The program uses a triangle inequality algorithm which sets up an upper bounds (maximum distance) and a lower bounds (lower distance) that is used for each pair of atoms, or distances from the starting structure. Through the use of this matrix, random conformations are generated.

These conformations were then submitted to the program Reject.[34] Reject compares distances between given atoms and based upon this comparison, duplicate conformations within an allowed tolerance are deleted. The atoms compared are shown in Figure 2 and the tolerance used was 0.2 Å. Thirteen conformations were kept, and then minimized to reduce any steric hindrances using two different programs, Chem-X and Discover.[35] The results from the minimization are given in Table 3, with the energies calculated by both programs. Both programs minimized the conformations in the gas phase due to the uncertainty in determining the dielectric constant and the solvent interactions.

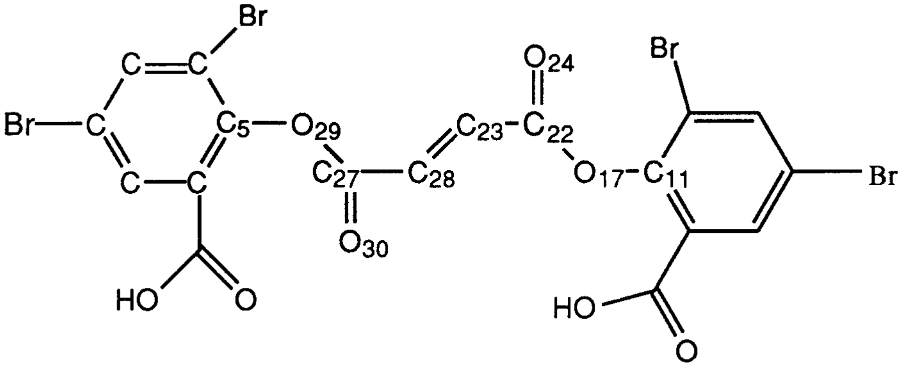
Nine out of the thirteen minimized conformations of DBSF, determined by either the Chem-X or Discover minimization, were a trans conformation (Table 3), the actual conformation of the reagent. It would seem reasonable that this low-energy configuration is most likely to occur when binding to the hemoglobin protein. The differences in the energy values of the minimized conformations from the two programs are due to the different force fields and parameters used by each of the programs.

Investigation of Hemoglobin Crosslinking Sites

An exploration of oxy and deoxy hemoglobin was undertaken in an effort to gain some familiarity with the two conformations of hemoglobin and the sites where crosslinking either occurs or is thought to occur. Therefore, using one

FIGURE 2

The DBSF Molecule with Atom Numbers as Used in the Reject Program


Br
Br
C5
O29
C27
O30
C28
C23
C22
O24
O17
C11
Br
Br
HO
O
HO
O

DBSF

TABLE 3

The Relative Minimized Energies of the DBSF Conformations Kept from the Reject Program.[a]

Conformation[b]	Chem-X (Kcal/mol)	Discover (Kcal/mol)
dbbfxm87	-18.47	63.95
dbbfxm99	-17.60	63.90
dbbfxm92 (cis)	-15.77	60.26
dbbfxm94	-12.37	71.09
dbbfxm86	-11.62	64.05
dbbfxm61	- 8.01	67.41
dbbfxm71	- 7.90	67.92
dbbfxm77 (cis)	- 7.74	69.66
dbbfxm88 (cis)	- 7.66	66.42
dbbfxm98	- 7.09	72.60
dbbfxm66	- 4.96	70.84
dbbfxm70	- 3.84	66.07
dbbfxm91 (cis)	12.07	69.70

[a] The differences in the numbers between the two programs is due to the different force fields and parameters used in each program.

[b] Unless otherwise specified, trans conformations are listed. The conformational names refer to the experimental codes.

conformation of diaspirin, dbbfxm87, and a minimization program, Discover, five different sites were explored in hemoglobin. This conformation of diaspirin was chosen because it was the lowest energy conformation calculated by Chem-X.

Diaspirin is known to crosslink oxyhemoglobin between the carbonyl carbons of diaspirin and the ζ-nitrogen atoms of the lysine residues positions at $\beta_1 82$-$\beta_2 82$.[15, 16] This compound is also known to crosslink in the same manner in deoxyhemoglobin between two lysines at the α99 sites.[36, 37] There are three other sites in deoxyhemoglobin, $\beta_1 82$-$\beta_2 82$, $\beta_1 82$-$\beta_2 132$, $\beta_1 82$-$\beta_2 1$, which are possible secondary sites for crosslinking to occur.[37] All the sites involve two lysine residues interacting with the fumarate except for the $\beta_1 82$-$\beta_2 1$ site; β1 is the N-terminal valine residue. Thus, the sole oxyhemoglobin site and the four deoxyhemoglobin sites were explored to elucidate why a crosslinker would bind in one site versus another.

Using the oxyhemoglobin and deoxyhemoglobin crystal structures, the fumarate was manually placed in the particular protein site being examined. Bonds were made between the carbonyl carbon of the fumarate and the nitrogen atoms of the lysines on both sides of either the alpha or beta chains except for the $\beta_1 82$-$\beta_2 1$ site in which bonds were made to the $\beta_2 1$ valine and the $\beta_1 82$ lysine amine. Since it would be too computationally intensive, and of minimal utility, to minimize the entire hemoglobin tetramer, only a subset of atoms of the beta chain or alpha chain within 10 Å of the alpha carbon of lysine β82 or α99 were considered. Atom potentials and charges were assigned by the Insight [38] program. The 10 Å subset was minimized in Discover allowing only the fumarate and lysine chains to move. The rest of the atoms were held fixed. The minimization used the conjugate gradients method[35] until the root mean square

(RMS) derivatives were less than 0.01. Table 4 lists energies for the minimized subsets of atoms.

Results and Discussion

Figures 3 to 7 show the minimized structures at the various crosslinking sites. The results of the minimization experiments of the different sites corroborates the experimental results from the crosslinking experiments. Comparison of Figure 3 (oxyhemoglobin, $\beta_1$82-$\beta_2$82) and Figure 4 (deoxyhemoglobin, $\alpha_1$99-$\alpha_2$99) to Figures 5-7 (deoxyhemoglobin $\beta_1$82-$\beta_2$82, $\beta_1$82-$\beta_2$132 and $\beta_1$82-$\beta_2$1, respectively), shows that the minimized conformations of the fumarates in the β-cleft of deoxyhemoglobin with β subunits crosslinked (Figures 5-7) are fully extended and the bond distances are slightly longer than normal bond lengths. The oxyhemoglobin at $\beta_1$82-$\beta_2$82 and deoxyhemoglobin $\alpha_1$99-$\alpha_2$99 crosslinks are more relaxed and the conformations are more folded.

From these results we suggest that the secondary crosslinking site in deoxyhemoglobin is the $\beta_1$82-$\beta_2$82 site. This conclusion was obtained after consideration of the distance between binding points in each site and when fumarate is bound, how it is oriented and how stretched the bonds are. The distance of the deoxy $\beta_1$82-$\beta_2$82 site were closer in length (9.33 Å) to the known crosslinking binding sites of deoxy $\alpha_1$99-$\alpha_2$99 (7.82 Å) and oxy $\beta_1$82-$\beta_2$82 (10.73 Å). Also, the energy of the minimized structure of the deoxy $\beta_1$82-$\beta_2$82 site (1.90 Kcal.) was lower then either of the deoxy $\beta_1$82-$\beta_2$1 (35.96 Kcal.) or $\beta_1$132-$\beta_2$82 (60.05 Kcal.) sites. An argument against the secondary binding site suggestion is that no crosslinked β82 in deoxyhemoglobin has ever been isolated. [39]

FIGURE 3

Minimization of crosslinked oxyhemoglobin: $\beta_1 82$-$\beta_2 82$ site.

The distances between each atom of the crosslink are measured.

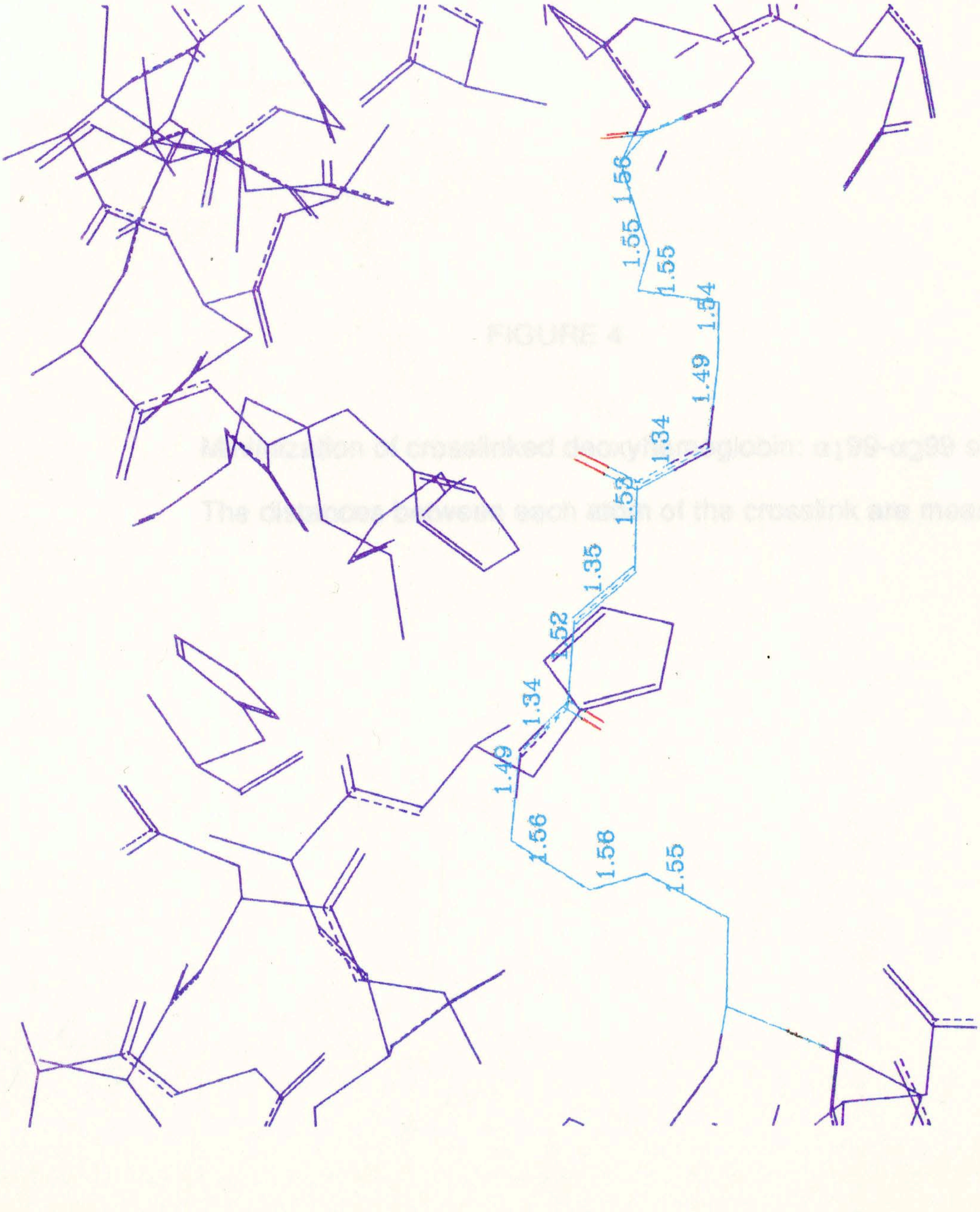

1.56
1.55
1.55
1.54
1.49
1.34
1.53
1.35
1.52
1.34
1.49
1.56
1.56
1.55

FIGURE 4

Minimization of crosslinked deoxyhemoglobin: $\alpha_1 99$-$\alpha_2 99$ site.

The distances between each atom of the crosslink are measured.

FIGURE 4

Minimization of crosslinked deoxyhemoglobin: $\alpha_1$99-$\alpha_2$99 site.

The distances between each atom of the crosslink are measured.

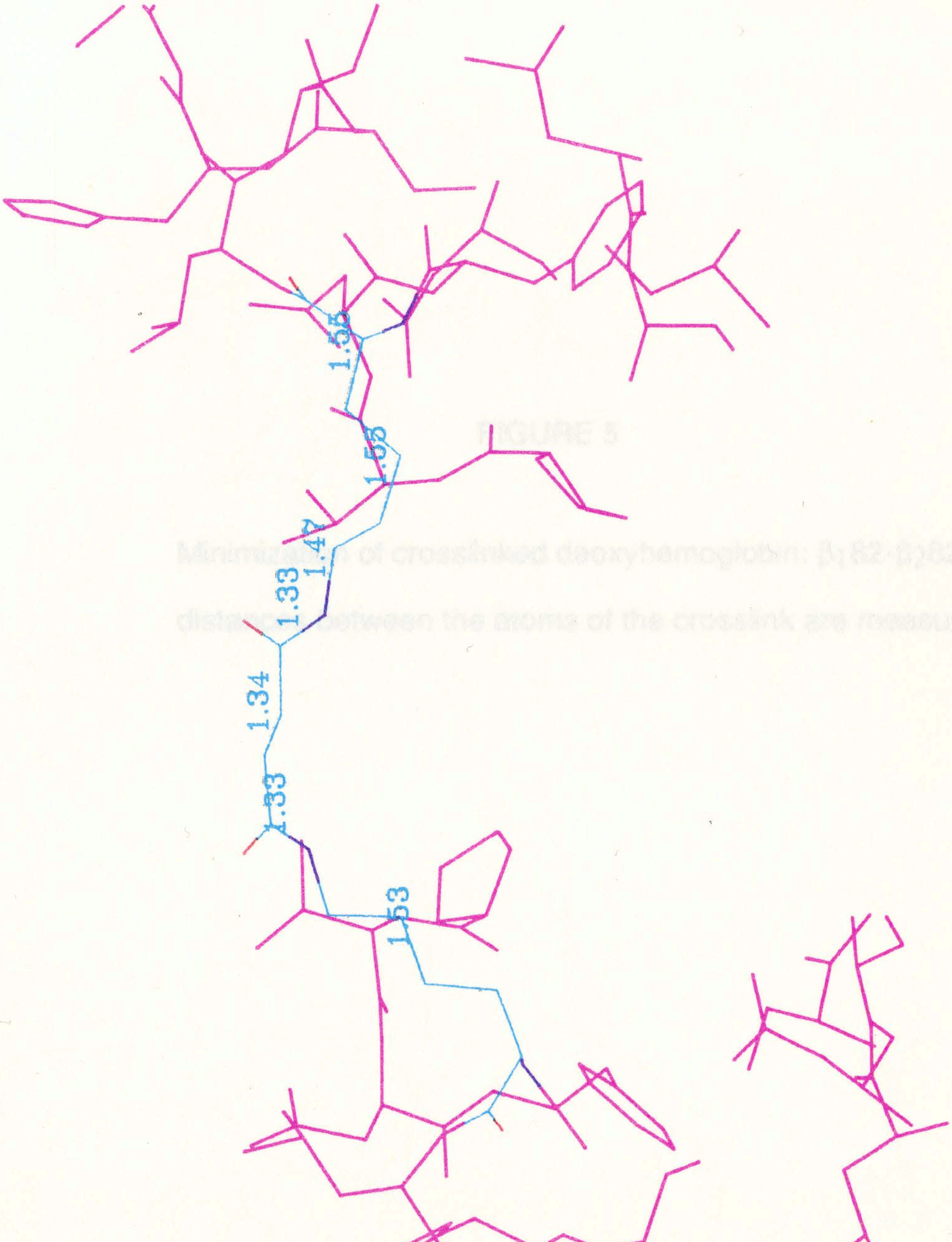

1.55
1.53
1.47
1.33
1.34
1.33
1.53

FIGURE 5

Minimization of crosslinked deoxyhemoglobin: $\beta_1$82-$\beta_2$82 site. The distances between the atoms of the crosslink are measured.

FIGURE 5

Minimization of crosslinked deoxyhemoglobin: $\beta_1$82-$\beta_2$82 site. The distances between the atoms of the crosslink are measured.

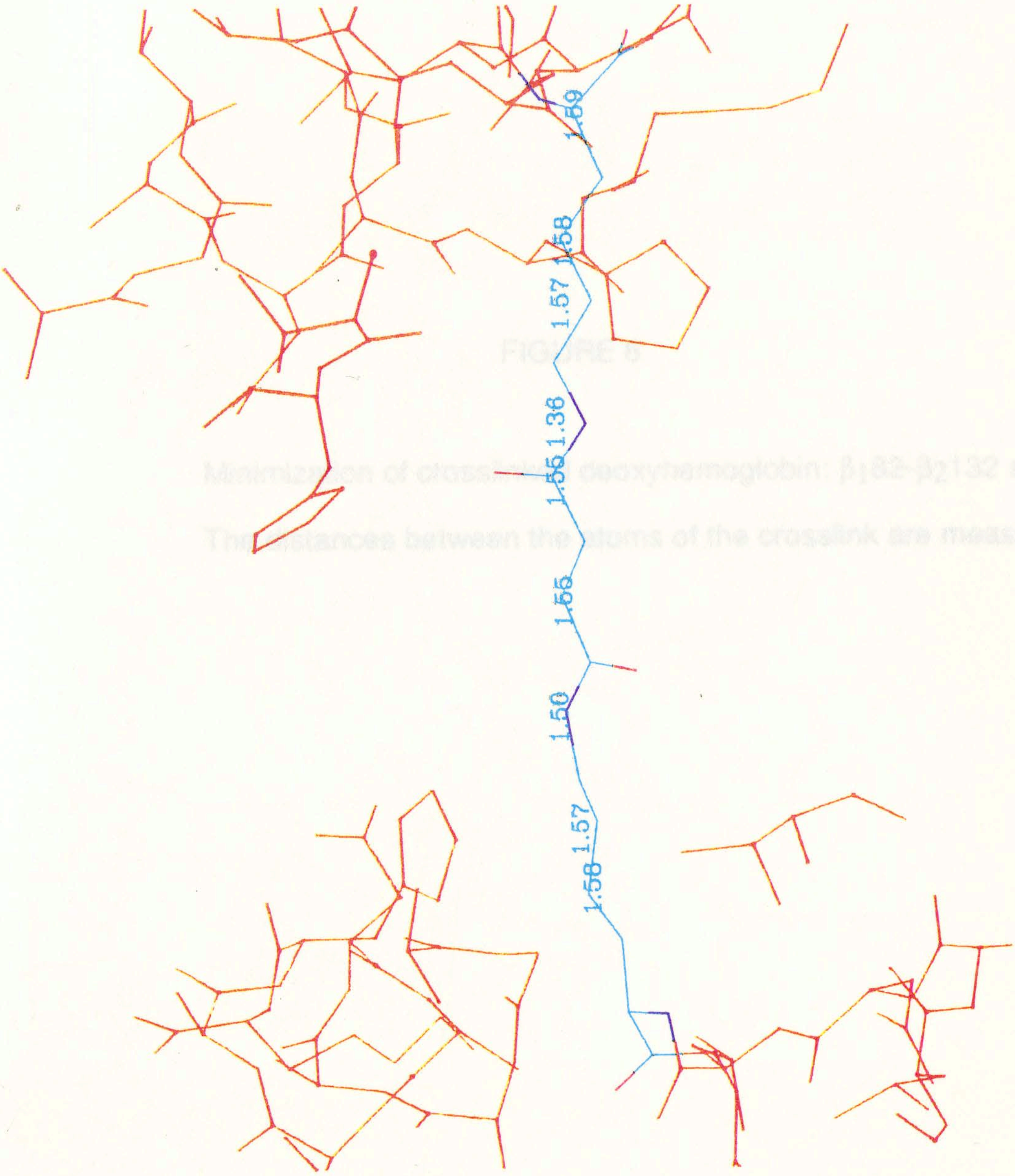

1.59
1.58
1.57
1.36
1.55
1.55
1.50
1.57
1.58

FIGURE 6

Minimization of crosslinked deoxyhemoglobin: $\beta_1 82$-$\beta_2 132$ site.

The distances between the atoms of the crosslink are measured.

FIGURE 6

Minimization of crosslinked deoxyhemoglobin: $\beta_1 82$-$\beta_2 132$ site.

The distances between the atoms of the crosslink are measured.

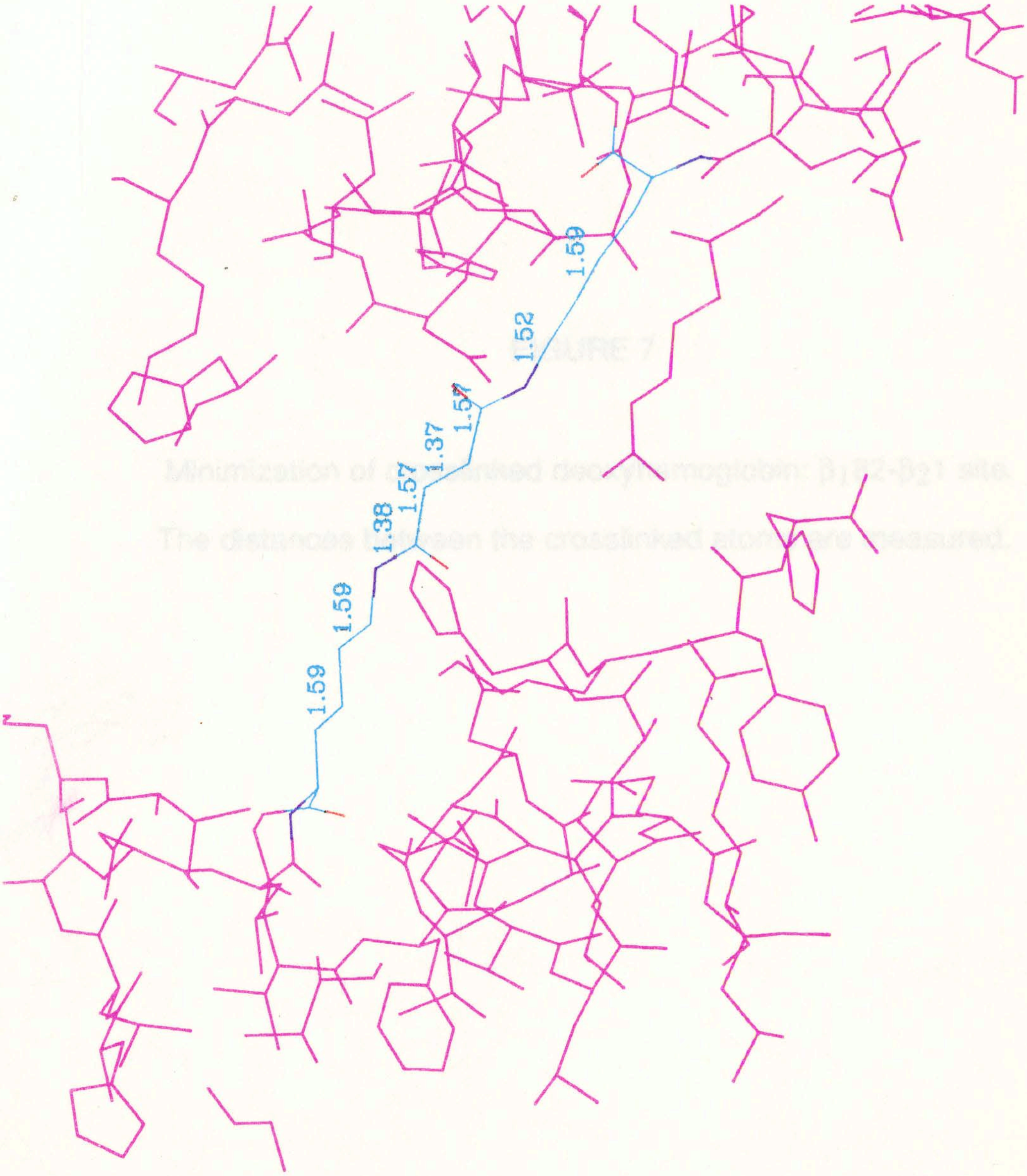

1.59
1.59
1.38
1.57
1.37
1.57
1.52
1.59

FIGURE 7

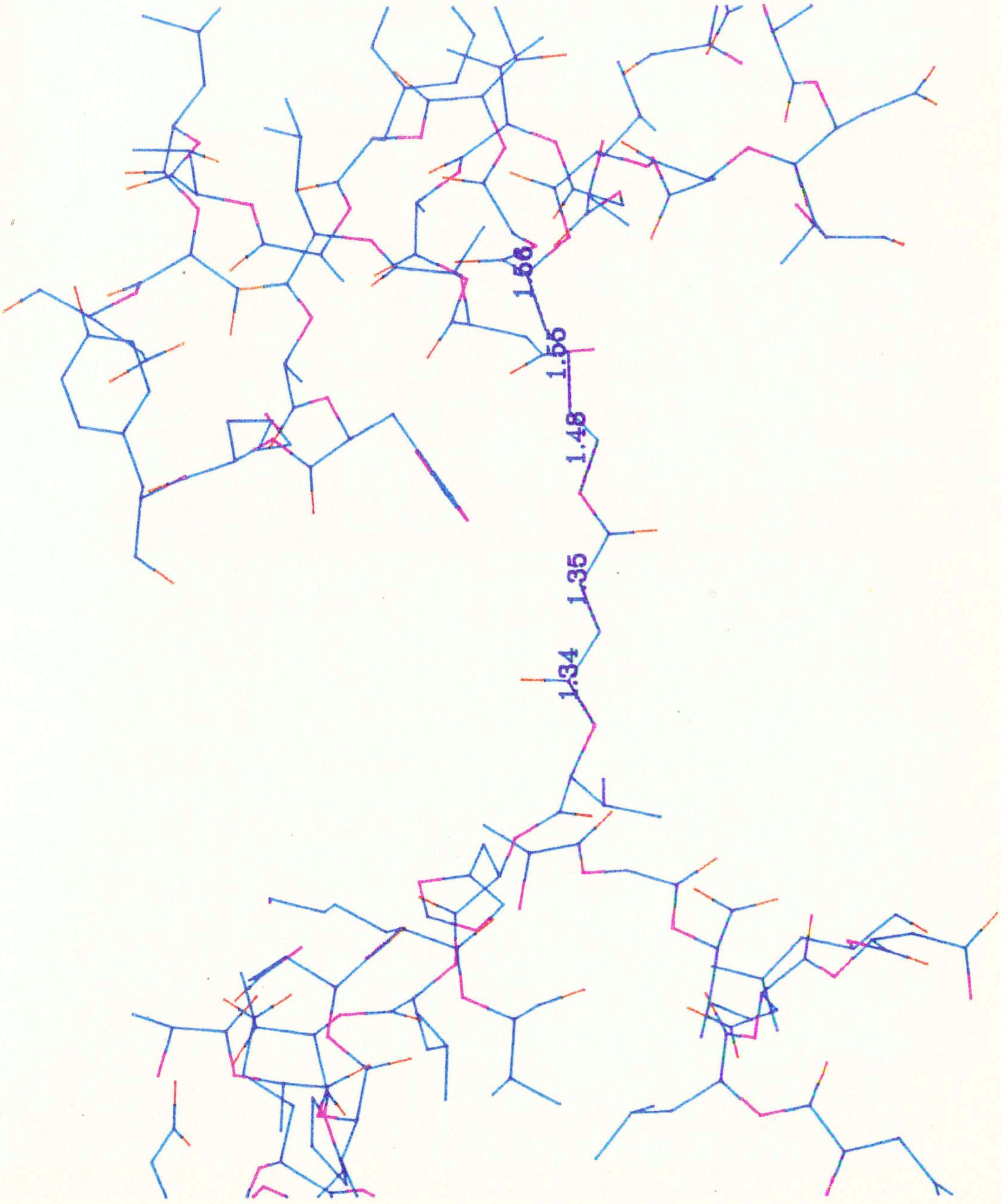
Minimization of crosslinked deoxyhemoglobin: $\beta_1 82$-$\beta_2 1$ site. The distances between the crosslinked atoms are measured.

FIGURE 7

Minimization of crosslinked deoxyhemoglobin: $\beta_1$82-$\beta_2$1 site.

The distances between the crosslinked atoms are measured.

The results of this portion of the research demonstrated why one molecule, DBSF, crosslinks at these sites. It also suggested a potential secondary site. Thus, this theoretical experimentation verified previous experimental work and provides insight regarding binding and crosslinking.



The results of this portion of the research demonstrated why one molecule, DBSF, crosslinks at these sites. It also suggested a potential secondary site. Thus, this theoretical experimentation verified previous experimental work and provides insight regarding binding and crosslinking.

TABLE 4

Energy of Minimized Structure and Measured Distances of X-ray Structures

SITE	FINAL ENERGY OF MINIMIZED STRUCTURE (KCAL.)	DISTANCE OF ζ AMINO OF ONE LYSINE TO ζ-AMINE OF OTHER LYSINE (Å)*
Oxy: $\beta_1$82-$\beta_2$82	-39.21	10.73
Deoxy: $\alpha_1$99-$\alpha_2$99	-78.63	7.82
Deoxy: $\beta_1$82-$\beta_2$82	1.90	9.33
Deoxy: $\beta_1$82-$\beta_2$1	35.96	11.40
Deoxy: $\beta_1$132-$\beta_2$82	60.05	13.91

*The distances measured are from the X-ray crystal structures from non-crosslinked hemoglobins before any minimizations were completed.

CHAPTER 3

MINIMIZATION OF OXY AND DEOXY HEMOGLOBIN CROSSLINKED WITH DBSF

Introduction

Chapter 2 discussed the orientation of the DBSF ligand, after crosslinking both deoxy and oxy hemoglobin at various sites. These calculations allowed only the fumarate and lysine chains to move during minimization with the remainder of the protein held fixed. It would be of interest to examine the crosslinked hemoglobins after minimization of the entire crosslinked protein and to determine where changes occurred in the protein.

Methodology

The X-ray crystal structure of deoxyhemoglobin[22] from the Protein Data Bank[31],(2HHB) 1.7 Å resolution, was used as the starting structure. DBSF was manually placed an approximately equal distance (3.32 Å and 3.26 Å) from the β82 lysine side chain amine groups using the Insight[35] program. Bonds were made between the β82 lysines and the ester carbonyls after the 3,5-dibromosalicyl rings were removed. The crosslinked protein was then minimized using the conjugate gradient algorithm in the Discover program for 10 X 100 iterations, holding the mainchain atoms (alpha carbons, carbonyls, and nitrogens) fixed and allowing only the sidechains to move. Discover also has a method of restraining, or tethering, the atoms of a protein to their initial coordinates. In order to minimize major movements of the protein a user-defined force constant, which controls the strength of the tethering, can be invoked and then decreased gradually in steps. Therefore, upon completion of this first set of minimizations, the mainchain was tethered with a force constant of 500 Kcal/Å for 1000 minimization iterations. The force constant was decreased to 250 Kcal/Å and another 1000 iterations were performed. This process was continued with decreasing force constants of 175, 100, 25, and 0 Kcal/Å with 1000 iterations at each force constant value. The total computer time was 27 hours on a VAX9000.

The oxyhemoglobin X-ray crystal structure[30] was also minimized with the DBSF ligand crosslinked at the β82 lysines. The same procedure as described above was used for deoxyhemoglobin. The cpu time for this minimization was 28 hours on a VAX9000.

A final "control" minimization experiment was to minimize both the native oxy and deoxyhemoglobin protein exactly as the model crosslinked proteins, as has previously been described This was performed so that a direct comparison could be made between the minimized crosslinked protein to the minimized uncrosslinked native protein.

Results of Minimization

The final crosslinked deoxyhemoglobin structure after minimization was superimposed upon the original native X-ray crystal structure[8] using the Insight program. An RMS deviation of 1.18 Å was found after superimposing the C-alpha atoms. A superposition of all backbone atoms gave an RMS deviation of 1.19 Å. Using the Abbott programs, Histplt and Distplt,[40, 41] the distance difference for each residue was calculated (Figure 8) and a histogram of the number of residues which occur at a specified interatomic distance was also calculated (Figure 9).

The minimized crosslinked and uncrosslinked deoxyhemoglobin proteins were superimposed upon each other with a carbon alpha RMS deviation of 1.23 Å. The β82 positions had moved more than most sections of the structure. However, the largest changes in the minimized crosslinked structure were not at the β82 positions. The same plots of the distance difference for each residue (Figure 10) and the histogram of the number of residues which are at certain interatomic distances were also calculated (Figure 11) for these two proteins.

The minimized oxyhemoglobin crosslinked structure was superimposed on the X-ray crystal structure[30] with an RMS deviation of 1.47 Å between all alpha carbons. There was also a 1.47 Å deviation between all backbone atoms. When only one α helix of the X-ray structure is compared with the crosslinked minimized structure, an RMS deviation of only 0.97 Å was measured. This suggests that the loop region residues have altered the most. The distance difference for each residue and the number of residues which occur at a specified interatomic distance were also calculated (Figures 12 and 13 respectively).

The superposition of the minimized β82 crosslinked oxyhemoglobin with uncrosslinked minimized oxyhemoglobin was completed with an RMS deviation of the C-alpha's being 1.36 Å. Figure 14 is the distance difference for each residue and Figure 15 is a histogram of the occurrence of residues at each interatomic distance. As in deoxyhemoglobin, the β82 residues did not show the greatest movement. Residue (55) of the α chain moved the most.

The RMS deviation measured between the crystal structure and the minimized crosslinked structure, and also for the minimized crosslinked and

FIGURE 8

Differences between C-alpha atom positions of β82 crosslinked and the X-ray structure of deoxyhemoglobin.

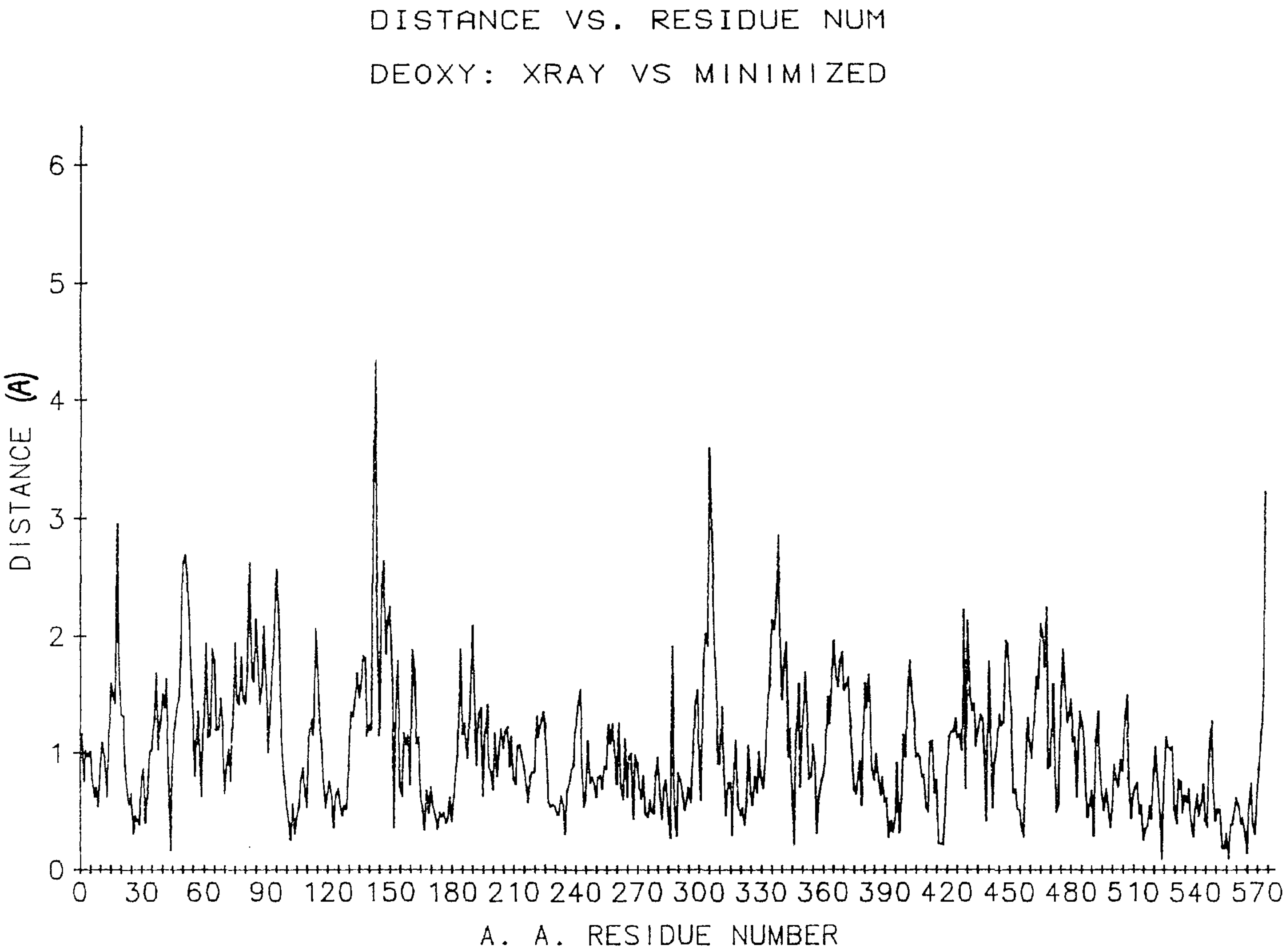

DISTANCE VS. RESIDUE NUM
DEOXY: XRAY VS MINIMIZED
DISTANCE (A)
0
1
2
3
4
5
6
0
30
60
90
120
150
180
210
240
270
300
330
360
390
420
450
480
510
540
570
A. A. RESIDUE NUMBER

FIGURE 9

Interatomic distances between the C-alphas of the β82 crosslinked and X-ray structure of deoxyhemoglobin.

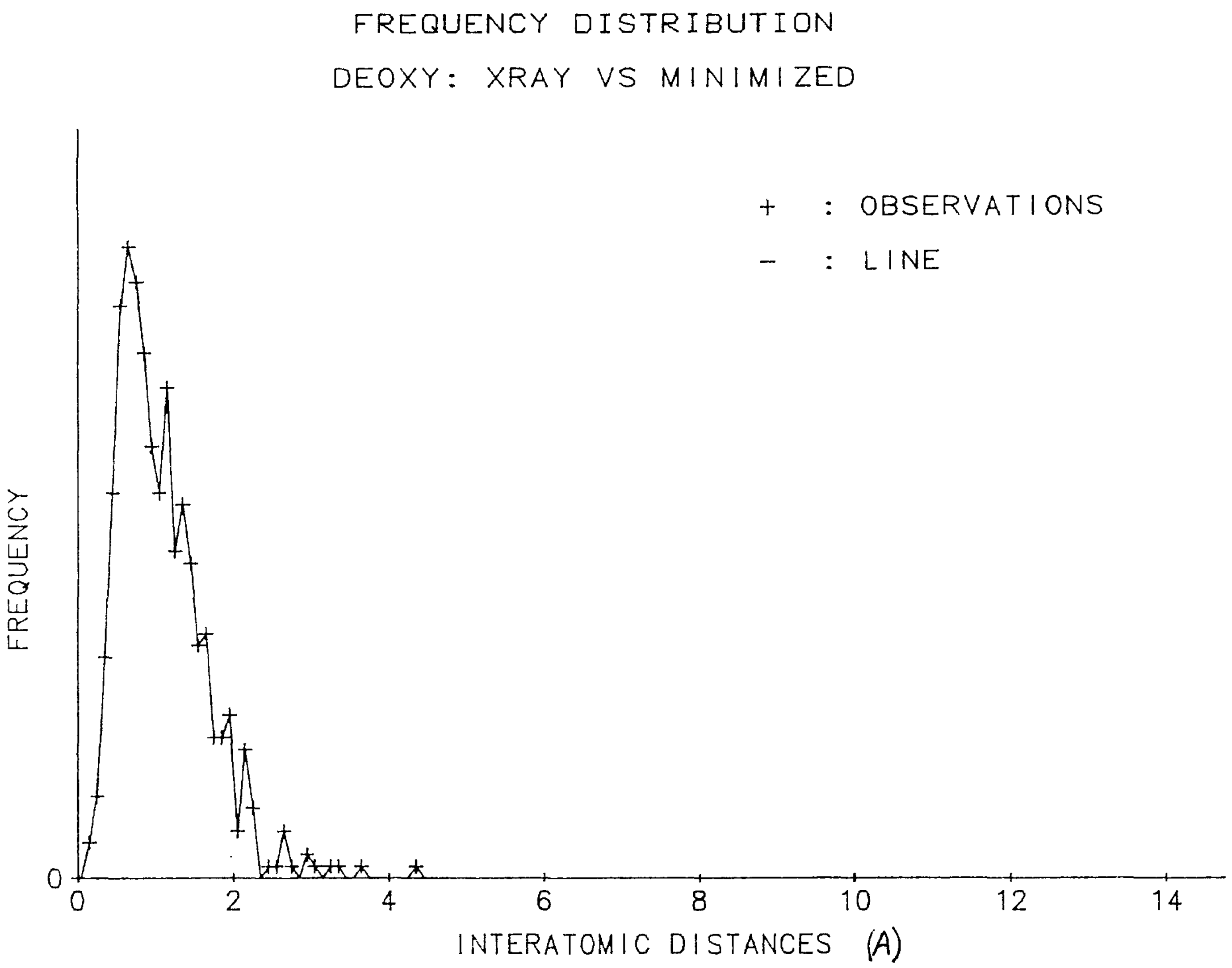

FREQUENCY DISTRIBUTION
DEOXY: XRAY VS MINIMIZED
+ : OBSERVATIONS
– : LINE
FREQUENCY
0
0
2
4
6
8
10
12
14
INTERATOMIC DISTANCES (A)

FIGURE 10

Differences between C-alpha atom positions of β82 crosslinked and minimized X-ray structure of deoxyhemoglobin.

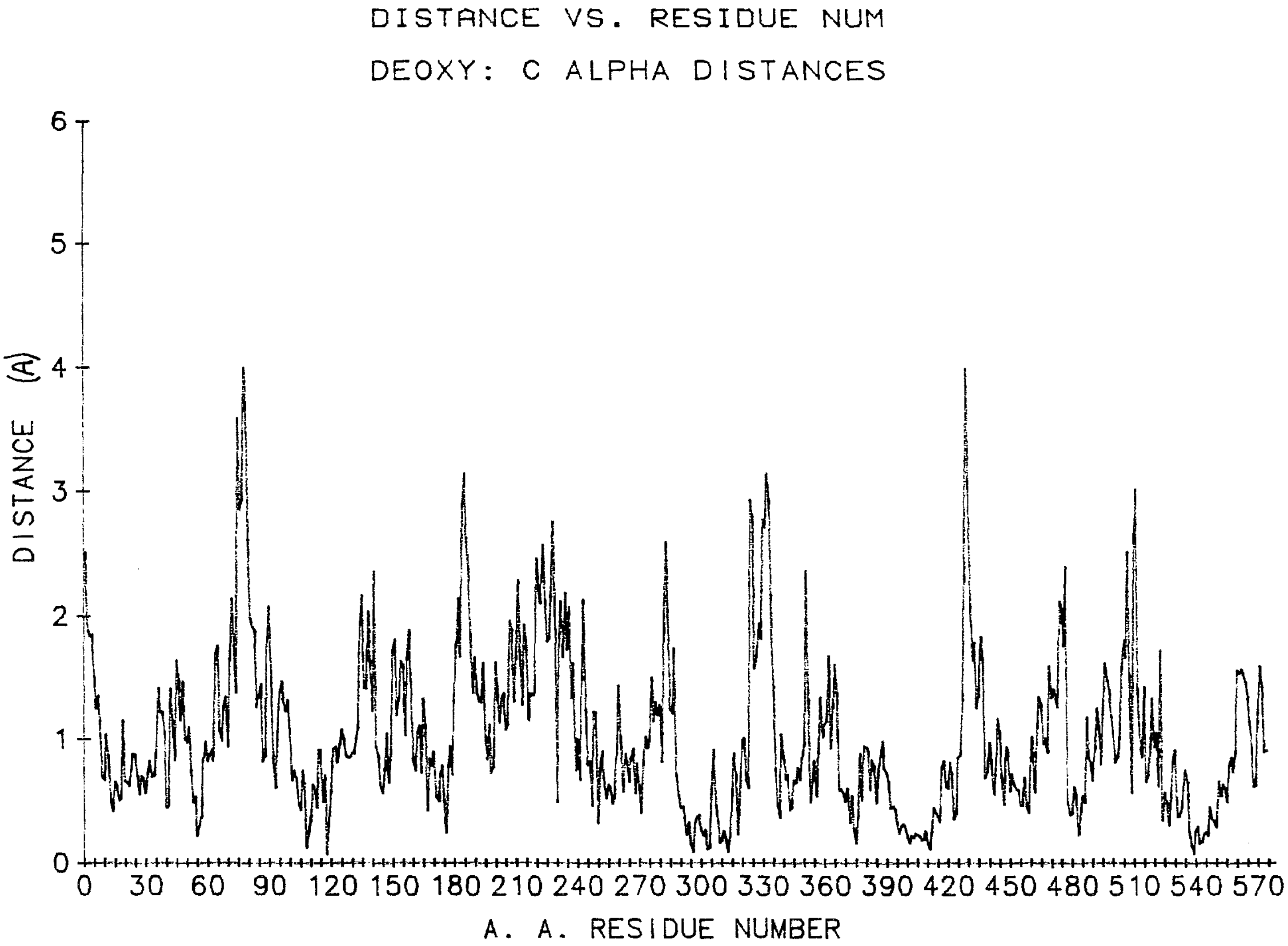

DISTANCE VS. RESIDUE NUM
DEOXY: C ALPHA DISTANCES
DISTANCE (A)
6
5
4
3
2
1
0
0
30
60
90
120
150
180
210
240
270
300
330
360
390
420
450
480
510
540
570
A. A. RESIDUE NUMBER

FIGURE 11

Interatomic distances between the C-alphas of the β82 crosslinked and minimized X-ray structure of deoxyhemoglobin.

FREQUENCY DISTRIBUTION

DEOXY: C ALPHA OF DEOXY

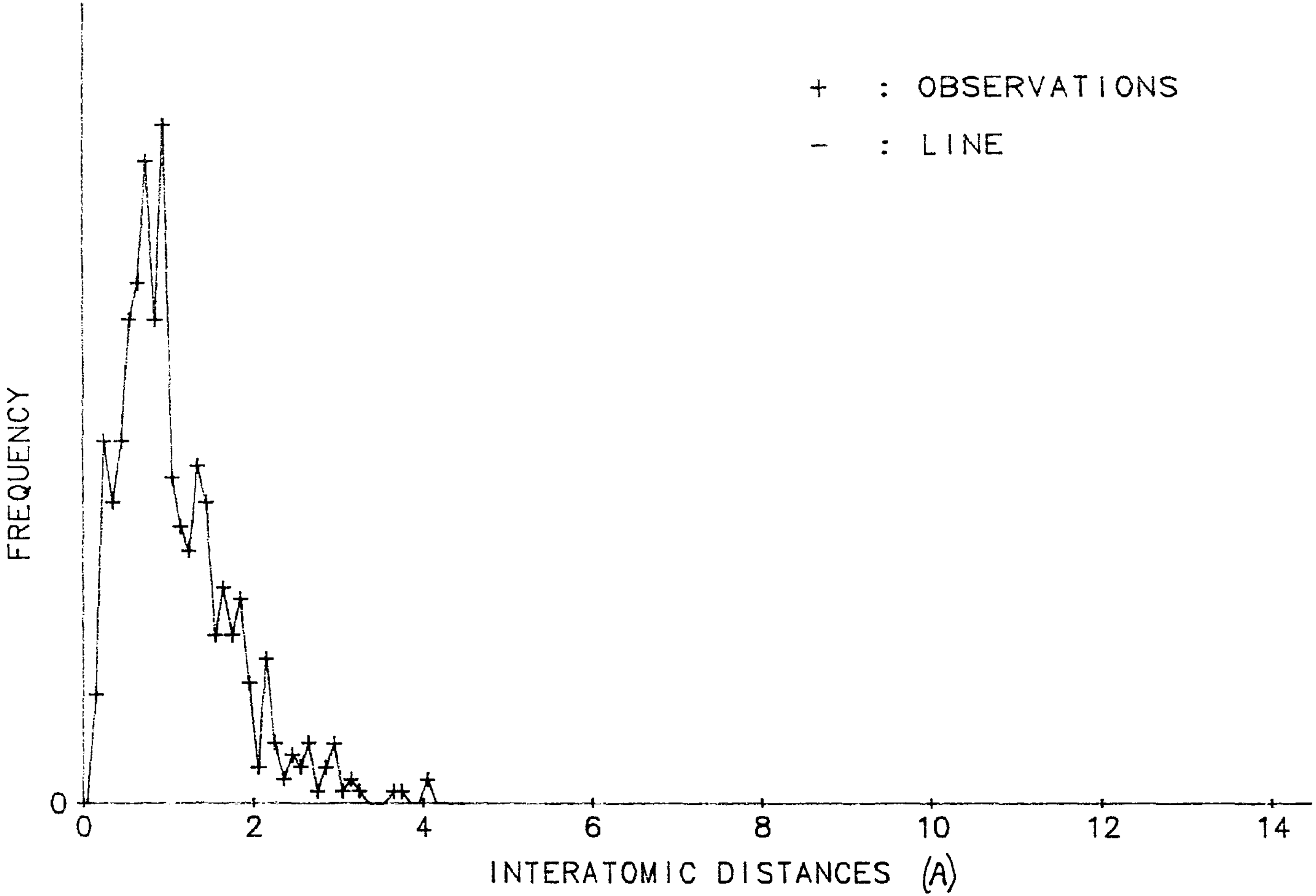


FIGURE 12

Differences between C-alpha atom positions of β82 crosslinked and the X-ray structure of oxyhemoglobin.

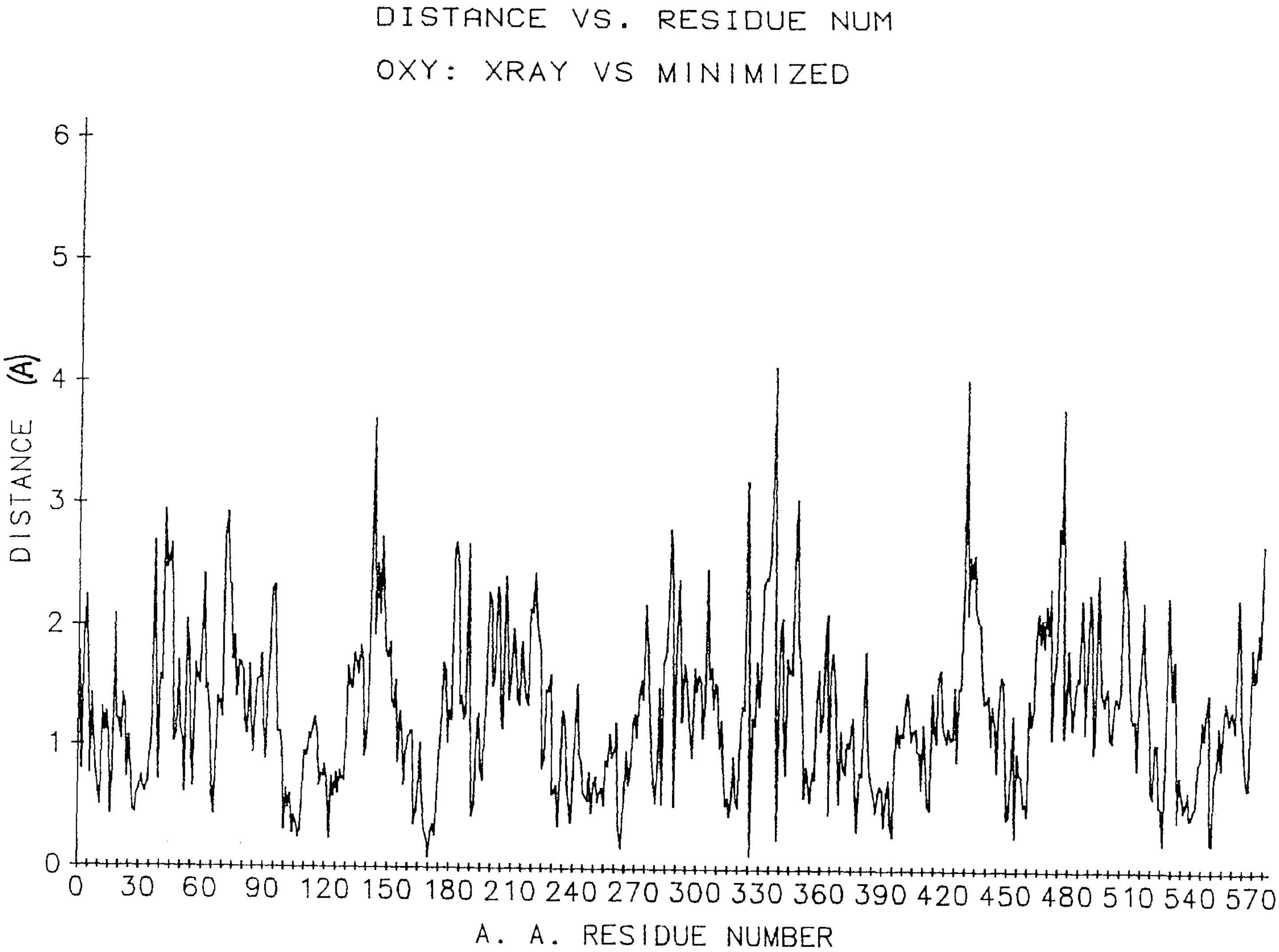

DISTANCE VS. RESIDUE NUM
OXY: XRAY VS MINIMIZED
DISTANCE (A)
0
1
2
3
4
5
6
0 30 60 90 120 150 180 210 240 270 300 330 360 390 420 450 480 510 540 570
A. A. RESIDUE NUMBER

FIGURE 13

Interatomic distances between the C-alphas of the β82 crosslinked and X-ray structure of oxyhemoglobin.

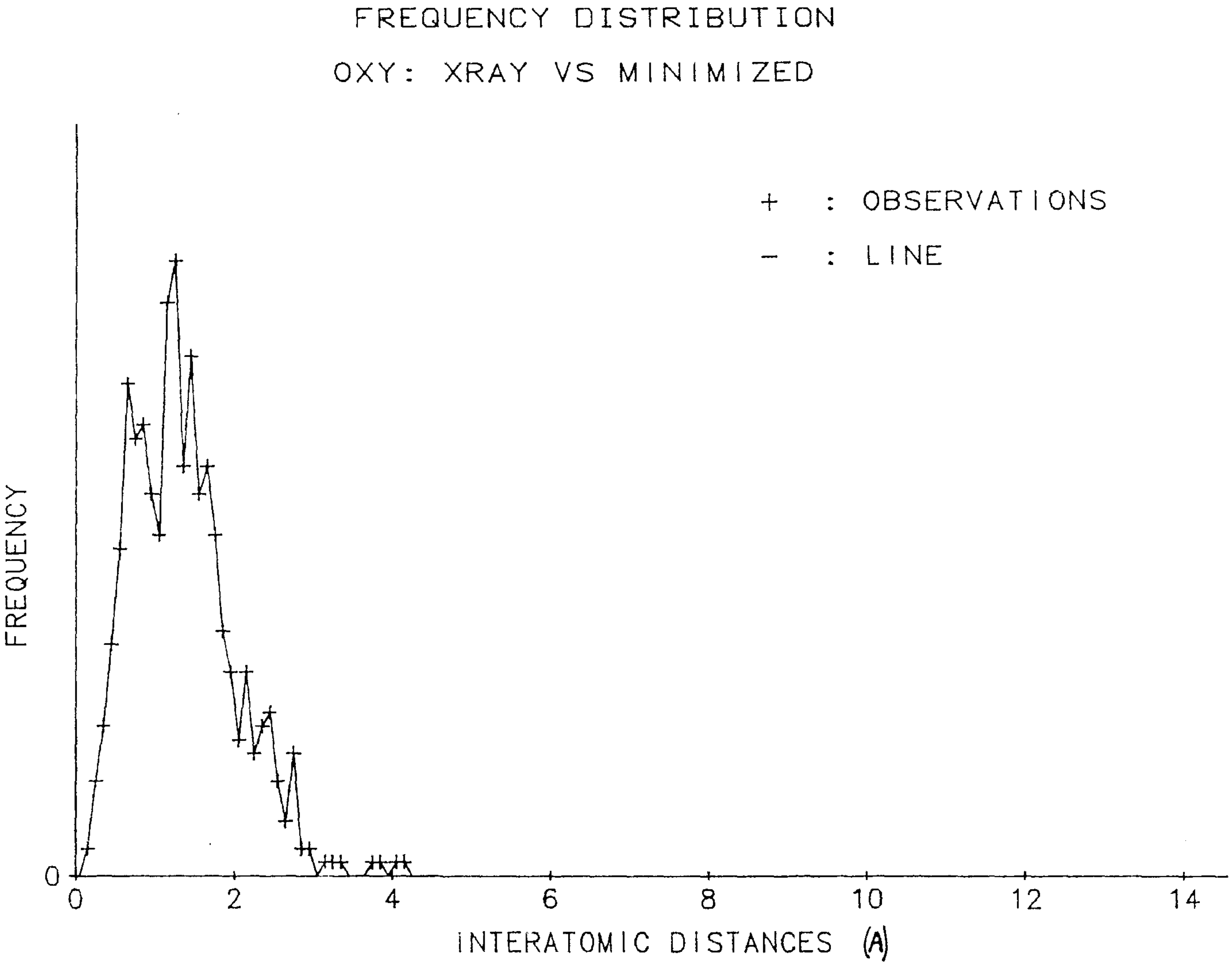

FREQUENCY DISTRIBUTION
OXY: XRAY VS MINIMIZED
+ : OBSERVATIONS
– : LINE
FREQUENCY
0
0
2
4
6
8
10
12
14
INTERATOMIC DISTANCES (Å)

FIGURE 14

Differences between C-alpha atom positions of β82 crosslinked and the minimized X-ray structure of oxyhemoglobin.

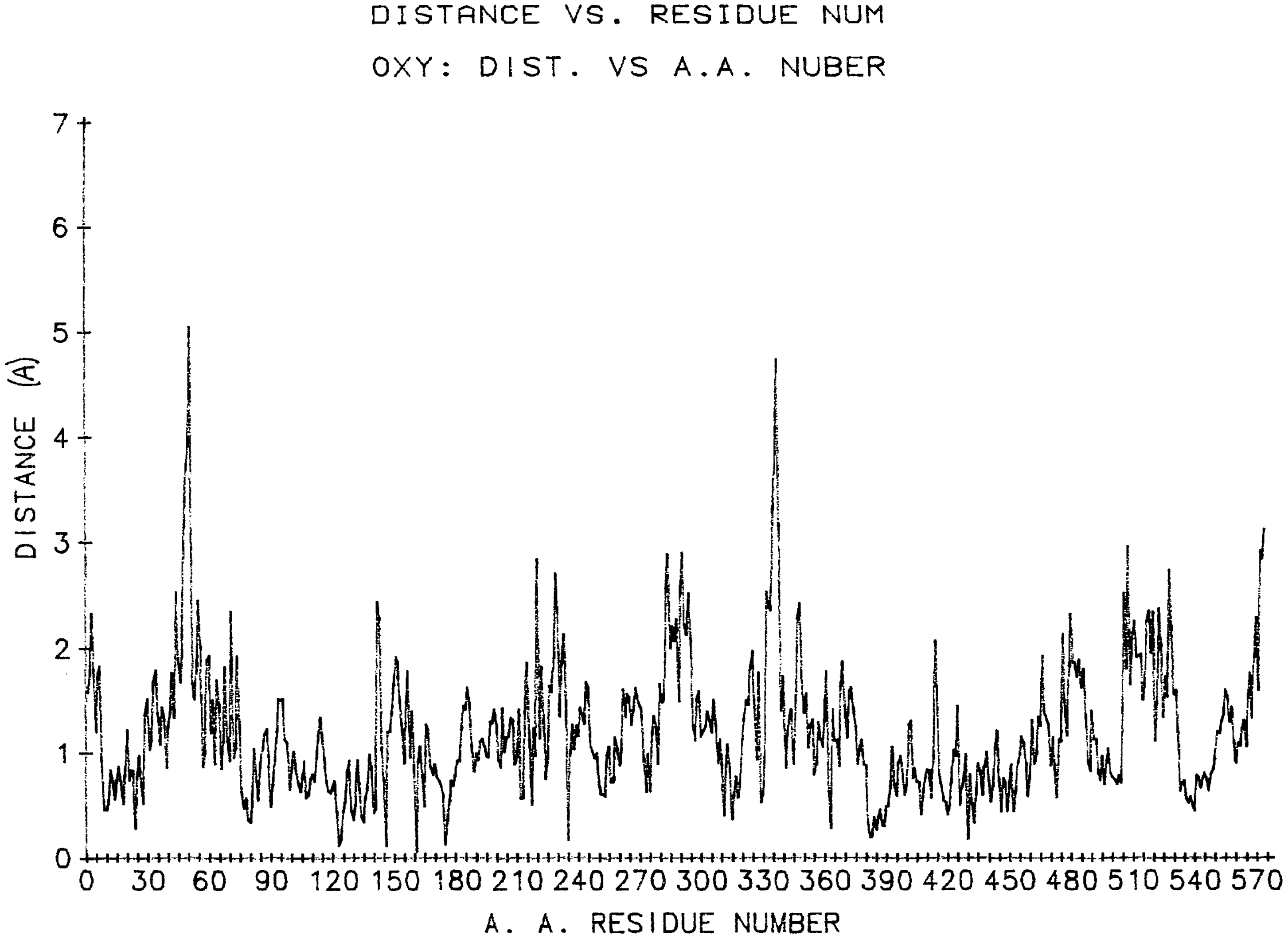
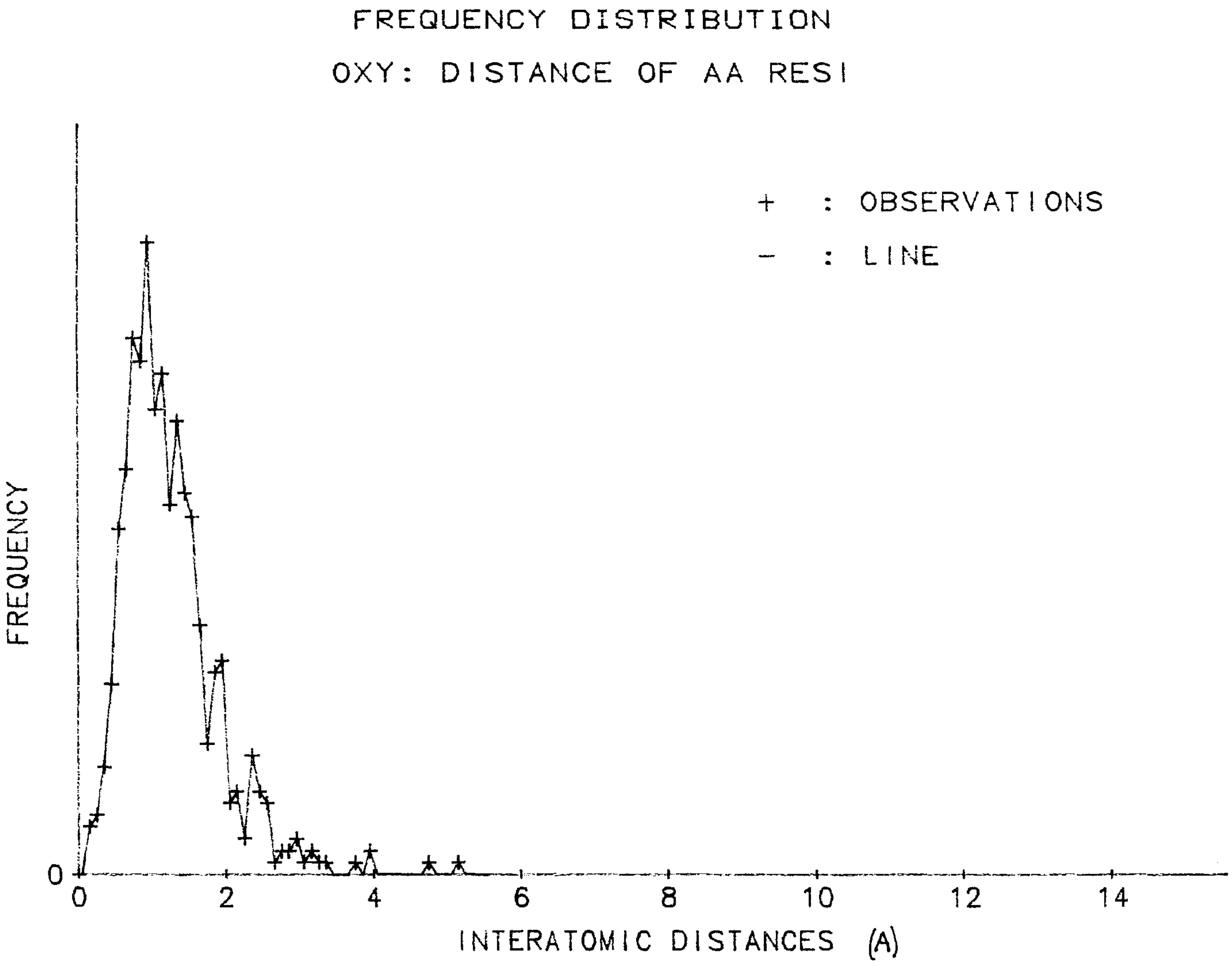

DISTANCE VS. RESIDUE NUM
OXY: DIST. VS A.A. NUBER
DISTANCE (A)
0
1
2
3
4
5
6
7
0
30
60
90
120
150
180
210
240
270
300
330
360
390
420
450
480
510
540
570
A. A. RESIDUE NUMBER

FIGURE 15

Interatomic distances between the C-alphas of the β82 crosslinked and the minimized X-ray structure of oxyhemoglobin.


FREQUENCY DISTRIBUTION
OXY: DISTANCE OF AA RESI
+ : OBSERVATIONS
— : LINE
FREQUENCY
0
0
2
4
6
8
10
12
14
INTERATOMIC DISTANCES (A)

uncrosslinked proteins, were acceptable for both oxyhemoglobin and deoxyhemoglobin.[42] A small RMS deviation (for example 0.2 Å) would indicate only a small change has occurred after minimization. However, hemoglobin is large and has different structural features (α helicies).[3] These secondary structure features would be expected to maintain their conformations. The loop residues between these features would be expected to vary more after minimization. The minimization did not include solvent interactions which would aid in holding the loop regions and end residues in a fixed position. We were not overly concerned that there was some movement of residues, since the RMS deviation was less than 5.0 Å. Also, the crosslinked deoxyhemoglobin structure did not minimize to a conformation similar to the oxyhemoglobin X-ray structure. The RMS deviation of the crosslinked deoxyhemoglobin structure to the X-ray structure of oxyhemoglobin for the alpha carbons was 2.56 Å and for the mainchain atoms (carbon alphas, carbonyls, and nitrogens) was also 2.56 Å. Therefore, the deoxyhemoglobin structure did not minimize to the oxyhemoglobin conformation.

One interesting observation for deoxyhemoglobin is that there was a greater movement of residues for the crosslinked protein when compared with the uncrosslinked minimized structure (Figure 10) rather than with the X-ray structure (Figure 8). This was just the reverse situation for oxyhemoglobin when Figures 12 and 14 are compared. At present, there is no reasonable explanation of why there is this difference. The RMS deviations were reasonable for any of these comparisons. Preliminary X-ray crystallographic results for the deoxy β82 crosslinked hemoglobin[43] also indicate that this structure is more similar to normal deoxyhemoglobin than it is to oxyhemoglobin. This is consistent with the results presented here. A complete comparison of these two models will be done when the final X-ray coordinates are available.

CHAPTER 4

MOLECULAR DYNAMICS EXPERIMENTS

Introduction

There are a number of groups who have studied hemoglobin using molecular dynamics experiments.[42, 44-48] Michael Johnson and coworkers,[42] along with Martin Karplus and associates,[44] used molecular dynamics to study the movement of sickle and hemoglobin A in order to compare flexibility of the two structures. This chapter will describe the molecular dynamics experiments that were performed to decipher the possible mode of interaction of a crosslinker, DBSF, with oxyhemoglobin. This mechanism is believed to be typical for many other crosslinking reactions.

Methodology

A molecular dynamics experiment was utilized in order to investigate the possible movement of the ligand in the binding site. The programs employed were Insight[35] and Discover. The DBSF ligand was manually oriented in the BPG binding site ($\beta_1$82-$\beta_2$82) of oxyhemoglobin[30] using the Insight program. One of the carbonyl carbons of the ligand was bonded to the nitrogen of lysine β82 as if it had reacted at this site. A subset was created that was 15 Å from the center of the β82-β82 site. This subset is further subdivided into three volumes when running the molecular dynamics: 1) the residues 15 - 13 Å from the center of the β82 oxyhemoglobin site were held rigid, 2) the residues from 13 - 8 Å were tethered (allowed to move slightly but somewhat restrained to their original position), and 3) the residues within 8 Å to the center were allowed to move freely. These constraints allowed the ligand and protein within 8 Å from the center to move and change conformation in order to avoid repulsive contacts and to simulate motion that occurs when DBSF binds in this site. All hydrogens were used in the molecular dynamics; the ionizable residues were used in the charge states seen at pH 7.0. In addition, if creation of this 15 Å subset resulted in partial residues, the entire residue was included in the subset.

The attached ligand and the side chains that were allowed to move in the oxyhemoglobin 8 Å subset were minimized for 100 iterations. This was in order to remove repulsive contacts between the ligand, which had been manually fitted into the protein, and the protein itself. The temperature of the system was allowed to adjust to 300 K after 1000 iterations of molecular dynamics equilibration. Discover has an algorithm that adjusts temperature by scaling velocities. If the temperature is far from the temperature set for equilibrium, the velocities are increased vigorously until the temperature is met. The temperature was kept constant using another algorithm that varies the temperature slightly, as if the system were coupled to a heat bath, for the remainder of the dynamics calculations. The molecular dynamics experiment used the Verlet algorithm.[35] The initial experiment was set up so that the dynamics ran for 1000 iterations using a time step of 1 femtosecond, for a total of 1 picosecond. Every tenth iteration was written out to a file which was later loaded into the Insight program to make a movie for a visual depiction of the atom movements.

Modifications on this Experiment

The initial dynamics experiment was modified several times in order to address specific mechanistic and procedural questions. In the first modification, the temperature was gradually increased by 20^o increments from the initial temperature of 0 K, and allowed to run for 100 iterations after each increment until a temperature of 300 K was reached. The 1000 iterations of molecular dynamics then followed this gradual equilibration. This experiment determined whether there would be a change in the motion and distance of the lysine β82 to the attached ligand if the temperature was gradually equilibrated or set at a specific temperature.

In a second modification the charge on the β82 lysine amino group that was interacting with the bound DBSF ligand was varied. In the initial experiment, the lysine amine was positively charged while the acid substituent on the ligand was negatively charged, simulating a physiological pH of 7.0. Therefore, the charged species would be attracted toward each other. In a separate experiment, the lysine residue on the protein was deprotonated so it has a neutral charge and the molecular dynamics experiment was recalculated.

Another modification to the molecular dynamics experiment lengthened the number of iterations from 1,000 to 100,000 with both the charged and neutral ligands bound to one β82 lysine. Every 100th iteration was written to a file for each run. This experiment explored the frequency of the bound ligand coming in close contact with the crosslinking β82 lysine.

The last modification to this experiment changed the subset specification on the charged lysine experiment in order to determine whether a larger subset of moving residues would result in a different answer. A new subset was created to give a 25 Å size, which was further subdivided into three volumes. The three volumes were: 1) 25 - 16 Å from the center was held rigid, 2) the residues from 16 - 13 Å were tethered, and 3) 13 Å to the center was allowed to move freely. This experiment gave a 5 Å radial increase to the volume of residues which were allowed to move freely rather than being tethered, compared to the initial experiment.

Results

The molecular dynamics experiments with DBSF bound to one side of oxyhemoglobin (β82) enables one to determine how the bound ligand could interact with the lysine β82 of the other subunit. The results of the experiment between the gradually equilibrated dynamics run versus a rapidly equilibrated system showed that the gradually equilibrated run allowed the bound ligand to interact more closely (Figure 16) with the β82 lysine than the fixed temperature equilibration (Figure 17). The gradual equilibration allows a better resolution of problems caused by bad contacts between the reagent and the protein and is probably a truer measure of the physiological state of the protein-ligand interactions. Therefore, all further molecular dynamics experiments were allowed to attain the equilibration temperature gradually, even at the expense of increased computer time. The computer time for the calculation increased on going from the fixed temperature regime (8 hours 52 minutes) to the slowly equilibrated system (11 hours 28 minutes) on a VAX 9000 computer.

The second modification, which varied the charge on the β82 lysine amino group interacting with the DBSF ligand bound to the other β82 residue, showed that the charged species were attracted toward each other. The two groups that would make the crosslink are the charged lysine β82 amino group and the ester carbonyl. The nearest distance between these groups was close

FIGURE 16

Molecular dynamics with gradually equilibrated temperature of bound DBSF on oxyhemoglobin. After each 20^{o} increment, 100 iterations of dynamics were performed until reaching 300 K. 1,000 iterations of molecular dynamics followed the gradual equilibration. The distance measured is between the bound ligand and the β82 lysine on the other subunit.

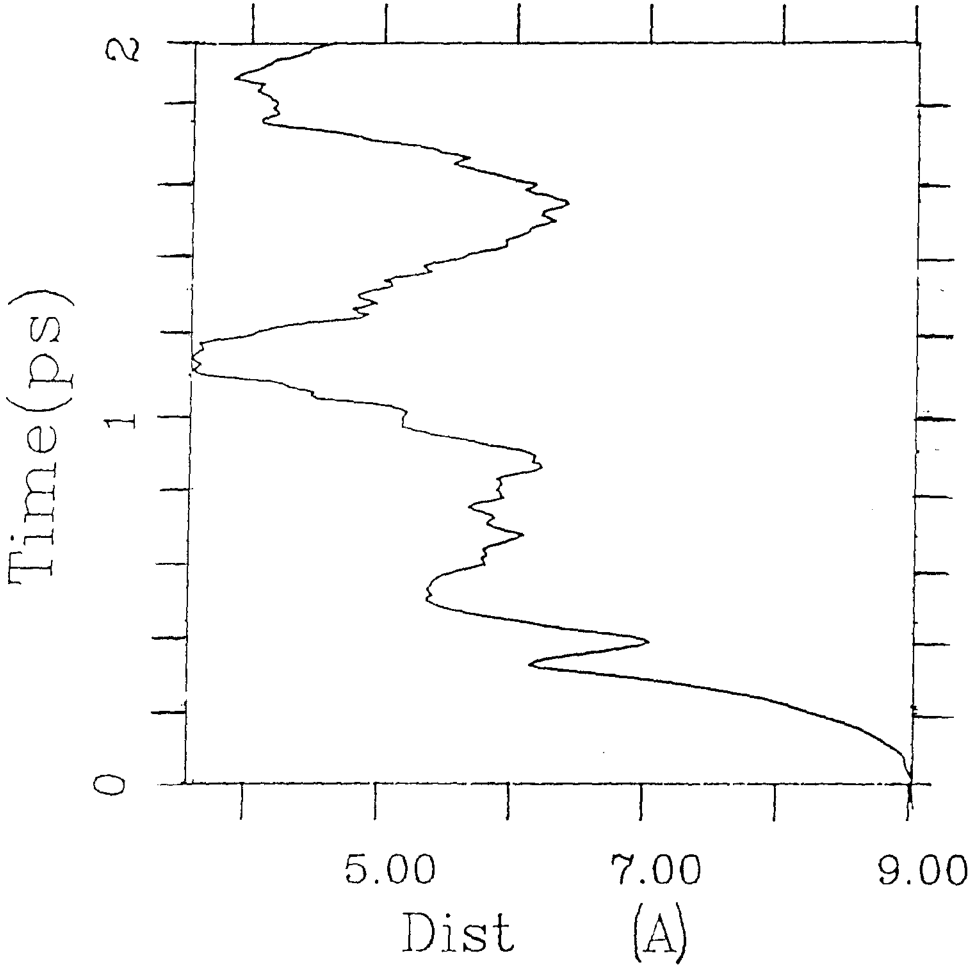
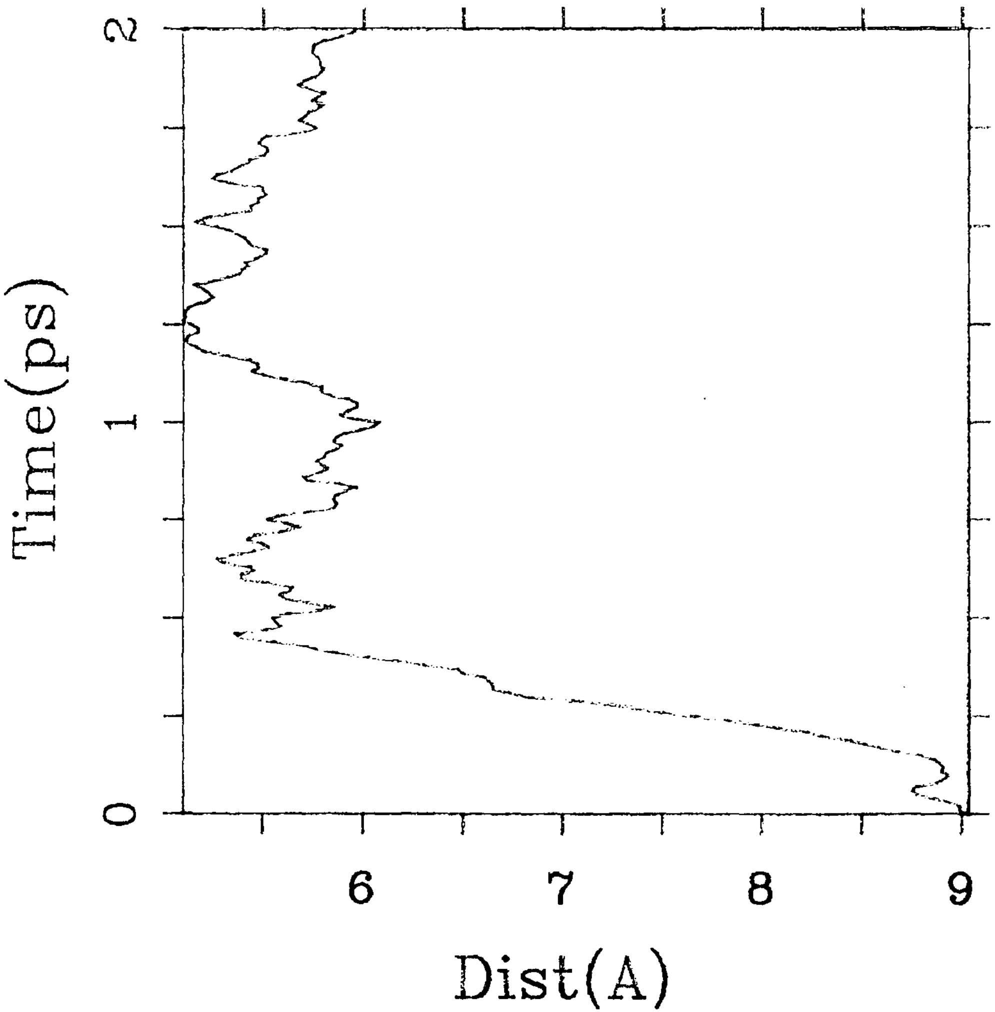

2
1
0
Time(ps)
5.00
7.00
9.00
Dist (A)

FIGURE 17

Molecular dynamics at fixed temperature of bound DBSF on oxyhemoglobin. The temperature was set at 300° and 1,000 iterations of molecular dynamics were performed.


Time(ps)
0
1
2
6
7
8
9
Dist(A)

enough (3.5 Å)[49] for the crosslinking to occur. The distance averaged between3.5 - 6.0 Å. It appears that the carboxylate on the aromatic ring attracts the lysine β82 and brings it sufficiently close for the crosslinking to occur (Figure 16).

This experiment was run three times and the closest contact between the neutral lysine β82 and the ester carbonyl was 4.11 Å. This is too great a distance for a crosslinking to occur (Figure 18).[49] Therefore, if the mechanism for crosslinking begins when one β82 lysine reacts with one ester carbonyl of DBSF, the negatively charged carboxylate group on the DBSF could aid the crosslinking with the other β82 lysine, by increasing the probability that the two reacting groups will be sufficiently close for reaction. It should be noted that the NH_3^+ lysine must still deprotonate prior to making the new bond.

The third modification lengthened the number of molecular dynamics iterations from 1,000 to 100,000 with experiments for both the charged and neutral lysine β82. In the 100,000 iteration experiment, the neutral ligand averaged a distance of between 12 - 16 Å between the β82 lysine and the ester carbonyl. The closest approach was approximately 5.5 Å between the ligand and the lysine (Figure 19). The distance between the carbonyl and the charged lysine in the 100,000 iteration experiment averaged a distance of 5.0 - 6.5 Å (Figure 20) with the closest contact being 4.8 Å. The negatively charged ligand was attracted to the positively charged lysine as evidenced by the shorter distance of interaction. This result revealed that this interaction could occur frequently if this is the mechanism for crosslinking. It should be emphasized that both the short and longer molecular dynamics experiments agreed there is a greater possibility for the charged lysine to interact with the negatively charged ligand than the neutral lysine amine.

FIGURE 18

Molecular dynamics for 1,000 Iterations: Measuring the distance between the neutral β82 lysine nitrogen and ester carbonyl of bound DBSF.

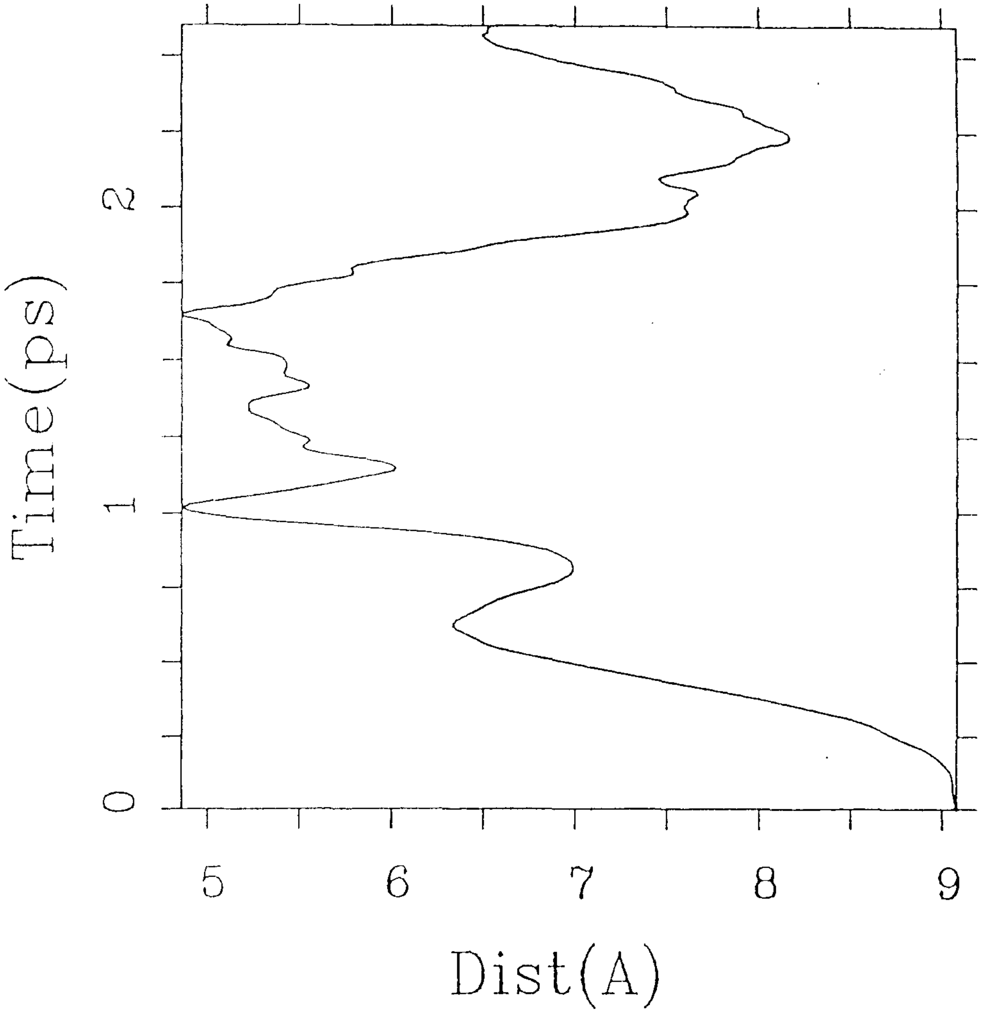

Time(ps)
0
1
2
5
6
7
8
9
Dist(A)

FIGURE 19

Molecular dynamics for 100,000 iterations: Measuring the distance between the neutral β82 lysine nitrogen and ester carbonyl of bound DBSF.

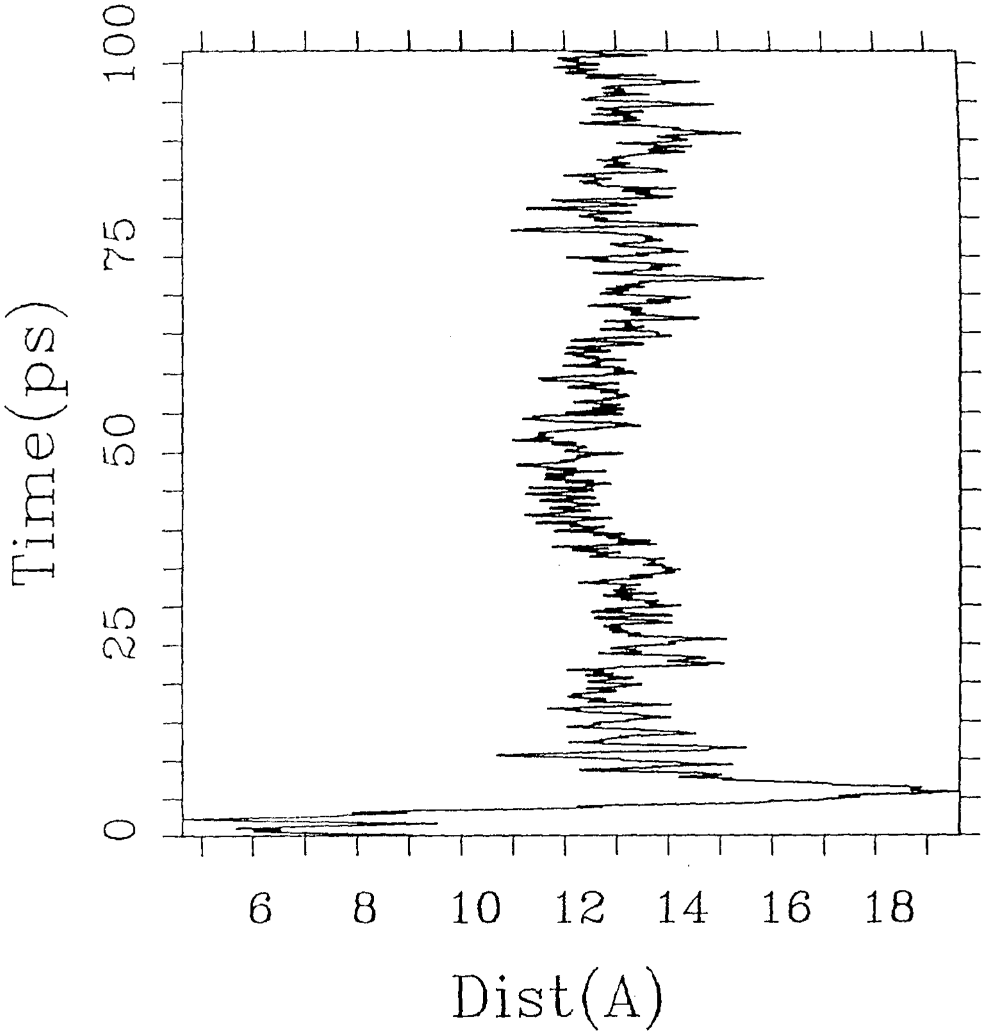
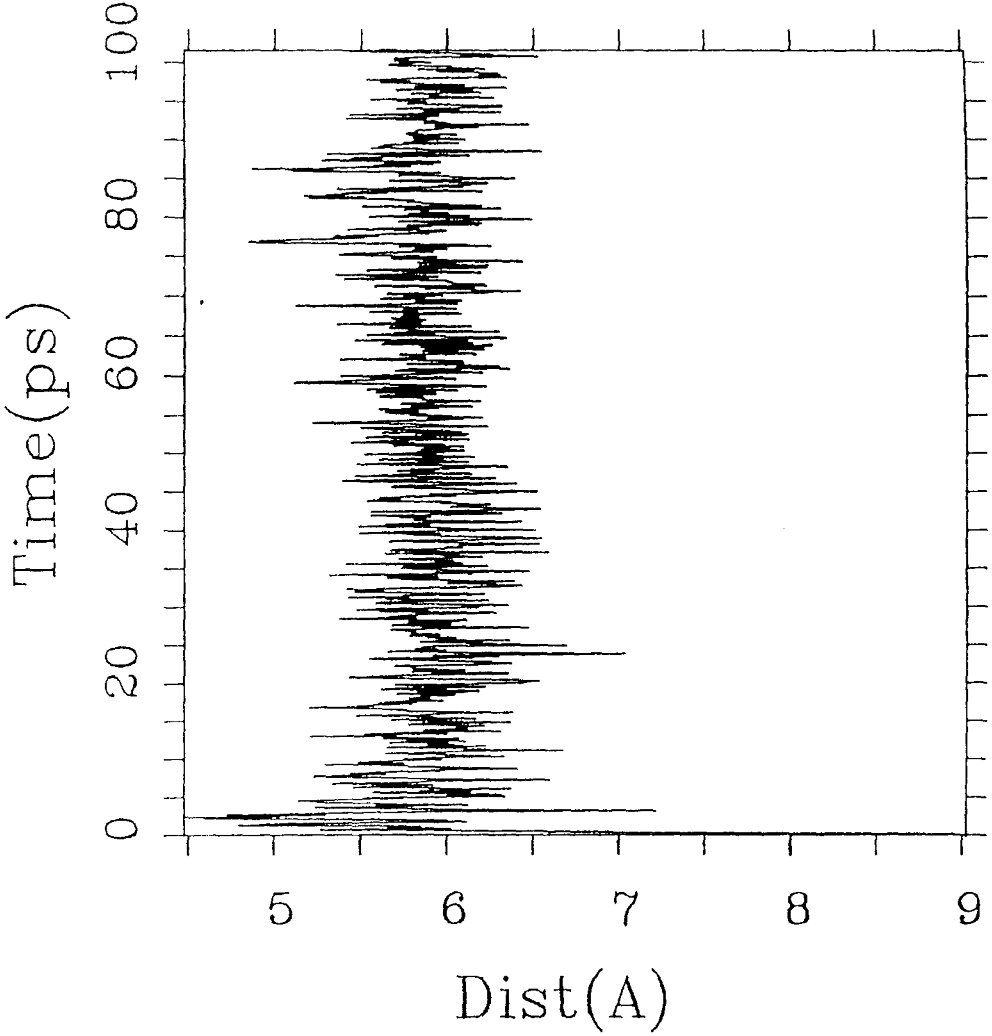

Time(ps)
0
25
50
75
100
6
8
10
12
14
16
18
Dist(A)

FIGURE 20

Molecular dynamics for 100,000 iterations: Measuring the distance between the protonated β82 lysine nitrogen and the salicylic acid.


Time(ps)
100
80
60
40
20
0
5
6
7
8
9
Dist(A)

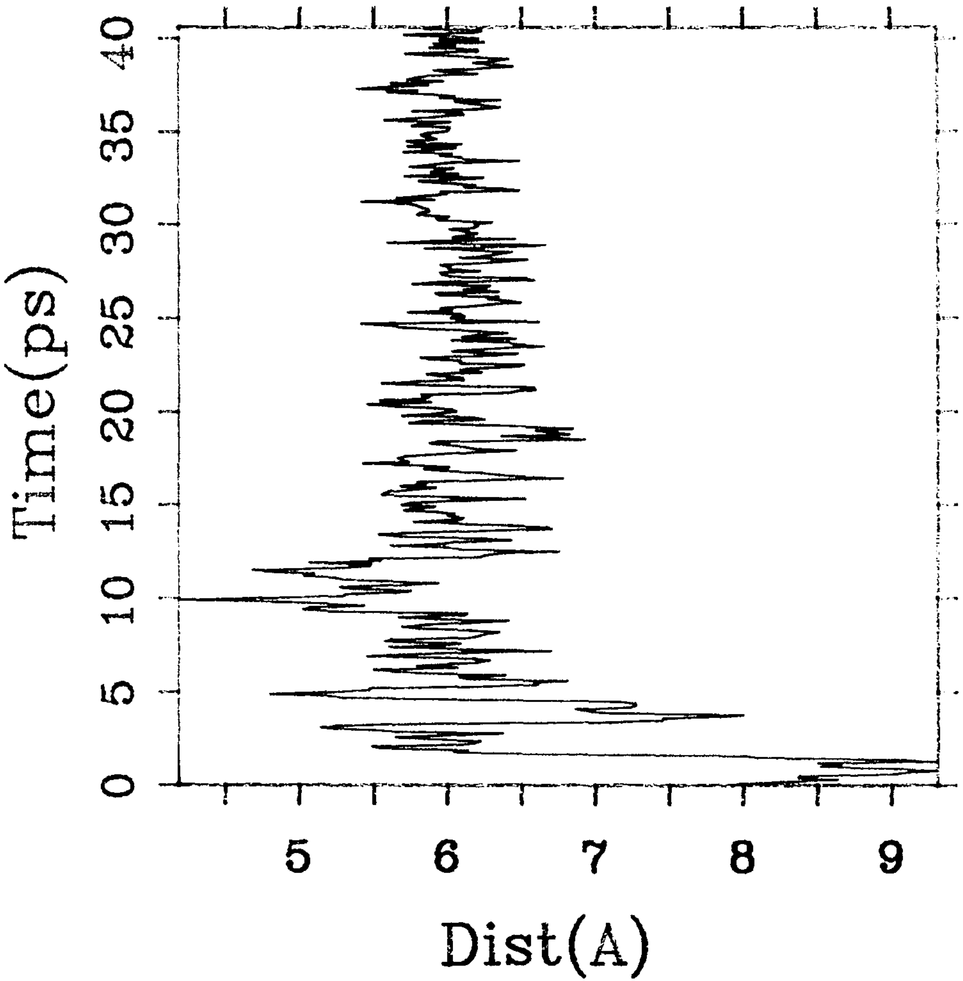
At this time, it can not be explained why there is a variation in the distances of interaction when comparing the shorter molecular dynamics runs and the longer one. One possible way to resolve this would be to write the molecular dynamics coordinates to a file more frequently than every 100 iterations. However, this was not done because the trajectory file where the coordinates were sampled every 100 iterations was already very large.

In the last modification, the subset of atoms considered in the molecular dynamics experiments with a charged lysine was increased from 15 Å to 25 Å. We originally intended to perform 100,000 iterations, but unfortunately the VAX 9000 crashed after about 40,000 iterations and the program was restarted for another 60,000 iterations. Thus the total iterations is 100,000 but the results will be shown in two parts: part a is the 40,000 iteration piece and part b is the 60,000 iteration portion of the trajectory. The closest approach between the lysine amine and the ester carbonyl was 4.19 Å for part a (Figure 21) and 3.91 Å for part b (Figure 23). The interesting result is that the distance from the lysine amine to the acid oxygen on the dibromosalicyl part was only 2.5 Å for both part a and b (Figures 22,24).

The mechanism of crosslinking is thought to occur in a two step mechanism: 1) one side of DBSF reacts with one β82 lysine of oxyhemoglobin and then, 2) the other β82 lysine comes in close contact due to electrostatic interactions with the acid to complete the crosslink. However, at pH 7.0, the lysine would be charged and only the uncharged species would attack the carbonyl of the substrate. Therefore, we speculate that the carboxylate of the salicylate leaving group not only electrostatically attracts and orients the reactive lysine, but also deprotonates the ζ-amino group, making it a better nucleophile. Both of these factors would increase the specificity and the speed of the crosslinking reaction.

FIGURE 21

Molecular dynamics for 40,000 iterations: Subset size is 25 Å measuring the distance between the protonated β82 lysine nitrogen and ester carbonyl of bound DBSF.



Time(ps)
0
5
10
15
20
25
30
35
40
5
6
7
8
9
Dist(A)

FIGURE 22

Molecular dynamics for 40,000 iterations: Subset size is 25 Å measuring the distance between the protonated β82 lysine nitrogen and the salicylic acid.

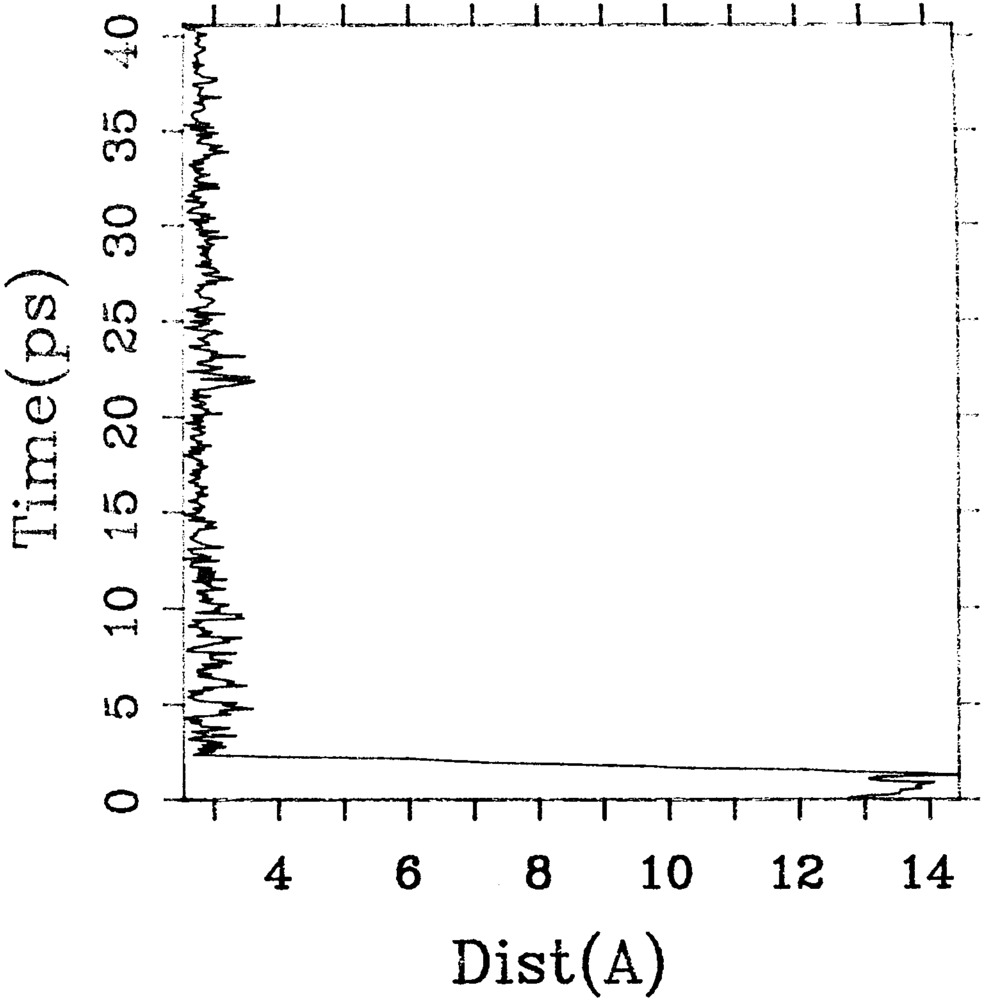

Time(ps)
0
5
10
15
20
25
30
35
40
4
6
8
10
12
14
Dist(A)

FIGURE 23

Molecular dynamics for 60,000 iterations: Subset size is 25 Å measuring the distance between the protonated β82 lysine nitrogen and ester carbonyl of bound DBSF.

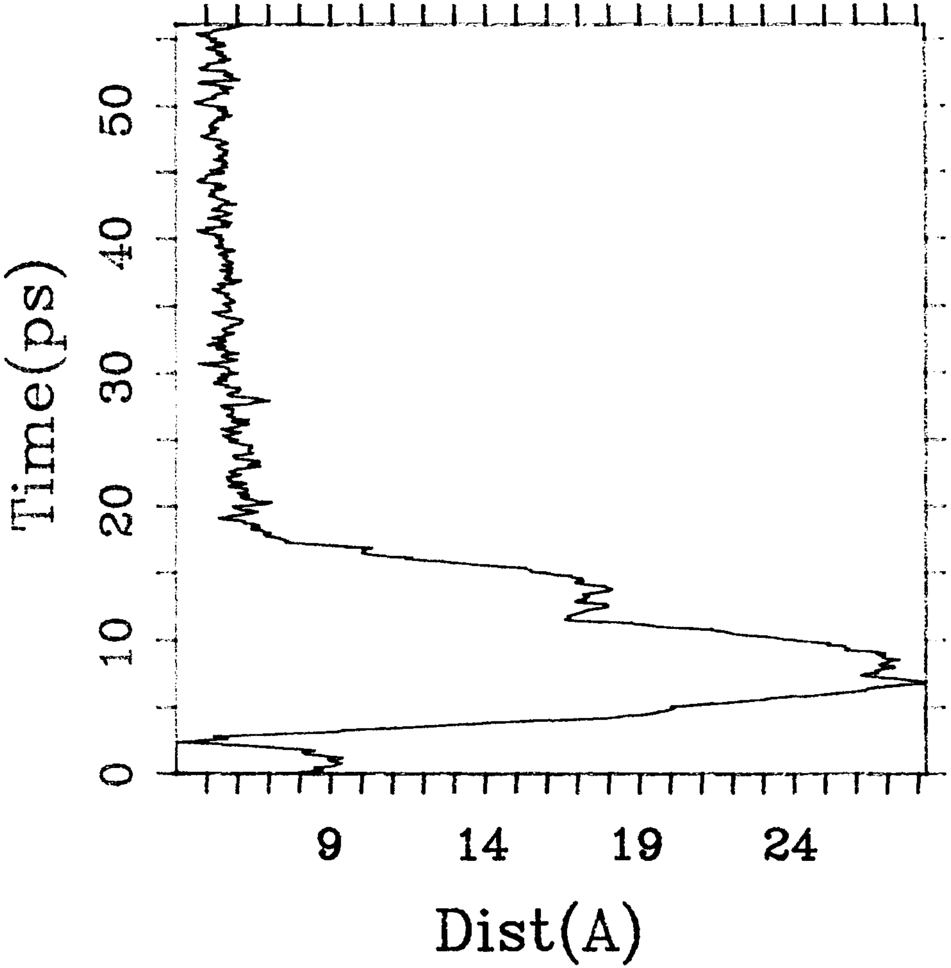
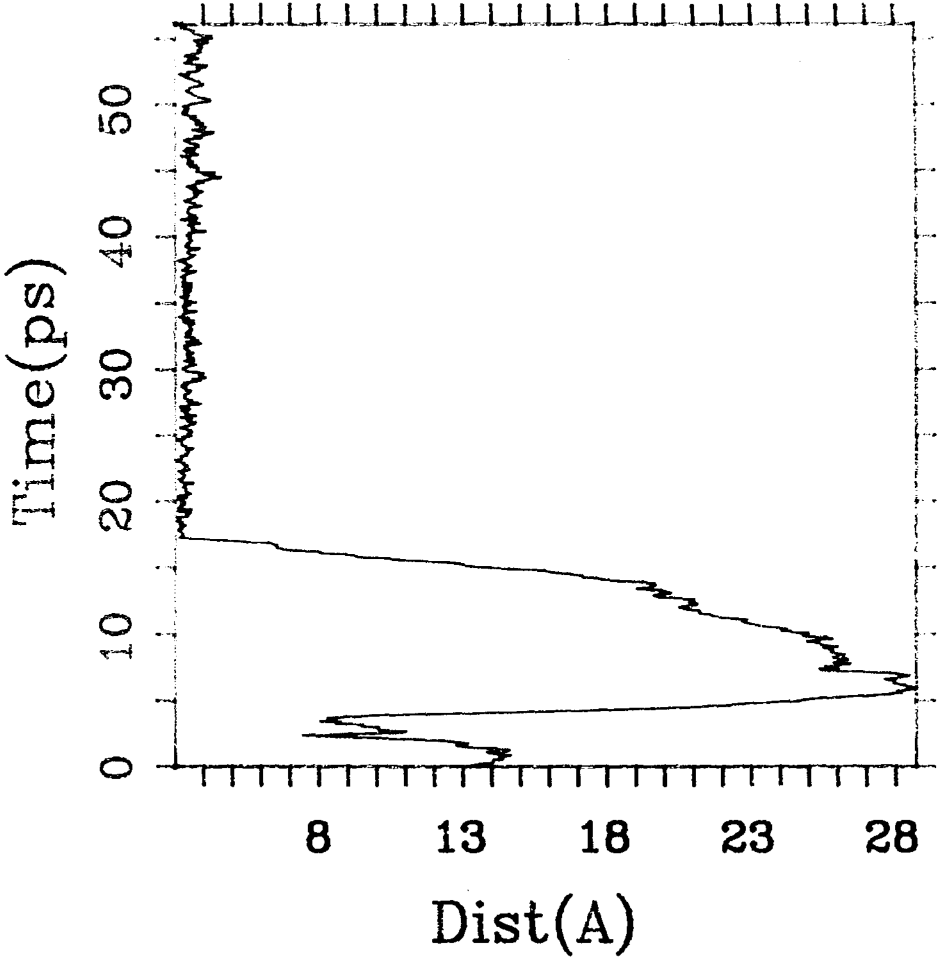

Time(ps)
0
10
20
30
40
50
9
14
19
24
Dist(A)

FIGURE 24

Molecular dynamics for 60,000 iterations: Subset size is 25 Å measuring the distance between the protonated β82 lysine nitrogen and the salicylic acid.


Time(ps)
0
10
20
30
40
50
8
13
18
23
28
Dist(A)

CHAPTER 5

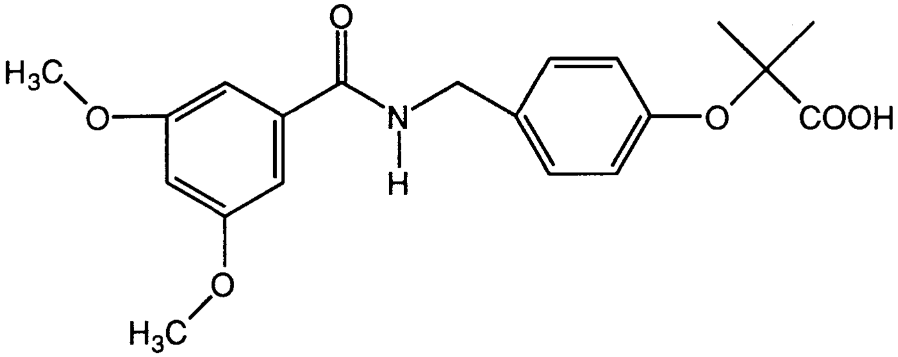
THE DEVELOPMENT OF A PHOTOAFFINITY CROSSLINKER FOR HEMOGLOBIN

Introduction

The purpose of this portion of research was to design a molecule using molecular modeling techniques that would crosslink hemoglobin when irradiated by light. Chapters 1-4 described the characterization of the different crosslinking sites in hemoglobin and the possible mode of interaction. This chapter will describe the process involved in the development of a new crosslinker.

Dock

There were several problems which needed to be addressed in order to develop a new crosslinker. The first problem was to find a computer program that could reproduce the binding of a known ligand in either deoxy or oxyhemoglobin. Abraham and associates examined the binding of bezafibrate and its analogues **8,10-12**[28] in the α99 site of deoxyhemoglobin. This paper included a description of the ligand binding sites and mentioned that the X-ray coordinates were available from the authors. We obtained one set of coordinates for 3,5-dimethoxybezafibrate, **14,** from Abraham in the Protein Data Bank format (PDB coordinates: protein plus ligands bound). A second set of coordinates of the bezafibrate analogues **8**,**10**,**11** mentioned in this article were obtained at a later date in PDB format.



14

These coordinates were converted into an ASCII file, which was subsequently converted into PDB format using Chem-X so that it could be read into Insight. Our intention was to take a conformation of one of the bezafibrate analogues, and using the Dock program[50-52] to reproduce the position of the ligand in the α99 deoxyhemoglobin site.

In order to use the Dock program, several steps must be completed before the ligand can be docked into the protein site. Initially, the program Sphgen[50-52] generates clusters of spheres which characterize the receptor site. A second program, Distmap[50-52] or Chemgrid,[50-52] creates a grid of the receptor site so that a score could be calculated for the fit of the ligand within the receptor, using either contact or forcefield scoring. In the next step, the ligand is placed into the receptor site, and the fit evaluated based on either 1) contact or surface interactions, 2) contact scoring with Delphi electrostatic scoring, 3) force-field scoring (considers both steric and electrostatic interactions), or 4) a combination of contact and forcefield scoring.

The protein coordinates, a site region (a subset of the protein) where the ligand may interact, and the ligand coordinates are used as input to the program.

The sizes for the regions chosen are either based upon the atoms the program could accommodate (the protein specification) or the region that best describes the interaction site of the ligand with the protein (the site subset). A larger site region increases the computer time, and also places spheres in regions where a ligand could not be accommodated, for example, on the outside of the protein.

The PDB coordinates used were those supplied by Abraham for **14**. All the waters and the heme groups were removed from the deoxyhemoglobin protein. The whole protein could not be used since the Dock program has a limit of 3,000 atoms. Therefore, a region of 25 Å from where one of the ligands binds, containing 2,653 atoms (Figure 25) was used as the protein. The site subset, the region where the ligand is to be docked, contains the atoms within 15 Å from where the ligand was found to bind (Figure 25). Charges were placed on the ligand, **14**, and written as a Sybyl mol2 file[53] (a file type which contains charges on the atoms to be used in docking the ligand) using the conformation as found in the X-ray coordinates. The ligand was moved away from the site where it was found to bind so as not to bias the results. The protein, subset and ligand were input into the Dock program and the binding orientations found were scored using both the contact and the forcefield method.

The same procedure was followed for compound **10b** and for DBSF **6** in oxyhemoglobin but using only the forcefield method of scoring.

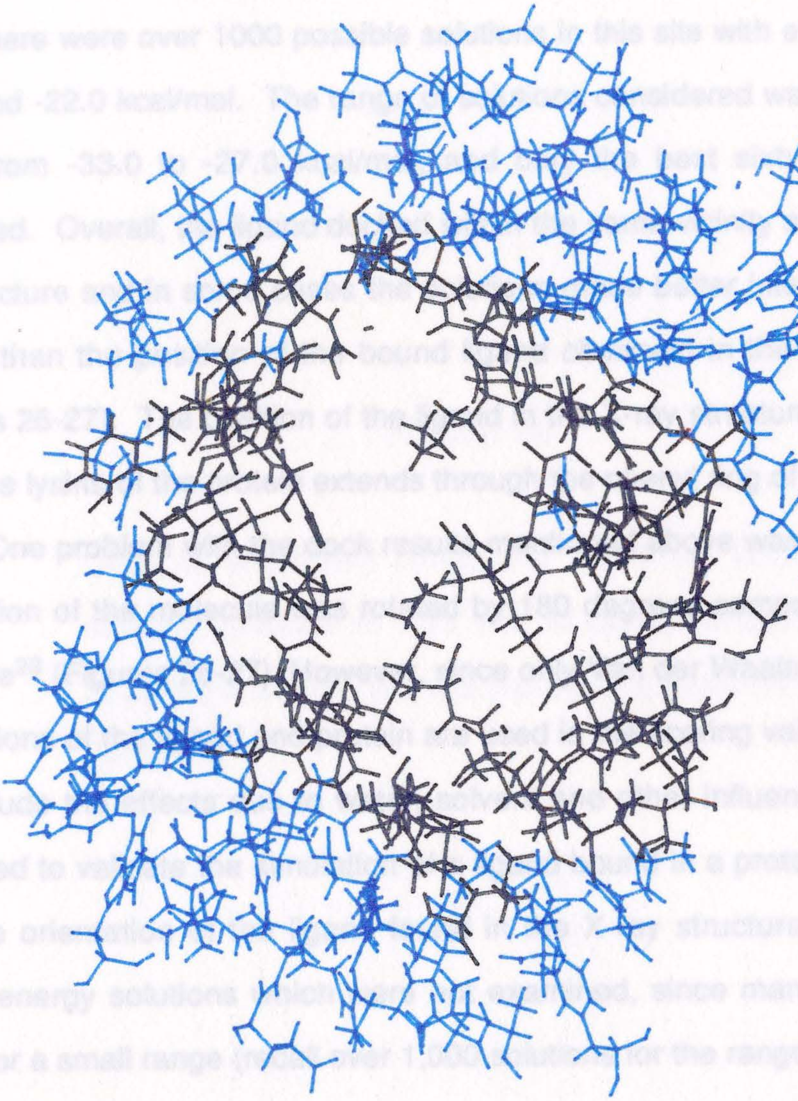
Results of the Docking Procedure

The dock procedure of the 3,5-dimethoxybezafibrate **14** analogue using contact scoring placed the ligand in the active site of the protein. The ligand fit into the open volume of the active site, however, the hydrogen bond interactions were not taken into account.

FIGURE 25

Region used in the Dock program: 25 Å for the protein, 15 Å subset where BZF was found in Deoxyhemoglobin. The 25 Å subset is colored blue. The 15 Å subset is colored brown.

The dock run of 3,5-dimethoxybezafibrate 14 using the forcefield scoring method showed very promising results, when compared to the PDB coordinates sent to us by Abrahams. The forcefield energies ranged from -33.0 kcal/mol to 224.0 kcal/mol with -33.0 kcal/mol energy being the best fit of the ligand in the site. There were over 1000 possible solutions in this site with energies between -33.0 and -22.0 kcal/mol. The range of [illegible] considered was then narrowed to be from -33.0 to -27.0 kcal/mol and [illegible] the best sixty solutions were examined. Overall, the ligand docked [illegible] the same site as found in the X-ray structure and in some cases the [illegible] better interactions with the protein than the [illegible] bound ligand [illegible] the crystal structure (Figures 26-27). The position of the ligand in the X-ray structure is questionable since the lysine of the protein extends through the [illegible] ring of the ligand.



One problem with the dock results [illegible] above was that the docking orientation of the molecule was rotated by 180 degrees compared to the X-ray structure[28] (Figures [illegible]). However, since only van der Waals and electrostatic interactions of the ligand and protein are used in the scoring value and this does not include the effects due to water, solvent and other influences, this method appeared to validate the orientation the ligand bound in a protein. It is possible that the orientation of the ligand found in the X-ray structure could be in the higher energy solutions which were not examined, since many solutions were found for a small range (note over 1,000 solutions for the range of -33.0 to -22.0 kcal/mol). The forcefield scoring method can only approximate the true interactions.

It should be mentioned that the αβ site being examined has a C2 symmetry axis in which Abraham[28] found a ligand bound to both halves of the protein. The Dock program gave a result in which a ligand was found to fit in

The dock run of 3,5-dimethoxybezafibrate **14** using the forcefield scoring method showed very promising results, when compared to the PDB coordinates sent to us by Abrahams. The forcefield energies ranged from -33.0 kcal/mol to 224.0 kcal/mol with -33.0 kcal/mol energy being the best fit of the ligand in the site. There were over 1000 possible solutions in this site with energies between -33.0 and -22.0 kcal/mol. The range of solutions considered was then narrowed to be from -33.0 to -27.0 kcal/mol, and only the best sixty solutions were examined. Overall, the ligand docked within the same vicinity as found in the X-ray structure and in some cases the solutions made better interactions with the protein than the position of the bound ligand observed in the crystal structure (Figures 26-27). The position of the ligand in the X-ray structure is questionable since the lysine of the protein extends through the phenyl ring of the ligand.

One problem with the dock results mentioned above was that the docking orientation of the molecule was rotated by 180 degrees compared to the X-ray structure[28] (Figures 26-27). However, since only Van der Waals and electrostatic interactions of the ligand and protein are used in the scoring value and this does not include the effects due to water, solvent and other influences, this method appeared to validate the simulation of a ligand bound in a protein. It is possible that the orientation of the ligand found in the X-ray structure could be in the higher energy solutions which were not examined, since many solutions were found for a small range (recall over 1,000 solutions for the range of -33.0 to -22.0 kcal/mol). The forcefield scoring method can only approximate the true interactions.

It should be mentioned that the α99 site being examined has a C2 symmetry axis in which Abraham[28] found a ligand bound in both halves of the protein. The Dock program gave a result in which a ligand was found to fit in

FIGURE 26

Dock of dimethoxy compound **14**: X-ray structure versus the docked structure in α99 site of deoxyhemoglobin. The X-ray structure is colored red and the docked molecule is colored blue in the figure.

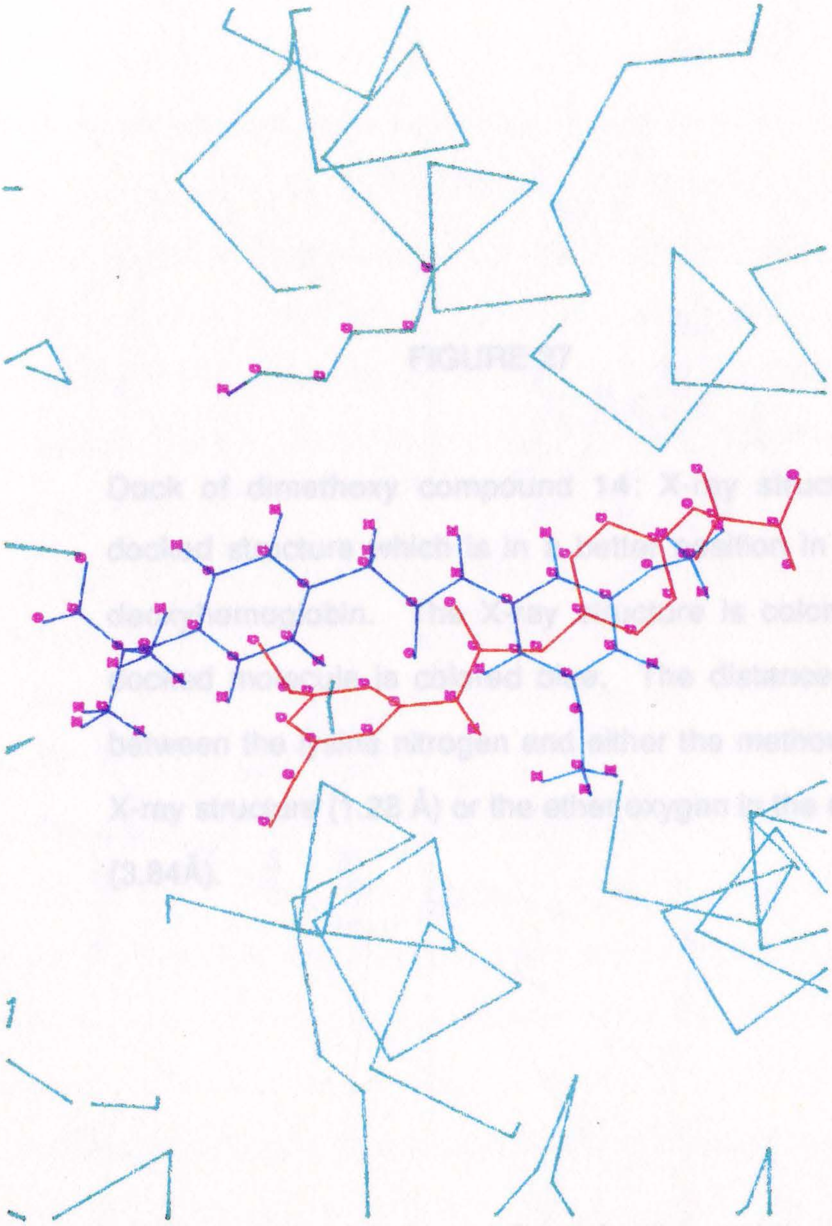


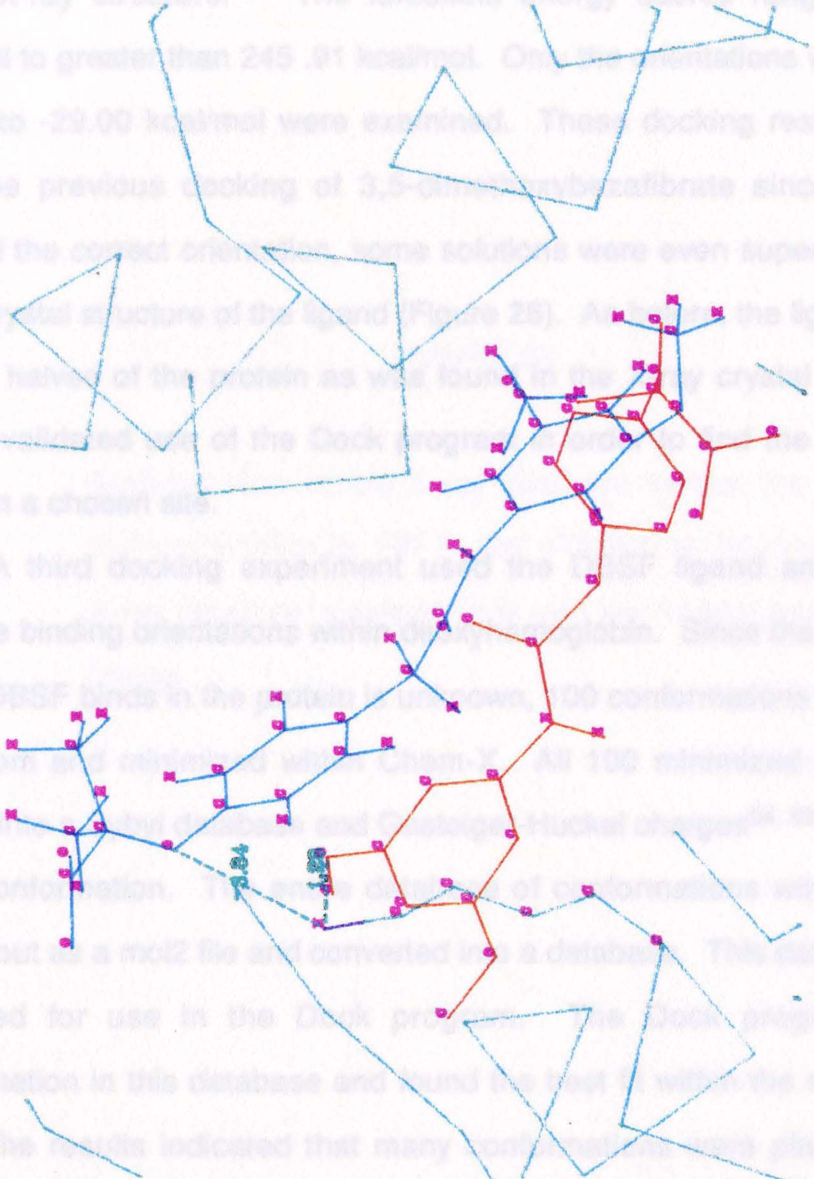
FIGURE 27

Dock of dimethoxy compound **14**: X-ray structure versus the docked structure which is in a better position in the α99 site of deoxyhemoglobin. The X-ray structure is colored red and the docked molecule is colored blue. The distance was measured between the lysine nitrogen and either the methoxy oxygen in the X-ray structure (1.28 Å) or the ether oxygen in the docked molecule (3.84Å).

either half of the protein site around the [illegible]. This program does not have the capability of placing two ligands in the site at the same time. So it was gratifying to find both possibilities present in the 50 solutions that were examined.

The docking of 3,5-dichlorobezafibrate analogue 15b into deoxyhemoglobin was also completed using the conformation of the ligand seen in the X-ray structure.[28] The forcefield energy scores ranged from -32.87 kcal/mol to greater than 245 .91 kcal/mol. Only the orientations with energy from -32.87 to -29.00 kcal/mol were examined. These docking results were better than the previous docking of 3,5-dimethoxybezafibrate since these results showed the correct orientation, some solutions were even superimposed on the X-ray crystal structure of the ligand (Figure 26). As before, the ligand was placed in both halves of the protein as was found in the X-ray crystal structure. This further validated use of the Dock program in order to find the placement of a ligand in a chosen site.

A third docking experiment used the DBSF ligand and examined its possible binding orientations within deoxyhemoglobin. Since the conformation in which DBSF binds in the protein is unknown, 100 conformations were generated in Dgeom and minimized within Chem-X. All 100 minimized structures were loaded into a sybyl database and Gasteiger-Huckel charges[34, 35] were placed on each conformation. The entire database of conformations with charges was written out as a mol2 file and converted into a database. This database was then specified for use in the Dock program. The Dock program took each conformation in this database and found the best fit within the specified protein site. The results indicated that many conformations were placed in the area where the crosslinking is known to occur experimentally (Figure 28). This was the first docking experiment in which the conformation of the ligand was



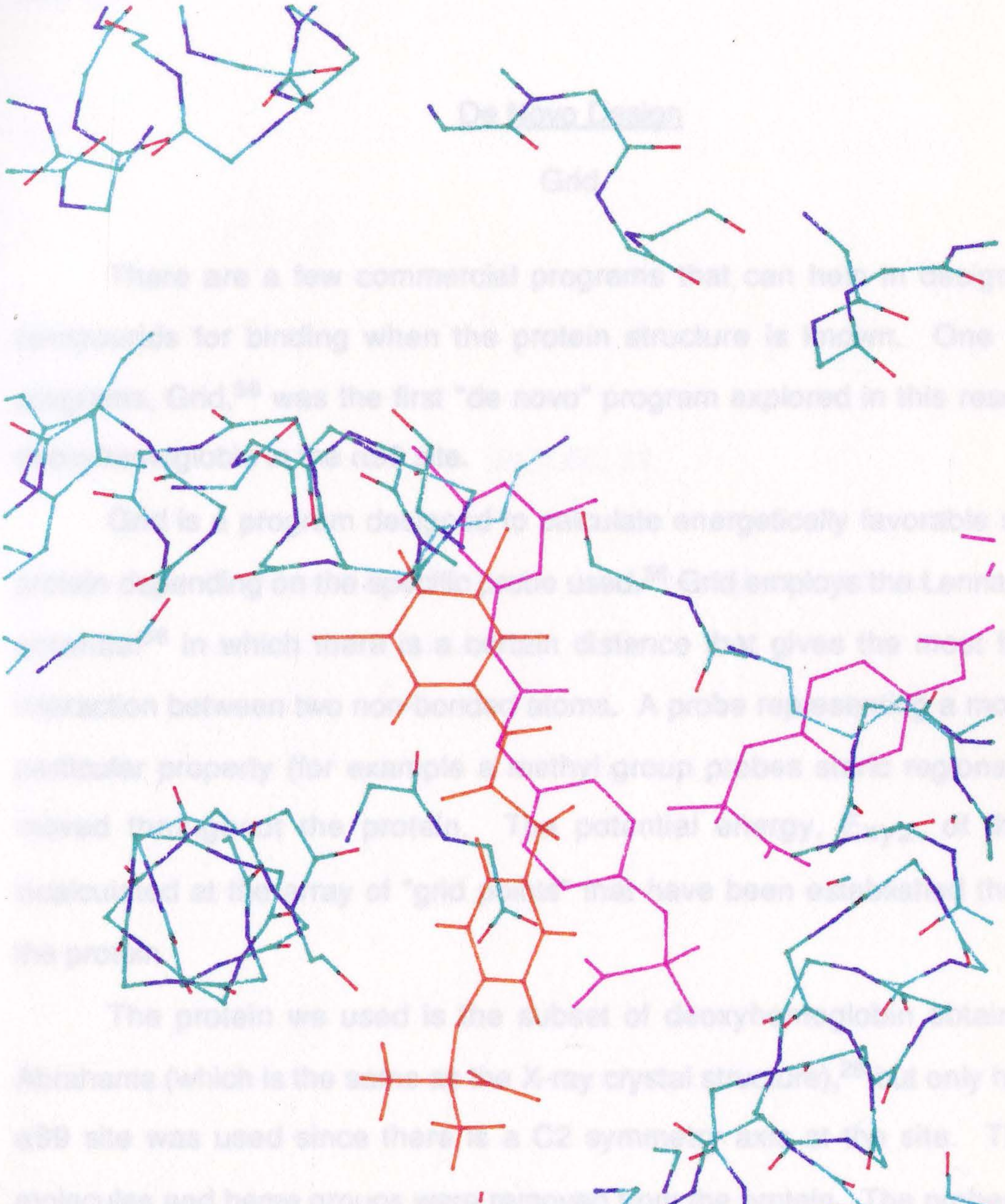
either half of the protein site around the α99 lysine. The program does not have the capability of placing two ligands in the site at the same time. So it was gratifying to find both possibilities present in the 60 solutions that were examined.

The docking of 3,5-dichlorobezafibrate analogue **10b** into deoxyhemoglobin was also completed using the conformation of the ligand seen in the X-ray structure.[28] The forcefield energy scores ranged from -32.87 kcal/mol to greater than 245 .91 kcal/mol. Only the orientations with energy from -32.87 to -29.00 kcal/mol were examined. These docking results were better than the previous docking of 3,5-dimethoxybezafibrate since these results showed the correct orientation, some solutions were even superimposed on the X-ray crystal structure of the ligand (Figure 28). As before, the ligand was placed in both halves of the protein as was found in the X-ray crystal structure. This further validated use of the Dock program in order to find the placement of a ligand in a chosen site.

A third docking experiment used the DBSF ligand and examined its possible binding orientations within deoxyhemoglobin. Since the conformation in which DBSF binds in the protein is unknown, 100 conformations were generated in Dgeom and minimized within Chem-X. All 100 minimized structures were loaded into a Sybyl database and Gasteiger-Huckel charges[54, 55] were placed on each conformation. The entire database of conformations with charges were written out as a mol2 file and converted into a database. This database was then specified for use in the Dock program. The Dock program took each conformation in this database and found the best fit within the specified protein site. The results indicated that many conformations were placed in the area where the crosslinking is known to occur experimentally (Figure 29). This was the first docking experiment in which the conformation of the ligand was

FIGURE 28

Superposition of the X-ray structure versus the docking of 3,5-dichlorobezafibrate **10b** in the α99 site of deoxyhemoglobin. The X-ray structure is colored pink and the docked molecule is colored orange.



unknown, and thus gave very encouraging results showing that the program would place the ligand where crosslink could occur. This is further validation that this program would give a good idea of the location that a molecule is likely to bind and could then crosslink within the site.

De Novo Design

Grid

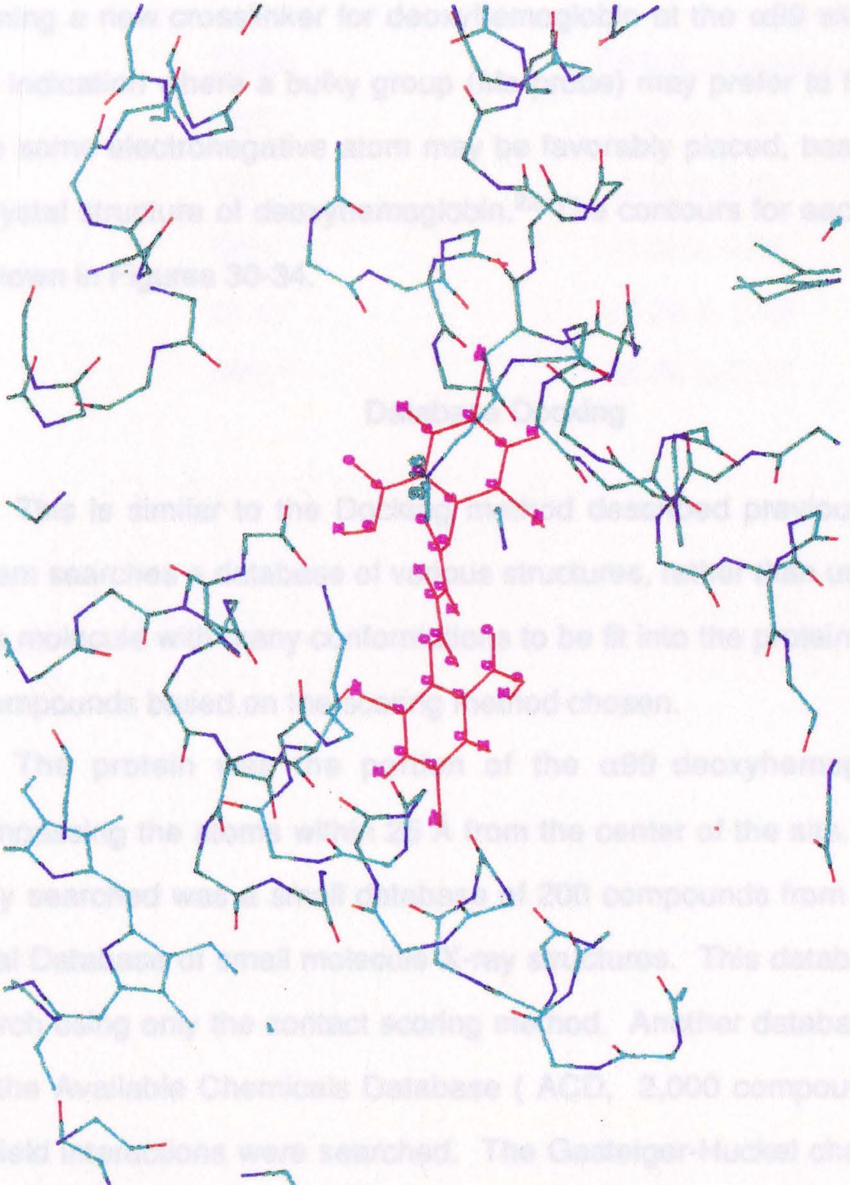
There are a few commercial programs that can help in designing new compounds for binding when the protein structure is known. One of these programs, Grid,[56] was the first "de novo" program explored in this research for deoxyhemoglobin in the α99 site.

Grid is a program designed to calculate energetically favorable sites in a protein depending on the specific probe used.[56] Grid employs the Lennard-Jones potential[56] in which there is a certain distance that gives the most favorable interaction between two non-bonded atoms. A probe representing a molecule or particular property (for example a methyl group probes steric regions) is then moved throughout the protein. The potential energy, E_{xyz}, of the probe iscalculated at the array of "grid points" that have been established throughout the protein.

The protein we used is the subset of deoxyhemoglobin obtained from Abrahams (which is the same as the X-ray crystal structure),[28] but only half of the α99 site was used since there is a C2 symmetry axis at the site. The water molecules and heme groups were removed from the protein. The probes used in these calculations were the water, hydroxyl, methyl, carboxy, and protonated amine probes. An array of energy values were calculated with each of the

FIGURE 29

Docking of DBSF into the deoxyhemoglobin $\alpha99$ site. DBSF is the red colored molecule.



probes and the energy values visualized using isoenergetic contours in Insight.

Results from Grid

The contours using each of the probes were examined and the range of energies listed for each probe (Table 5). These contours are very helpful in designing a new crosslinker for deoxyhemoglobin at the α99 site since it gives some indication where a bulky group (Me probe) may prefer to be or not be, or where some electronegative atom may be favorably placed, based upon the X-ray crystal structure of deoxyhemoglobin.[22] The contours for each of the probes are shown in Figures 30-34.

Database Docking

This is similar to the Docking method described previously. The Dock program searches a database of various structures, rather than using a database of one molecule with many conformations to be fit into the protein site, and ranks the compounds based on the scoring method chosen.

The protein was the portion of the α99 deoxyhemoglobin tetramer encompassing the atoms within 25 Å from the center of the site. The database initially searched was a small database of 200 compounds from the Cambridge Crystal Database of small molecule X-ray structures. This database allowed for a search using only the contact scoring method. Another database was created from the Available Chemicals Database (ACD, 2,000 compounds)[57] in which forcefield interactions were searched. The Gasteiger-Huckel charges had been calculated for these compounds.

TABLE 5

The Range of Energies for Each of the Probes Employed in the Grid Program.

Probe	Range of Energy (Kcal.)
H_2O	- 9.69 to 5.00
OH^-	- 8.54 to 5.00
CH_3	- 4.29 to 5.00
COO^-	-19.29 to 5.00
NH_3^+	-25.26 to 5.00

FIGURE 30

Contour from Grid for the water probe. The red contour are places in the α99 deoxyhemoglobin site where water or a hydrophilic group would like to be placed. The energy range for this probe is from -9.7 to 5.0 Kcal. and these contours are at -6.5 Kcal..

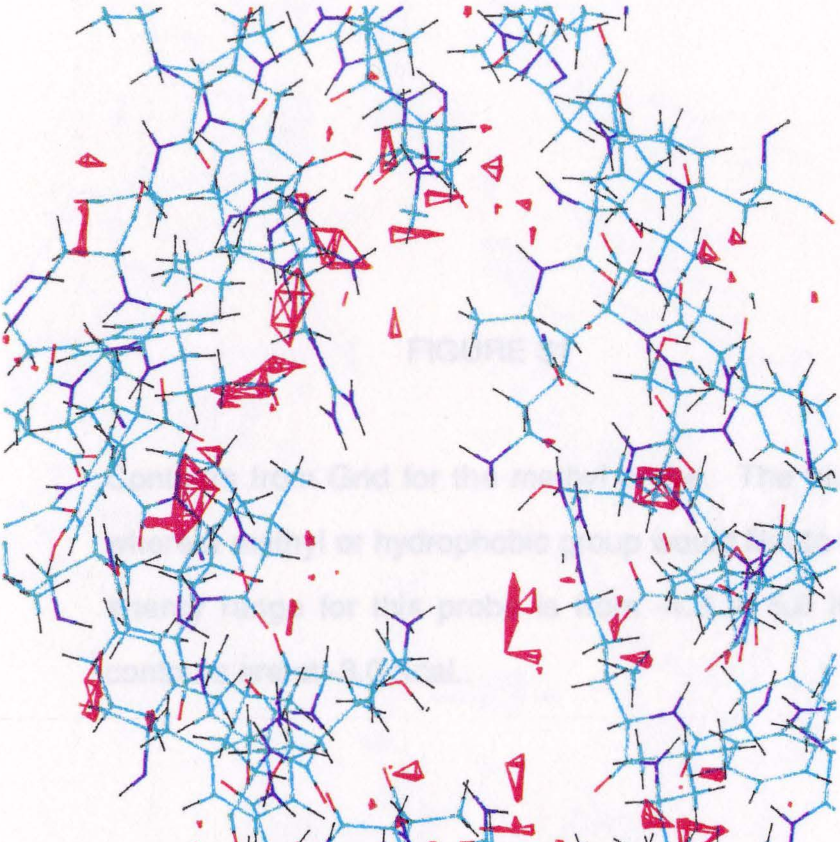


FIGURE 31

Contours from Grid for the methyl probe. The blue contours are where a methyl or hydrophobic group would like to be placed. The energy range for this probe is from -4.3 to 5.0 Kcal. and these contours are at -3.0 Kcal..

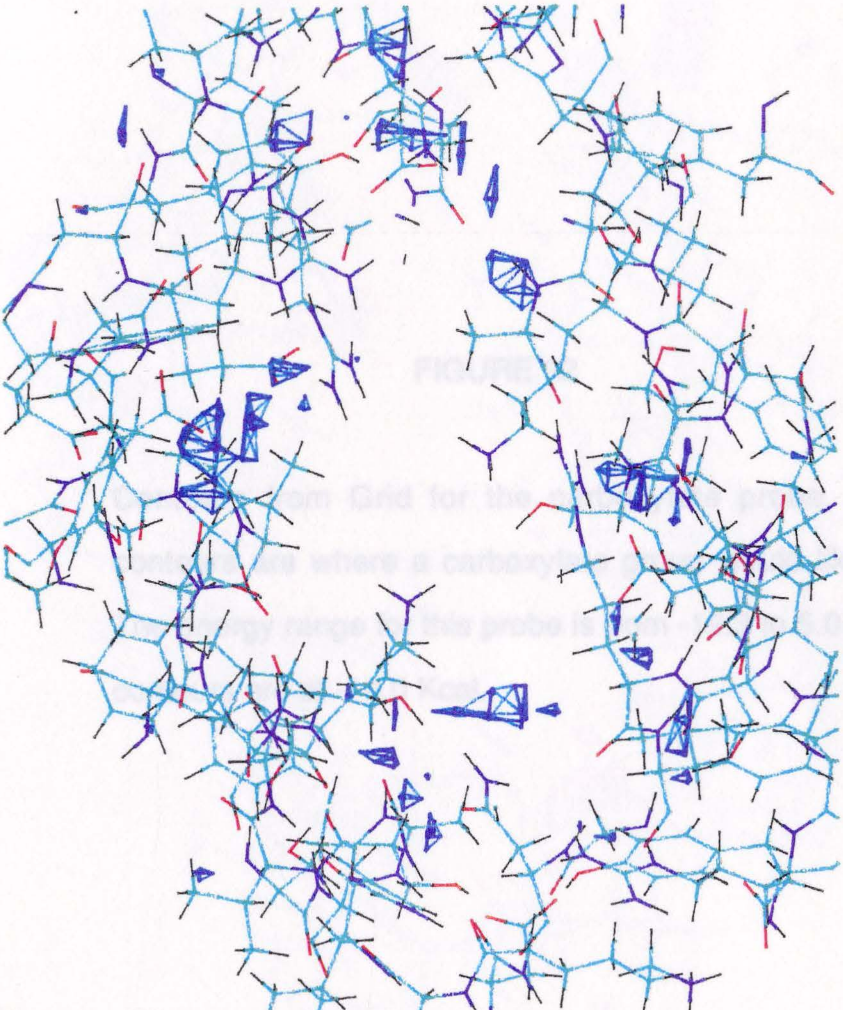


FIGURE 32

Contours from Grid for the carboxylate probe. The magenta contours are where a carboxylate group would like to be placed. The energy range for this probe is from -19.3 to 5.0 Kcal. and these contours are at -10.0 Kcal..

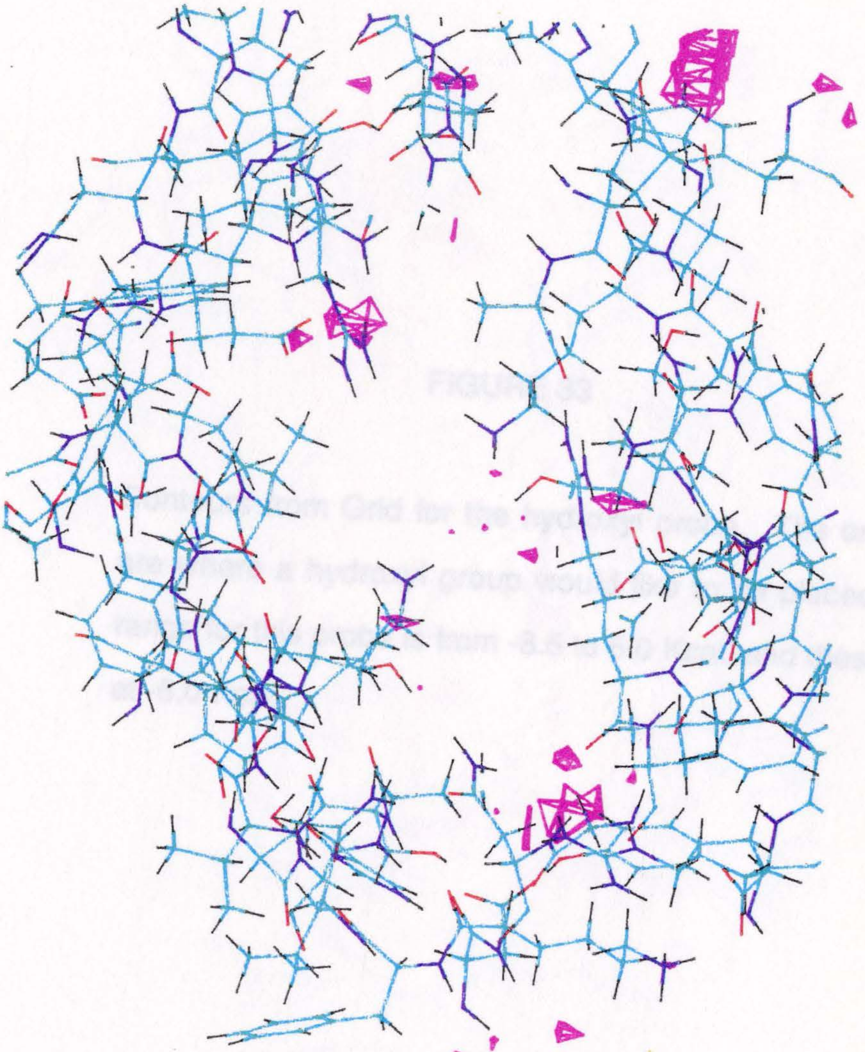


FIGURE 33

Contours from Grid for the hydroxyl probe. The orange contours are where a hydroxyl group would like to be placed. The energy range for this probe is from -8.5 to 5.0 Kcal. and these contours are at -6.0 Kcal..

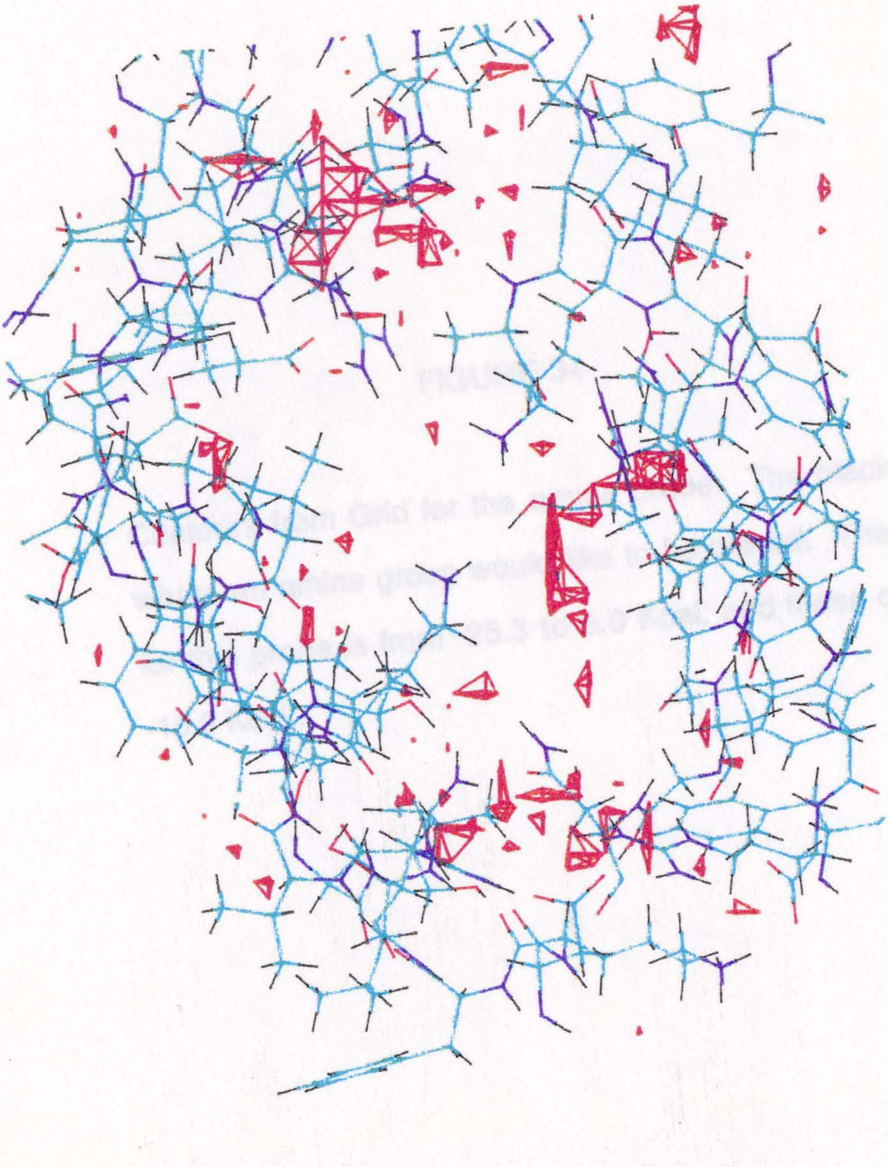
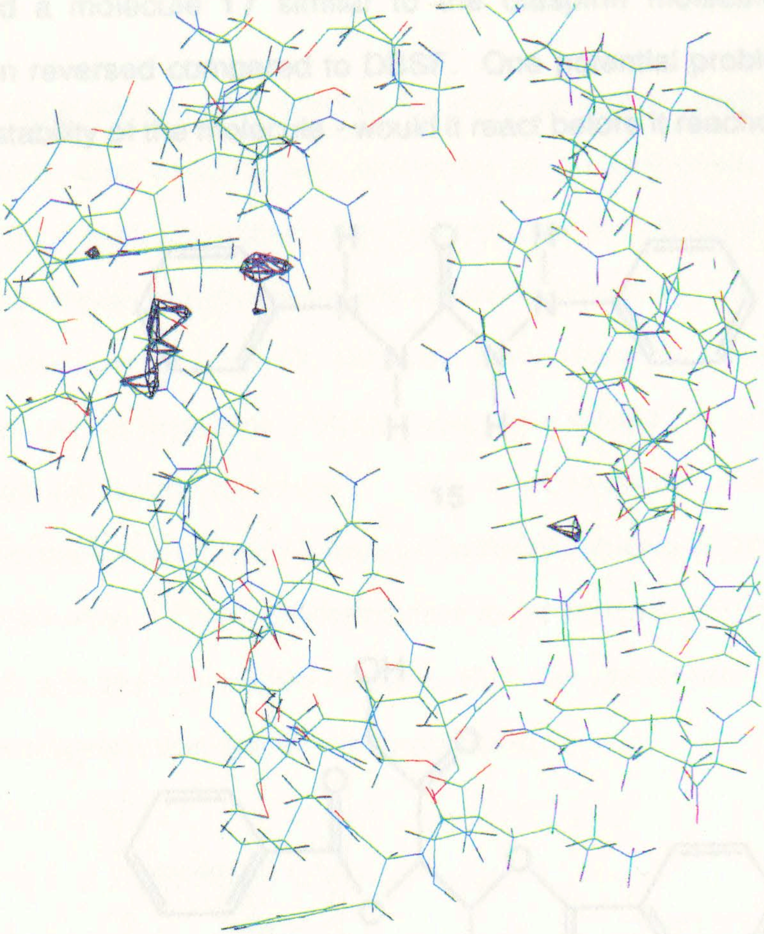


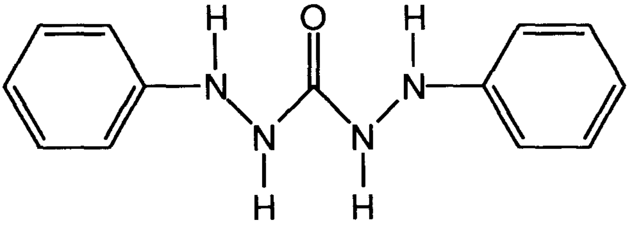
FIGURE 34

Contours from Grid for the amine probe. The black contours are where an amine group would like to be placed. The energy range for this probe is from -25.3 to 5.0 Kcal. and these contours are at -15.0 Kcal..

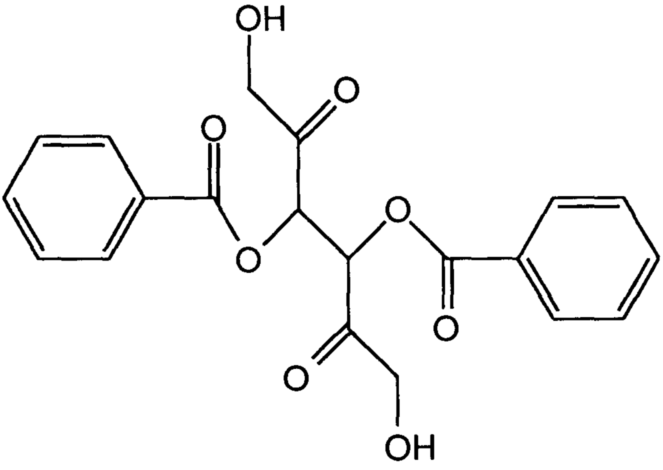


Results of Database Docking

Using contact or forcefield scoring, the Dock program placed a number of molecules in the α99 deoxyhemoglobin site. Most molecules were not of interest since they were too large in size or very toxic. Two molecules that were of interest are **15** and **16**, both of which came from the ACD database, are shown below. **16** was thought to be too large to fit into the site of interaction, however it suggested a molecule **17** similar to the diaspirin molecule with the ester orientation reversed compared to DBSF. One potential problem with **17** is the possible stability of the molecule - would it react before it reaches the desired

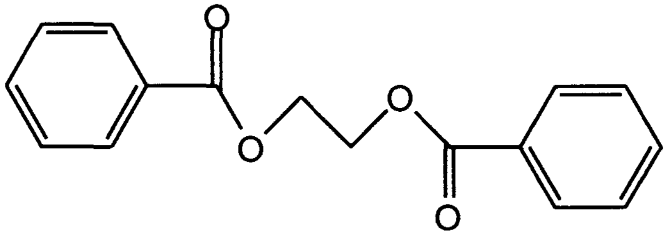


15



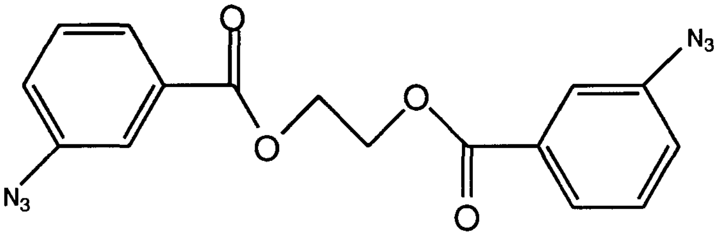
16

crosslinking site? Despite this potential problem, **17** was Docked into the α99 deoxyhemoglobin site.

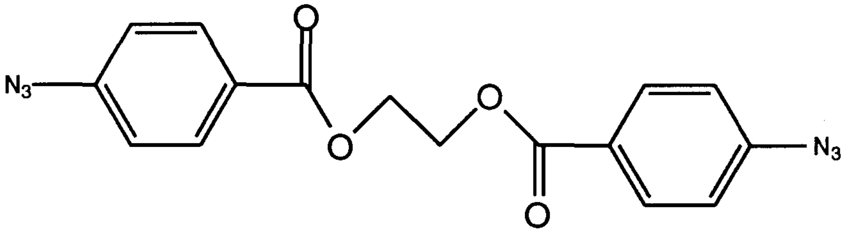


17

Azide groups were added in either the meta and para positions on both of the phenyl rings, thus creating two molecules to be examined, **18** and **19**. 150 conformations were generated with Dgeom for each of **18** and **19.** We attempted to minimize these conformations with Chem-X. However, the azide group could not be minimized in Chem-X because there were no parameters available, therefore, an isocyanate (NCO) was substituted in its place, and all conformations were minimized. The conformations with the isocyanate substituent were loaded into Sybyl and Gasteiger-Huckel charges were placed on each conformation. These conformations for each molecule were written out into a mol2 file and then converted into a file that was converted to a Dock database. The Dock program was run for both molecules.



18



19

Both Dock runs found a small number of conformations of the ligand which might interact in the α99 site of deoxyhemoglobin. But overall, it didn't seem that either molecule would have a driving force which would help to direct these molecules into the α99 site or keep it at that site. Therefore, these molecules were not pursued any further.

Ludi

Ludi[58, 59] is a program, incorporated into Insight, which is designed for use in *de novo* design of new ligands for a protein. There are three steps in using Ludi: (1) calculation of *interaction sites* within the area of the protein that is chosen to be the binding site, (2) a search in the fragment library for pieces that will fit the protein site and (3) attaching or linking these fragments to form a new ligand. Each of these steps will be described in more detail.

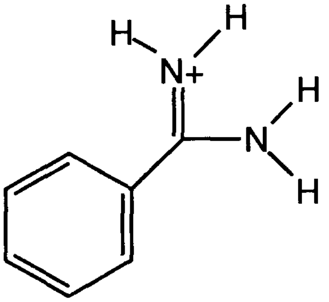
The polar atoms of the protein can form favorable hydrogen-bonds from acceptors and donors positioned within a small area near each of these polar atoms. The possible locations of these acceptors and donors within these areas are the interaction sites. The X-ray crystal structure for small molecules revealed that there was not just one specific point for hydrogen bonding to occur, but rather a range of interaction points. There are four types of interaction sites that are created by Ludi: (1) H-donor, (2) H-acceptor, (3) lipophilic-aliphatic and (4) lipophilic-aromatic. Hydrogen bond interactions are represented by the H-donor and acceptor, while hydrophobic interactions are represented by the remaining two types of interactions.

The second step in this process is fragment fitting. Ludi contains a library of fragments through which it searches to find those that can interact at the interaction sites. The fragments are chosen based upon the square of the distances between the atoms of the fragments and the interaction sites.[60] Once a fragment meets these criteria, Ludi performs a root mean square superposition using the Kabsch algorithm.[60] A fragment is acceptable when the RMS value is within user specifications (usually 0.2 Å to 0.6 Å). Ludi also ensures that there are no steric or repulsive electrostatic interactions between the fragment and the protein. Once the fragment has passed these requirements, the coordinates are then stored in PDB format to be recalled later.

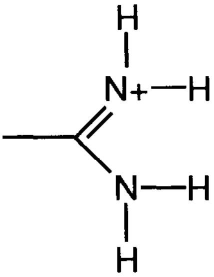
The final step in this procedure is the linking of the accepted fragments together or onto an already existing molecule. Ludi can position a fragment onto a hydrogen of the core fragment in an orientation that would be favorable for a bond to be made. A modified version of Ludi called Autobuild[61] can pick one acceptable fragment at random and make a bond. The number of fragments to be attached can be specified, varying from one to three. The program is iterative; a fragment can be added or built on to a ligand and the process repeated.

Ludi and Dock Experimentation

A subset of the deoxyhemoglobin protein was used in the Ludi run. The $\alpha 99$ region was of interest, therefore a subset of the protein, 20 Å from one of the α99 lysine nitrogens, was created. Ludi searched within a radius of 12 Å from this same α99 lysine atom. Fragments with an RMS of 0.3 Å or less were accepted. The search produced 89 possible fragments that fit within this area. Two fragments, **20** and **21**, were of interest because multiple hydrogen bonds were made with the protein.

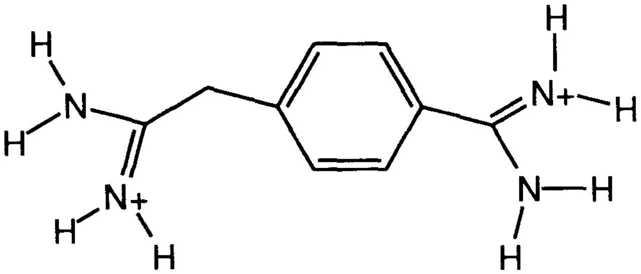


20



21

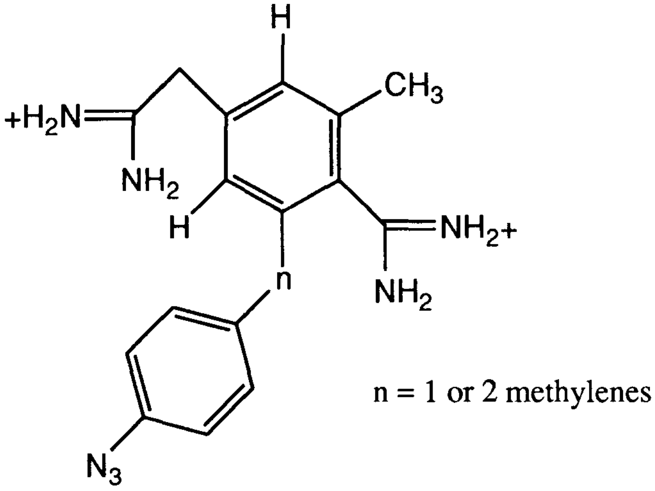
20 made hydrogen bonds to aspartate C126 and tyrosine D35 and **21** had hydrogen bonds to glutamate D101 or aspartate D94. These fragments were then connected by a methylene group creating **22** and the molecule was minimized to remove any steric repulsions within the α99 site. 200 conformations of the bisamidine, **22,** were generated with Dgeom and minimized in Chem-X. All



22

200 conformations were added to a Sybyl database and Gasteiger-Huckel charges were calculated for each conformation. These were all written into a mol2 file and converted into a Dock database. A Dock run was then submitted where the bisamidine database was used to place the molecule in the protein binding site.

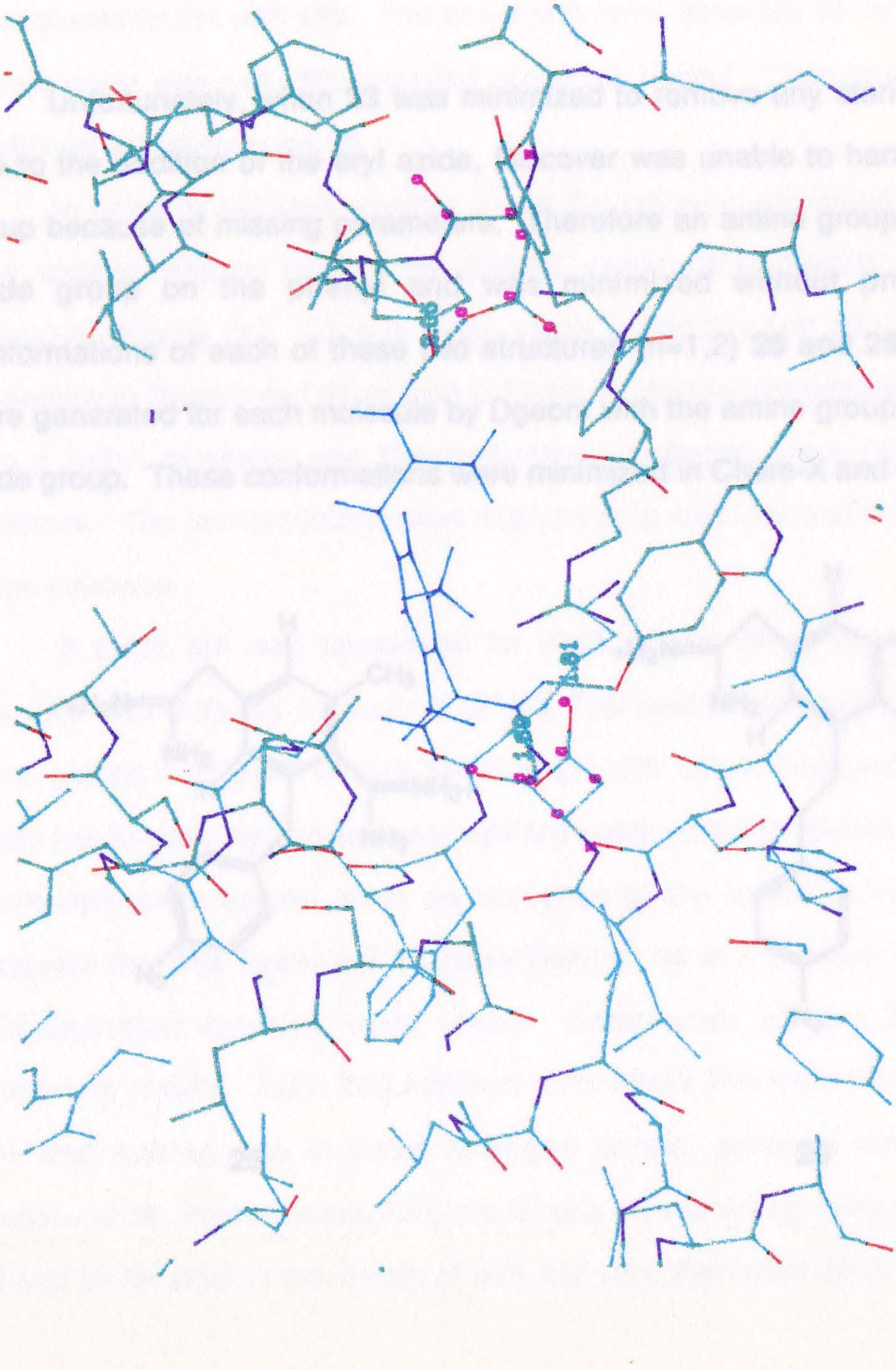
The results of this Dock run were very encouraging since most of the conformations were placed into the α99 site making many of the hydrogen bonds bisamidine so that photochemically-induced crosslinking would occur. After reviewing the literature,[62-65] it was decided that an aryl azide would be a good candidate. The aryl azide was added onto the ligand along with a methyl group which was to represent a second crosslinking portion to be added later to the bisamidine **23**. Gasteiger-Huckel charges were assigned to the ligand. Insight could not assign charges to the azide group. With the help of Dr. Tetsuro Oie, appropriate charges for an aryl azide were devised as shown on **24**.

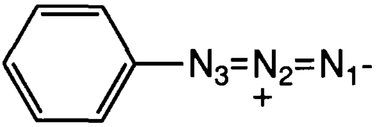


23

FIGURE 35

Dock of bisamide portion into the deoxyhemoglobin α99 site. The ligand is forming hydrogen bonds to the aspartic acid (2.05 Å) on one side and an aspartic acid (1.69 Å) and tyrosine residue (1.81 Å) on the other side.

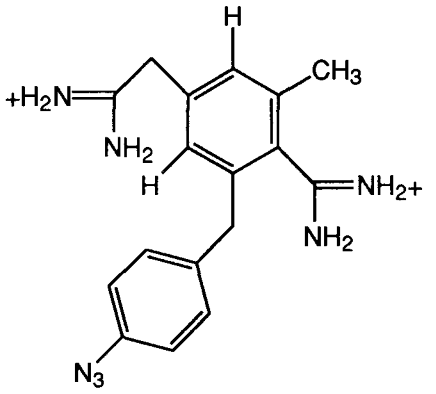

1.91

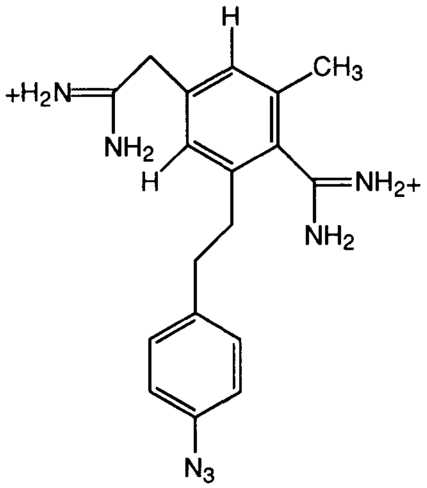


Charges on the nitrogens are as follows: $N_1 = -0.213$, $N_2 = 0.091$, and $N_3 = -0.036$.

24

Unfortunately, when **23** was minimized to remove any steric interactions due to the addition of the aryl azide, Discover was unable to handle the azide group because of missing parameters. Therefore an amine group replaced the azide group on the phenyl and was minimized without problem. 200 conformations of each of these two structures (n=1,2) **25** and **26** respectively, were generated for each molecule by Dgeom with the amine group replacing the azide group. These conformations were minimized in Chem-X and loaded into a





25

26

Sybyl database where Gasteiger-Huckel charges were added. The conformations were written out into two separate mol2 files and converted into files that could be read by Insight. The Dock program was used to determine whether the compounds would fit into the α99 site.

The results from these Dock runs showed that a number of conformations were placed in the α99 site. The major difference between **25** and **26** was that the molecule with n=2 (**26**) extended out of the pocket. Therefore, further work continued with compound **25**.

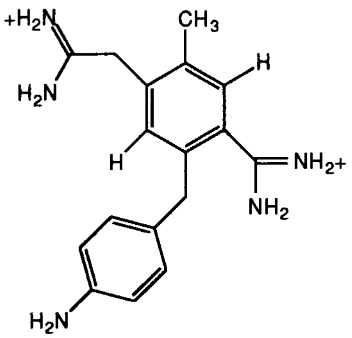
In order to examine whether this is the optimum placement of the azide, and amidine groups around the phenyl, seven other related ligands were examined (Figure 36). Each of these seven molecules had 200 conformations generated in Dgeom and minimized in Chem-X. All the conformations were input into a Sybyl database and Gasteiger-Huckel charges were placed on each molecule. The conformations were output into a mol2 file and converted into a Dock database.

A Dock run was completed for each of the seven compounds. The outcome was different for each of **27-33**. The best Dock results were from **30**. Dock placed this ligand several times in the α99 site of deoxyhemoglobin, and made two to three hydrogen bonds with the residues within the site. This result is particularly encouraging since an attraction of the ligand to the binding site suggests that this ligand will be more likely to be in the active site where the photoactivated crosslink could occur. Compounds **27** and **31** also gave interesting results. Each had some conformations that were placed in the α99 site and making one to three hydrogen bonds, although not as many as compound **30.** Nevertheless, they would also be interesting molecules to pursue. **32** and **33** did dock in the vicinity of α99, but very often were docked sideways or

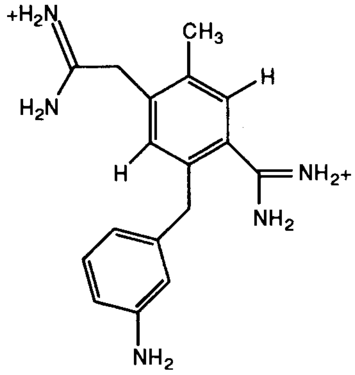
FIGURE 36

7 substituted bisamidines docked into the deoxyhemoglobin $\alpha99$ site.

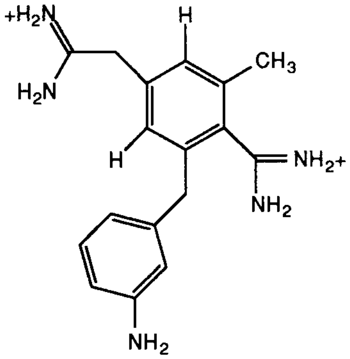
Figure 36


+H2N
CH3
H
H2N
H
NH2+
NH2
H2N

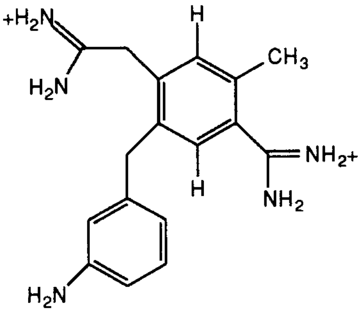
27


+H2N
CH3
H
H2N
H
NH2+
NH2
NH2

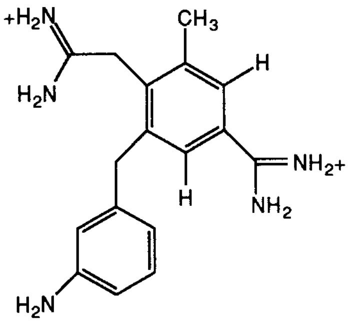
28


+H2N
H
CH3
H2N
H
NH2+
NH2
NH2

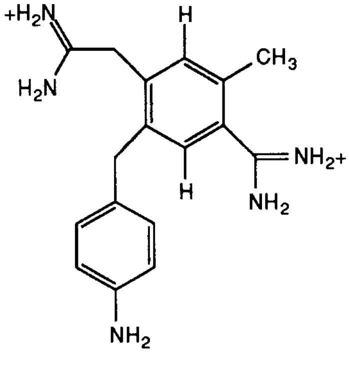
29


+H2N
H
CH3
H2N
NH2+
H
NH2
H2N

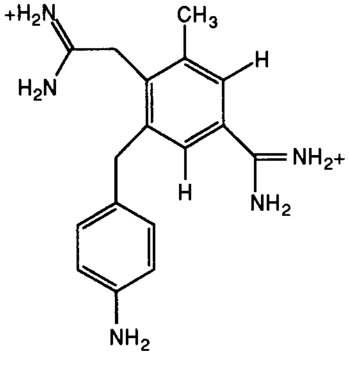
30


+H2N
CH3
H
H2N
NH2+
H
NH2
H2N

31


+H2N
H
CH3
H2N
NH2+
H
NH2
NH2

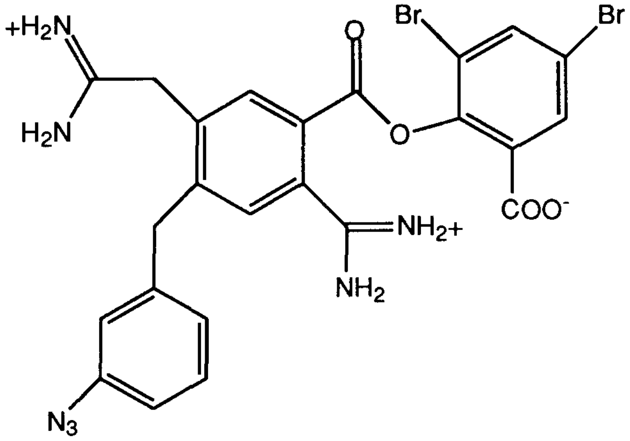
32


+H2N
CH3
H
H2N
NH2+
H
NH2
NH2

33

backward with the aryl azide group in toward the center of the cavity rather than rotated 180°. **27** and **28** had some well-placed conformations but they were not making the desired hydrogen bonding interactions.

The last step of the *de novo* design was to take a conformation of **30** and add the dibromosalicyl portion to form the new crosslinker **34.** In order to obtain the best assurance that **34** would bind in the α99 deoxyhemoglobin site, 200 conformations were generated and minimized using Dgeom and Chem-X respectively, with an amine group in place of the azide for the minimization. These conformations were loaded into a Sybyl database and the amine group was changed to an azide group. For each of these 200 conformations, the azide group was rotated 180° and this conformation was also stored in the database. Therefore, the database contained 400 conformations of the ligand. Gasteiger-Huckel charges were assigned for the atoms of each conformation and all conformations were written into a mol2 file. This mol2 file was converted into a Dock database and Dock was run.

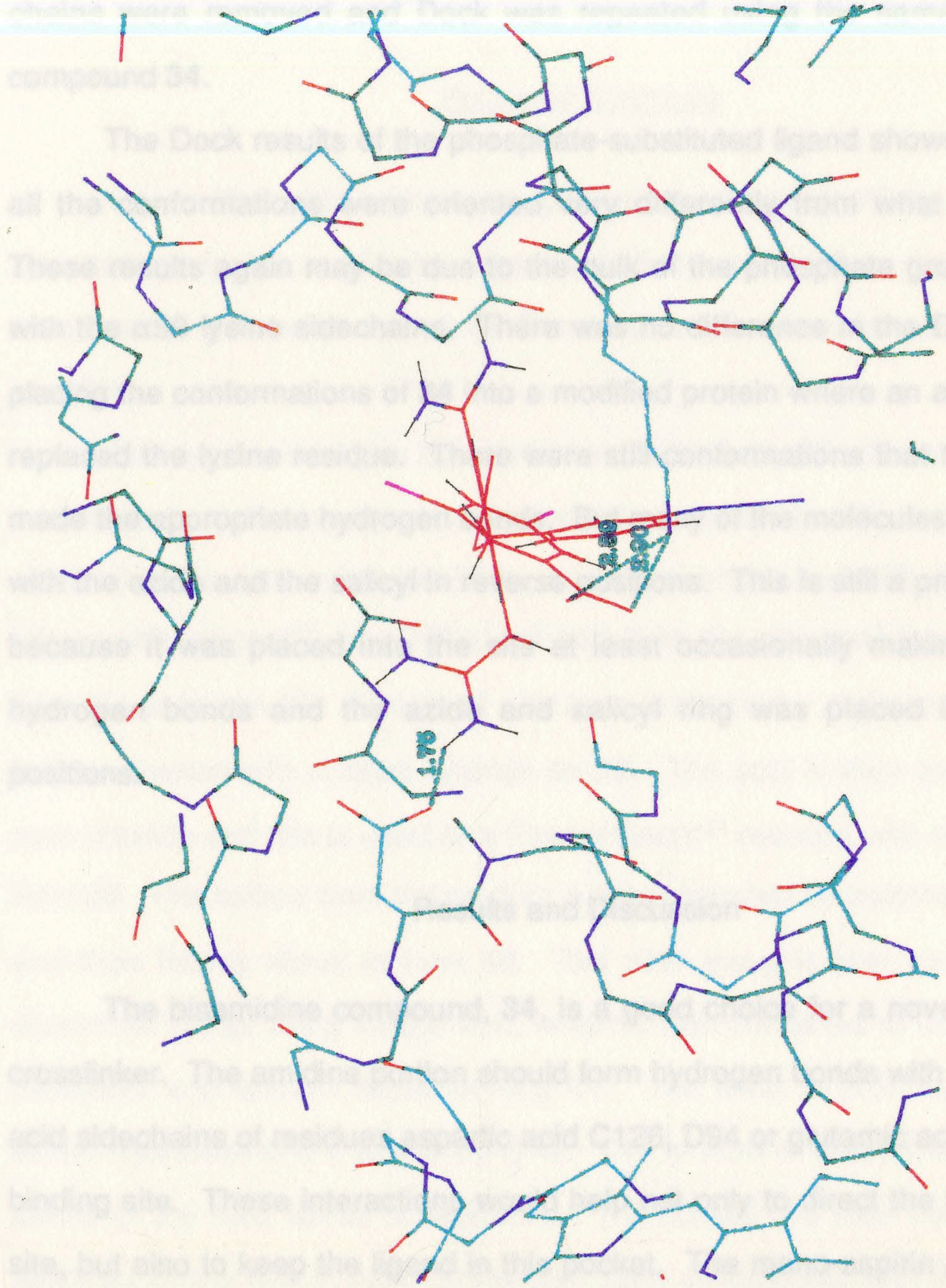


34

There were some interesting results in which ligand **34** was oriented in the protein site as had been designed (Figure 37). But there were many conformations in which the ligand was oriented reversing the positions of

FIGURE 37

Docking of proposed molecule **34** into the α99 deoxyhemoglobin site.


2.60
2.90
1.76

the azide and the salicyl groups. This could be due to the size of the salicyl portion negatively interacting with the α99 lysine side chains. Therefore, two things were modified: first the mono aspirin was replaced with a phosphate group and the procedure repeated with this compound. And secondly, since the lysine sidechains can move and we are dealing with a rigid protein site, the α99 lysine chains were removed and Dock was repeated using the same database of compound **34**.

The Dock results of the phosphate-substituted ligand showed that almost all the conformations were oriented very differently from what was desired. These results again may be due to the bulk of the phosphate group interacting with the α99 lysine sidechains. There was no difference in the Dock results of placing the conformations of **34** into a modified protein where an alanine residue replaced the lysine residue. There were still conformations that fit the site and made the appropriate hydrogen bonds. But many of the molecules were oriented with the azide and the salicyl in reverse positions. This is still a promising ligand because it was placed into the site at least occasionally making the correct hydrogen bonds and the azide and salicyl ring was placed in the correct positions.

Results and Discussion

The bisamidine compound, **34**, is a good choice for a novel photoaffinity crosslinker. The amidine portion should form hydrogen bonds with the carboxylic acid sidechains of residues aspartic acid C126, D94 or glutamic acid D101 in the binding site. These interactions would help not only to direct the ligand into the site, but also to keep the ligand in this pocket. The mono-aspirin portion should crosslink with an α99 lysine residue. Then, with irradiation, the ligand should crosslink with the residues near by. This crosslinker has the unique combination of two different methods of forming covalent bonds between the ligand and the protein. This molecule, which has been designed to interact specifically with one site of hemoglobin, represents an attempt to use the methods of molecular modeling to suggest new compounds to help develop a better blood substitute.

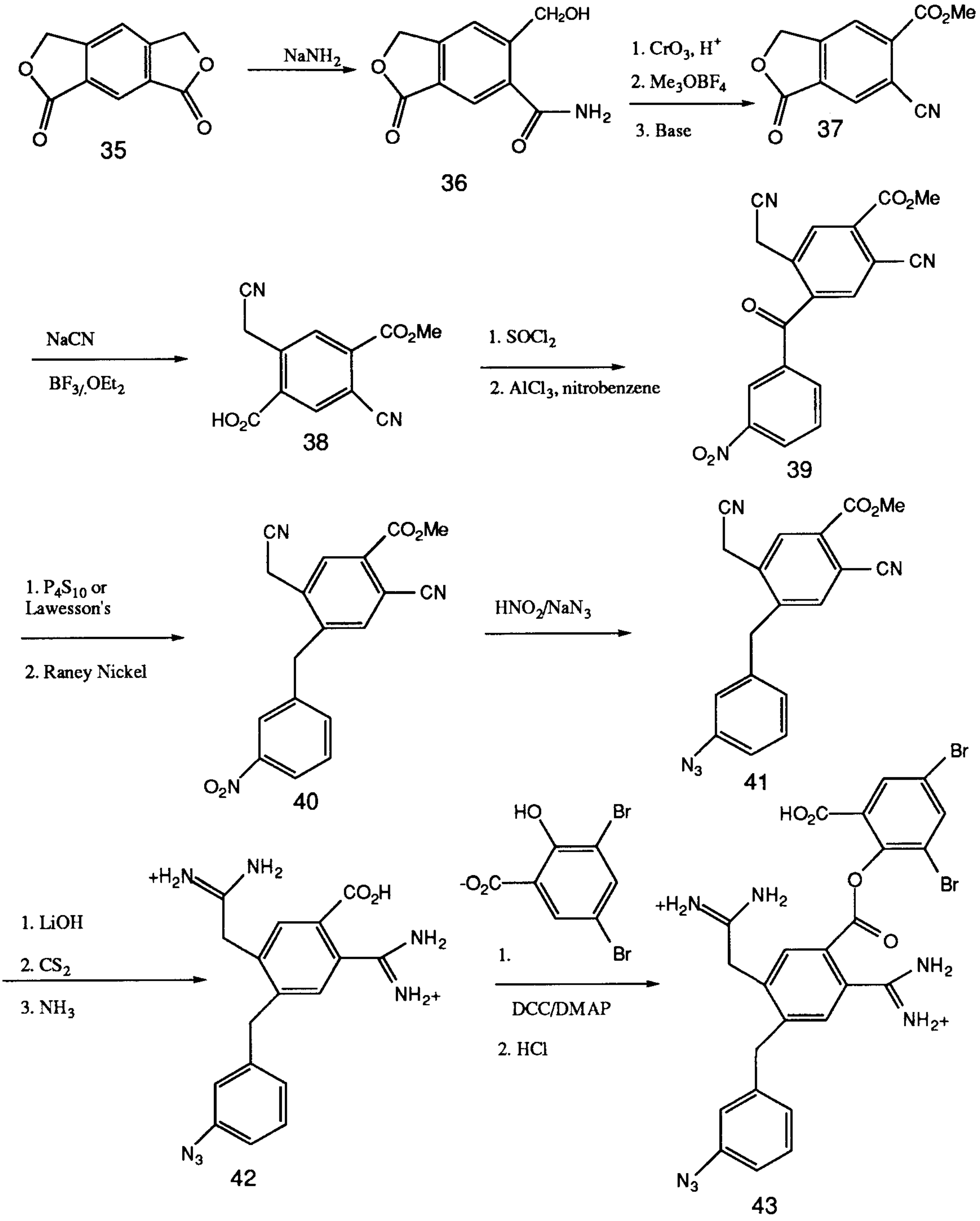
Proposed Synthesis

Figure 38 depicts a proposed synthesis for the photoaffinity crosslinker **34**. The strategy for the synthesis of the bisamidine is to introduce the most reactive functionality, the phenyl ester, last. The amidine groups are introduced as late as possible.

The synthesis begins by reaction of the bisester **35** with one equivalent of sodamide in order to prepare only the monoamide **36**. The benzyl alcohol is oxidized to the carboxylic acid **37** by acidic chromium trioxide[66]. Treatment with Meerwein's reagent[67] (Me_3OBF_4) will both form the ester and the methyl imidate from the amide on **37**. Treatment with base will form the nitrile on **37**. The ester is then opened with sodium cyanide on **38**. The acid is then converted to the acid chloride and this is used in a Friedel-Crafts[68] reaction with nitrobenzene to form **39**. The ketone from the product is deoxygenated by treatment with P_4S_{10} and then Raney nickel to form **40**. The nitro group is then converted to the diazonium group, using nitrous acid, which is then converted to the aryl azide by treatment with sodium azide forming **41**. The ester is then hydrolyzed with lithium hydroxide to the acid forming **42**. This is done to prevent formation of the amide in the next series of reactions. The nitriles are then converted to the amidines by any one of many techniques, such as treatment with carbon disulfide and ammonia to give compound **42**. The remaining task is to form the phenyl ester from dibromosalicylic acid and the benzoic acid using dicyclohexylcarbodiimide and dimethylaminepyridine as catalyst forming **43**. The salicylic acid may need prior protection as the tert-butyl ester. Treatment with hydrochloric acid should cleave the acid-sensitive t-butyl ester and form the bisamide dihyrochloride **43**.

FIGURE 38

Proposed Synthesis of Photoaffinity Crosslinker 34


35
NaNH2
36
CH2OH
NH2
1. CrO3, H+
2. Me3OBF4
3. Base
37
CO2Me
CN
NaCN
BF3/.OEt2
38
HO2C
1. SOCl2
2. AlCl3, nitrobenzene
39
O2N
1. P4S10 or Lawesson's
2. Raney Nickel
40
HNO2/NaN3
41
N3
1. LiOH
2. CS2
3. NH3
42
+H2N
NH2+
CO2H
HO
Br
-O2C
1.
DCC/DMAP
2. HCl
43

CHAPTER 6

CONCLUSION

Chapter 2 examined the binding of a crosslinker, DBSF, at the active site of hemoglobin. A complete conformational search and minimization of DBSF was completed in order to better understand the types of conformations this molecule can achieve. Secondly, the sites where crosslinking is known, or thought to occur, in both deoxy and oxyhemoglobin were examined using minimization experiments of DBSF bound within the active sites of these proteins. In these minimization experiments, only the bound DBSF was allowed to move. It was through these experiments that an understanding was achieved of why crosslinking occurs at the $\beta_1 82$-$\beta_2 82$ site of oxyhemoglobin and the $\alpha_1 99$-$\alpha_2 99$ site of deoxyhemoglobin. It was also concluded that the $\beta_1 82$-$\beta_2 82$ site in deoxyhemoglobin may be the secondary crosslinking site.

Another experiment involved the minimization of the whole protein of both oxy and deoxyhemoglobin while crosslinked with DBSF. This experiment allowed the sidechain residues of the protein to move together with the bound DBSF. The results of this experiment showed that the α helices do not change greatly, however the loop regions change significantly. This is consistent with preliminary X-ray crystallographic results on deoxy $\beta 82$ crosslinked hemoglobin.

The molecular dynamics experiments of chapter four examined the movement of a crosslinker bound to one lysine residue in oxyhemoglobin, but it also suggested a possible mode of interaction. Several modifications of the molecular dynamics experiments were made, such as lengthening the number of iterations of the experiment, changing the charges on the interacting lysines, and enlarging the subset so as to allow more residues to move. These experiments suggested a probable two step mechanism of crosslinking: 1) one side of DBSF binds to one β82 lysine, and 2) then the other β82 lysine comes in close contact with the remaining salicyl group because of the electrostatic interactions between the acid group on DBSF and the amino group of the lysine, to complete the crosslinking. Therefore, the molecular dynamics experiments demonstrated what may be happening in the protein in a more realistic manner rather than observing a static protein.

The last portion of this thesis was devoted to developing a new photoaffinity crosslinker for hemoglobin. This was accomplished using a variety of commercially available software programs. The initial problem was to find a program which could reproduce the binding of a known ligand in hemoglobin. This was accomplished using the Dock program with bezafibrate analogues being placed into the α99 site of deoxyhemoglobin. The procedure to develop a new crosslinker involved the use of such programs as Grid and Ludi. After the new ligand was developed, it was docked into the deoxy α99 site to examine the feasibility of binding in this site. The ligand was placed in the proposed site several times making the strong hydrogen bonds as initially proposed. This concluded an attempt to develop a new photoaffinity crosslinker for hemoglobin using molecular modeling techniques.

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