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**High-resolution X-ray structures of myoglobin- and
hemoglobin-alkyl isocyanide complexes**

Johnson, Kenneth Alan, Ph.D.

Rice University, 1993

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RICE UNIVERSITY

HIGH RESOLUTION X-RAY STRUCTURES OF MYOGLOBIN-
AND HEMOGLOBIN-ALKYL ISOCYANIDE COMPLEXES

by

KENNETH A. JOHNSON

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

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ABSTRACT

High resolution X-ray structures of myoglobin- and
hemoglobin-alkyl isocyanide complexes.

by

Kenneth A. Johnson

The structures of sperm whale myoglobin (Mb) and human hemoglobin (Hb) complexed with methyl, ethyl, n-propyl and n-butyl isocyanide (MNC, ENC, nPNC and nBNC, respectively) were determined by X-ray crystallography. The polar isocyano head groups of the alkyl isocyanides (RNC's) have similar affinities for heme iron, whereas the size and stereochemistry of the alkyl groups cause the different RNC ligands to cross varied steric barriers when entering or exiting the protein. Four Mb structures were determined at a resolution <2.0 Å: MbENC at a pH <6.0 and MbMNC, nPNC and nBNC at pH 7.0. In addition, MbENC data at pH 7.0 were collected to 2.0 Å. The low pH of the initial MbENC structure led to the discovery in ferrous myoglobin of a two-state histidine swinging gate for ligand entry and exit from the heme pocket. At neutral pH and with diatomic ligands (O_2 and CO), the side chain of His-E7 blocks ligand entry and exit from the heme pocket. At low pH, presumably by protonation of the histidyl ring, and also with triatomic or larger ligands bound, the histidyl ring can occupy a second stable conformation reached by a $>100^\circ$ rotation about its C_α - C_β bond. Relatively immobile side chains of amino acids in the heme pocket allowed the alkyl groups of MNC and ENC to lie in the hydrophobic interior of the heme pocket. The larger alkyl groups of nPNC and nBNC

projected into the hydrophilic exterior entrance to the heme pocket.

Data for the structures of Hb complexed with MNC, ENC, nPNC and nBNC (pH 6.7) were collected to $\sim 2.2\text{\AA}$. In α subunits, the alkyl moieties of the ligands lay approximately parallel to the heme plane. In β subunits, the alkyl groups lay at about a 45° angle from the heme plane. The alkyl groups of the ligands occupied the hydrophobic interior of the heme pocket in both subunits. Little disorder in His-E7 was observed in either subunit.

Ligand binding causes greater tertiary structure changes in the α and β subunits than in myoglobin. These changes in tertiary structure in response to isocyanide binding appear to cause the α and β subunits to have more of a T-state tertiary conformation than does the oxy complex, and may explain the lesser cooperative effect seen in isocyanide binding compared to oxygen binding.

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Emerson writes in Self Reliance: “The voyage of the best ship is a zigzag line of a hundred tacks. See the line from a sufficient distance, and it straightens itself to the average tendency.” The success of my journey through graduate school depended on the many who viewed my progress from a distance.

Dr. George Phillips, Jr., patiently instructed me in the art and practice of X-ray crystallography. He generously taught me how to collect data on a succession of diffractometers in his laboratory and arranged for me to use the area detector in Dr. Florante Quiocho’s laboratory at The Howard Hughes Medical Institute at Baylor College of Medicine. From Dr. Phillips, I’ve acquired a healthy skepticism concerning the analysis of X-ray crystal structures.

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Chapter 1.

Introduction

1.1. Physiological role of myoglobins and hemoglobins.

Myoglobins, hemoglobins and related heme proteins exhibit diverse functions. Myoglobin is a small globular protein (17.8 kD) that contains one bound prosthetic group, protoheme IX, and functions as the oxygen storage protein in vertebrate muscle. In more primitive organisms, myoglobins serve as transporters of oxygen and other metabolites. For example, the giant clam *Lucina pectinata* forms a symbiotic relationship with bacteria living in its gills. An unusual sulfide-reactive myoglobin is postulated to transport H₂S from the clam to the symbiotic bacteria (Kraus and Wittenberg, 1990). Leghemoglobin, found in plant root nodules, is thought both to scavenge oxygen to protect the nitrogen-fixing enzyme system from irreversible oxidation and to facilitate the diffusion of small amounts of oxygen for basal metabolism in the bacteroid mitochondria (Dickerson and Geis, 1983). Since all these myoglobins contain only protoheme IX as a cofactor, the physiological function of a particular myoglobin is governed solely by the protein structure surrounding the heme pocket.

Hemoglobin is the oxygen transport protein in vertebrate red blood cells. It is a tetramer composed of two heterodimers containing alpha (α) and beta (β) chains. Each subunit contains one protoheme IX and binds one dioxygen molecule. The intact tetramer exhibits cooperative binding of O₂ which facilitates oxygen release in capillaries surrounded by respiring tissue and uptake in alveolar capillaries.

1.2. Tertiary structure of myoglobin and hemoglobin.

For the first X-ray structures of proteins, those of horse hemoglobin (Fe III with bound water) (Perutz *et al.*, 1960) and sperm whale myoglobin (Fe III with bound water) (Kendrew *et al.*, 1960), Perutz and Kendrew received the Nobel prize in Chemistry in 1960. Their work described the basic tertiary structure of myoglobin and the α and β subunits of hemoglobin (Fig. 1.1a-c). The “myoglobin fold” is an arrangement of two terminal regions (NA and HC), 8 internal α -helices (A-H) and 7 short interspersed non-helical regions (AB, BC ... GH). This folding pattern creates a hydrophobic pocket for the binding of protoheme IX. All known myoglobins, and the subunits of multimeric hemoglobins in both the plant and animal kingdoms, exhibit this folding pattern (Riggs, 1991). The α subunits of tetrameric vertebrate hemoglobins are somewhat unique in that they lack the short D helix (4-5 residues). However, this loss of the D helix appears to be a functionally neutral deletion. In an engineered human hemoglobin in which the D-helix was removed from β -chains, the protein exhibited almost identical oxygen binding properties to that of the native protein (Komiyama *et al.*, 1991).

Two nomenclatures for amino acids in myoglobins and hemoglobins are commonly used. In the first, amino acids are named according to helical position (Fig. 1.2). Thus, His-E7 is the histidine residue at the seventh position in the E helix. This nomenclature allows the comparisons between globins of different lengths and non-homologous sequences. In the second system, amino acids are identified according to position in polypeptide chain. For example, His-E7 would also be designated His⁶⁴ in most myoglobins because it is the 64th residue from the N-terminus.

Figure 1.1a. Ribbon depiction of the tertiary structure of sperm whale myoglobin showing the eight helices (A-H), the heme group, the distal residues His-E7 and Phe-CD1, and the proximal His-F8. The heme fits between the E and F helices and is bound to the protein by a bond between the iron atom and the N ϵ of the distal histidine.

Figure 1.1b. Ribbon depiction of the tertiary structure of the α subunit of human oxyhemoglobin showing the seven helices (A-C,E-H), the heme group, the distal residues His-E7 and Phe-CD1, and the proximal His-F8. The α subunit lacks the short D helix found in most globins. The H helix is much shorter than in myoglobin.

Figure 1.1c. Ribbon depiction of the tertiary structure of the β subunit of human oxyhemoglobin showing the eight helices (A-H), the heme group, the distal residues His-E7 and Phe-CD1, and the proximal His-F8. The heme group is less planar than in oxymyoglobin and oxy α subunits.

			A1	A2	A3	A4	A5	A6	A7	A8	A9
1 VAL		2 LEU	3 SER	4 GLU	5 GLY	6 GLU	7 TRP	8 GLN	9 LEU	10 VAL	11 LEU
1 VAL		2 LEU	3 SER	4 PRO	5 ALA	6 ASP	7 LYS	8 THR	9 ASN	10 VAL	11 LYS
1 VAL	2 HIS	3 LEU	4 THR	5 PRO	6 GLU	7 GLU	8 LYS	9 SER	10 ALA	11 VAL	12 THR
A10	A11	A12	A13	A14	A15	A16		B1	B2	B3	B4
12 HIS	13 VAL	14 TRP	15 ALA	16 LYS	17 VAL	18 GLU	19 ALA	20 ASP	21 VAL	22 ALA	23 GLY
12 ALA	13 ALA	14 TRP	15 GLY	16 LYS	17 VAL	18 GLY	19 ALA	20 HIS	21 ALA	22 GLY	23 GLU
13 ALA	14 LEU	15 TRP	16 GLY	17 LYS	18 VAL			19 ASN	20 VAL	21 ASP	22 GLU
B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16
24 HIS	25 GLY	26 GLN	27 ASP	28 ILE	29 LEU	30 ILE	31 ARG	32 LEU	33 PHE	34 LYS	35 SER
24 TYR	25 GLY	26 ALA	27 GLU	28 ALA	29 LEU	30 GLU	31 ARG	32 MET	33 PHE	34 LEU	35 SER
23 VAL	24 GLY	25 GLY	26 GLU	27 ALA	28 LEU	29 GLY	30 ARG	31 LEU	32 LEU	33 VAL	34 VAL
C1	C2	C3	C4	C5	C6	C7					
36 HIS	37 PRO	38 GLU	39 THR	40 LEU	41 GLU	42 LYS	43 PHE	44 ASP	45 ARG	46 PHE	47 LYS
36 PHE	37 PRO	38 THR	39 THR	40 LYS	41 THR	42 TYR	43 PHE	44 PRO	45 HIS	46 PHE	47 ASP
35 TYR	36 PRO	37 TRP	38 THR	39 GLN	40 ARG	41 PHE	42 PHE	43 GLU	44 SER	45 PHE	46 GLY
			D1	D2	D3	D4	D5	D6	D7	E1	E2
48 HIS	49 LEU	50 LYS	51 THR	52 GLU	53 ALA	54 GLU	55 MET	56 LYS	57 ALA	58 SER	59 GLU
48 LEU	49 SER		50 HIS						51 GLY	52 SER	53 ALA
47 ASP	48 LEU	49 SER	50 THR	51 PRO	52 ASP	53 ALA	54 VAL	55 MET	56 GLY	57 ASN	58 PRO
E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14
60 ASP	61 LEU	62 LYS	63 LYS	64 HIS	65 GLY	66 VAL	67 THR	68 VAL	69 LEU	70 THR	71 ALA
54 GLN	55 VAL	56 LYS	57 GLY	58 HIS	59 GLY	60 LYS	61 LYS	62 VAL	63 ALA	64 ASP	65 ALA
59 LYS	60 VAL	61 LYS	62 ALA	63 HIS	64 GLY	65 LYS	66 LYS	67 VAL	68 LEU	69 GLY	70 ALA
E15	E16	E17	E18	E19	E20						
72 LEU	73 GLY	74 ALA	75 ILE	76 LEU	77 LYS	78 LYS	79 LYS	80 GLY	81 HIS	82 HIS	83 GLU
66 LEU	67 THR	68 ASN	69 ALA	70 VAL	71 ALA	72 HIS	73 VAL	74 ASP	75 ASP	76 MET	77 PRO
71 PHE	72 SER	73 ASP	74 GLY	75 LEU	76 ALA	77 HIS	78 LEU	79 ASP	80 ASN	81 LEU	82 LYS
		F1	F2	F3	F4	F5	F6	F7	F8	F9	FG1
84 ALA	85 GLU	86 LEU	87 LYS	88 PRO	89 LEU	90 ALA	91 GLN	92 SER	93 HIS	94 ALA	95 THR
78 ASN	79 ALA	80 LEU	81 SER	82 ALA	83 LEU	84 SER	85 ASP	86 LEU	87 HIS	88 ALA	89 HIS
83 GLY	84 THR	85 PHE	86 ALA	87 THR	88 LEU	89 SER	90 GLU	91 LEU	92 HIS	93 CYS	94 ASP

Figure 1.2. Sequence alignment of sperm whale myoglobin (1st row) and the α and β subunits (2nd and 3rd rows, respectively) of human hemoglobin (according to Dickerson and Geis, 1983, except for residues H23 through H26 of myoglobin which are listed as helical residues in the Protein Data Bank file for oxy and deoxy myoglobin).(continued on next page).

				G1	G2	G3	G4	G5	G6	G7	G8
96LYS	97 HIS	98 LYS	99 ILE	100 PRO	101 ILE	102 LYS	103 TYR	104 LEU	105 GLU	106 PHE	107 ILE
90 LYS	91 LEU	92 ARG	93 VAL	94 ASP	95 PRO	96 VAL	97 ASN	98 PHE	99 LYS	100 LEU	101 LEU
95 LYS	96 LEU	97 HIS	98 VAL	99 ASP	100 PRO	101 GLU	102 ASN	103 PHE	104 ARG	105 LEU	106 LEU
G9	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	GH1
108SER	109 GLU	110 ALA	111 ILE	112 ILE	113 HIS	114 VAL	115 LEU	116 HIS	117 SER	118 ARG	119 HIS
102SER	103 HIS	104 CYS	05 LEU	106 LEU	107 VAL	108 THR	109 LEU	110 ALA	111 ALA	112 HIS	113 LEU
107GLY	108 ASN	109 VAL	110 LEU	111 VAL	112 CYS	113 VAL	114 LEU	115 ALA	116 HIS	117 HIS	118 PHE
				H1	H2	H3	H4	H5	H6	H7	H8
120PRO	121 GLY	122 ASP	123 PHE	124 GLY	125 ALA	126 ASP	127 ALA	128 GLN	129 GLY	130 ALA	131 MET
114PRO	115 ALA	116 GLU	117 PHE	118 THR	119 PRO	120 ALA	121 VAL	122 HIS	123 ALA	124 SER	125 LEU
119GLY	120 LYS	121 GLU	122 PHE	123 THR	124 PRO	125 PRO	126 VAL	127 GLN	128 ALA	129 ALA	130 TYR
H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20
132ASN	133 LYS	134 ALA	135 LEU	136 GLU	137 LEU	138 PHE	139 ARG	140 LYS	141 ASP	142 ILE	143 ALA
126ASP	127 LYS	128 PHE	129 LEU	130 ALA	131 SER	132 VAL	133 SER	134 THR	135 VAL	136 LEU	137 THR
131GLN	132 LYS	133 VAL	134 VAL	135 ALA	136 GLY	137 VAL	138 ALA	139 ASN	140 ALA	141 LEU	142 ALA
H21	H22	H23	H24	H25	H26	HC1					
144ALA	145 LYS	146 TYR	147 LYS	148 GLU	149 LEU	150 GLY	151 TYR	152 GLN	153 GLY		
138SER	139 LYS						140 TYR	141 HIS			
143HIS	144 LYS						145 TYR	146 HIS			

Figure 1.2. (continued).

The heme is bound tightly between the E and F helices and forms a coordination bond to the N_ε of His-F8. Several highly conserved amino acids lie near the heme and are divided into proximal residues which are located on the His-F8 side of the heme and distal residues on ligand binding side (Fig. 1.3). An examination of the heme pocket structure reveals putative roles for these residues. Interior hydrophobic residues orient the heme's vinyl groups to the interior of the protein whereas polar surface residues interact with the propionate groups at the protein surface. Other residues interact with the bound ligand and play a role in regulating rate and affinity constants.

The distal histidine (His-E7) is a key residue in regulating ligand affinity in both myoglobin and hemoglobin. It sterically hinders bound carbon monoxide and can form a hydrogen bond to stabilize bound oxygen (S.E.V. Phillips, 1980; Phillips and Schoenborn, 1981; Mims *et al.*, 1983b; Kuriyan *et al.*, 1986). In myoglobin, this hydrogen bond has been seen by neutron diffraction in oxymyoglobin crystals (Phillips and Schoenborn, 1981). In hemoglobin, the presence of this hydrogen bond is not as certain. In the structure of human oxyhemoglobin (Shaanan, 1982, 1983), the distance between the N_ε of His-E7 and the second oxygen atom suggests the presence of a hydrogen bond in the α -chains but not in β -chains. However, there is some doubt concerning this interpretation. The oxyhemoglobin structure was refined to a model with an R-factor of only 21% and by current standards is still partially refined. At the resolution of 2.1 Å for the oxyhemoglobin X-ray data, the observed electron density for His-E7 and bound dioxygen is probably broad enough to allow a hydrogen bonding geometry in β subunits.

Two features which determine ligand affinity are seen in a comparison of the crystal structures of unliganded and liganded myoglobin and hemoglobin (Perutz,

Figure 1.3. The heme pocket of sperm whale oxymyoglobin showing the heme group and bound oxygen, several distal residues and the proximal histidine.

1970). The first is the movement of the iron atom into and out of the plane of the heme group towards the proximal histidine, His-F8. Hexacoordinate (bis-liganded) heme iron(II) has a low spin ($S=0$) electronic configuration and can fit in the plane of the pyrrole nitrogens. Pentacoordinate (unliganded) heme iron(II) is high spin ($S=2$) and too large to fit in the center of the porphyrin ring. The in-plane iron has more surface area accessible to an incoming ligand and thus a higher reactivity. The X-ray structures of deoxymyoglobin and deoxyhemoglobin show the iron position to be $> 0.3 \text{ \AA}$ below the plane of the heme; liganded structures of myoglobin and hemoglobin show the iron to be within $0.1 \text{ \AA}$ of the heme plane. In addition to the X-ray evidence, there is recent spectroscopic evidence for motion of the iron atom into the plane of the heme during ligand binding. Low temperature flash photolysis of MbCO complexes suggest that deoxymyoglobin exists in conformational substates in which the iron is in or below the plane of the porphyrin. In 'kinetic hole burning' experiments, the near IR band III spectrum of deoxymyoglobin is seen to shift to higher wavelengths as ligand binding occurs due to depletion of longer wavelength components. This red end of band III is assigned to deoxymyoglobin species in which the iron is more in the plane of the porphyrin (Xie *et al.*, 1991). There is an equilibrium between in plane and out of plane iron which appears to have a half-life of about 50 ps at low temperature but is too fast to measure at room temperature.

The second feature which regulates ligand affinity is the rearrangement of protein tertiary structure caused by ligand binding. On the distal side of the heme, the E-helix moves towards the E-F corner and the N_ϵ of the imidazole ring of His-E7 can form a hydrogen bond with bound O_2 but not with CO. On the proximal side of the heme, the F-helix moves in the direction of the F-G corner, being pulled

in this direction by movement of the iron atom into the plane of the heme. In myoglobin, the rms difference between the coordinates for backbone C_{α} atoms in deoxymyoglobin vs oxymyoglobin is $\sim 0.09 \text{ \AA}$. The differences are more than five times this value in both subunits of hemoglobin and cause the switch from the low affinity T quaternary conformation to the high affinity R-state. Thus, in hemoglobin the deoxy tertiary state is highly correlated with quaternary T-state although there is evidence for transition-like subunit conformations within the T-state tetramer which have affinities for ligands that are intermediate to those of completely T- and R- state tetramers. (Daugherty *et al.*, 1991).

1.3. Quaternary changes in hemoglobin.

The high resolution deoxy T-state structure (Fermi *et al.*, 1984) and the carbon monoxide (Baldwin, 1980; Derewenda *et al.*, 1990) and oxy (Shaanan, 1983) R-state structures of human hemoglobin reveal that the α - and β -subunits have distinct T and R tertiary and quaternary conformations. T-state deoxyhemoglobin contains pentacoordinate iron atoms and as a result, the hemes are domed and the iron atom is about $0.4 \text{ \AA}$ below the plane of the four pyrrole nitrogens. The plane of the histidyl ring of His-F8 is off normal to the heme plane. In β -chains, two distal residues, His-E7 and Val-E11, sterically hinder the ligand binding site. In the liganded R-state structures, the hemes are relatively planar with the iron atoms close to the plane of the hemes. The histidyl ring of His-F8 becomes normal to the heme plane by a $1 \text{ \AA}$ movement of the F-helix towards the FG corner. In the β -chains, the side chains of His-E7 and Val-E11 have been pulled away from the ligand binding site by movement of the E-helix. Thus, two types of barriers to ligand binding cause the low affinity of T-state hemoglobin. Ligands are

sterically hindered by the four pyrrole nitrogens because the iron atoms are below the plane of the porphyrin ring in both subunits. In addition, the distal histidine and valine side chains sterically block the ligand binding site in β subunits.

Perutz proposed a series of structural changes which occur upon ligand binding to T-state deoxyhemoglobin (Dickerson and Geis, 1983). These structural changes relay a signal from the heme group through the protein to the C-terminal salt bridges which stabilize the low affinity quaternary structure. In the T-state structure, the F helix pulls the iron out of the plane of the heme by means of the coordination bond to the N_ϵ of His-F8. Tight packing by side chains near the hemes stabilize this domed conformation. When a ligand binds, the iron is pulled into the plane of the porphyrin which in turn pulls the proximal histidine (His-F8) into a position with the imidazole ring normal to the plane of the heme. As a result, the F helix is pulled toward the FG corner through a hydrogen bond from the N_δ of the proximal histidine to the carbonyl oxygen of Leu-F4. The heme group also pushes down against Val-FG5 and weakens the hydrogen bond between the carbonyl oxygen of Val-FG5 and the penultimate C-terminal residue side chain in both chains (α Tyr-140 and β Tyr-145). These changes in the T-state tertiary structure upon ligand binding weaken the network of salt bridges and hydrogen bonds at the C-termini and favor the switch to the quaternary R-state by means of a 15° pivot of one $\alpha\beta$ dimer relative to the other as shown in figure 1.4. The $\alpha_1\beta_1$ and $\alpha_2\beta_2$ contact regions remain relatively rigid during the T to R conformational switch compared to the changes at the FG α_1 -C β_2 contact region which acts as a ball and socket joint for the pivot and the C α_1 -FG β_2 contact region which is a ratcheting two way switch. In the R-state conformation, the heme is no longer as tightly

Figure 1.4a. Ribbon depiction of the quaternary structure of the $\alpha\beta$ dimer of human hemoglobin. Two $\alpha\beta$ dimers combine to make one molecule of tetrameric hemoglobin.

Figure 1.4b. Ribbon depiction of the quaternary structure of the hemoglobin tetramer. One $\alpha\beta$ dimer pivots 15 degrees relative to the other dimer about an axis through the indicated point during the T- to R-state switch.

constrained by packing forces and is free to assume a planar conformation with the iron atom in the center of the porphyrin ring.

1.4. Functional studies of ligand binding to myoglobin and hemoglobin.

Structural studies of myoglobin and hemoglobin are not sufficient to answer the two key questions: how does protein structure regulate exact ligand affinity, and where in the protein are the pathways for ligand entry and exit? Three approaches have been taken to address these questions. Molecular dynamics studies have focused on obtaining the trajectories for ligands leaving the binding site and escaping to solvent. Site-directed mutagenesis has examined the specific roles of the various distal amino acids in ligand binding. The classical substrate analogue approach has used alkyl isocyanide ligands to probe size and flexibility of the distal heme pocket.

1.4.1. Molecular dynamic studies of ligand trajectories.

Several molecular dynamics studies have focused on determining the path of ligand escape from sperm whale myoglobin. The dynamic studies have been undertaken in part because X-ray structures of myoglobin revealed that the bound ligand was completely surrounded by protein and no obvious path to the solvent was apparent. An early study used a rigid protein molecule with the O₂ ligand modeled as a hard sphere with reduced radius of 2.8 Å (Case and Karplus, 1979). In this computational study, the major pathway was found to be between the side chains of His-E7, Thr-E10, and Val-E11. A minor pathway involved eventual escape of the ligand between the side chains of Leu-E4 and Phe-B14. For a ligand

of normal radius (4.2-4.9Å) in a rigid protein matrix, these two paths were calculated to have an kinetic barrier of ~100 kcal/mole which is an order of magnitude too high compared to the experimental values. The side chain of His-E7 appeared to be in a conformation with a broad potential well which was limited on one side by the side chain of Arg-CD3. Motions necessary to open a pathway were estimated to have a energetic cost of ~10 kcal/mole which is closer to the observed activation energies.

A further study focused on the pathway between His-E7 and Val-E11 (Kottalam and Case, 1988) by performing dynamics calculations over a range of temperatures. Only residues within 12 Å of the initial ligand position were modelled dynamically with the rest of the protein harmonically fixed in position. The ligands sampled most of the heme pocket and escaped on a 0.1 μs time scale. The activation energy for the transition state was estimated at 1 kcal/mole. Their simple model was that two residues opened a path just large enough for a ligand to squeeze through. In traversing this narrow channel, the ligand lost most of its rotational and translational freedom. In this model, geometric properties of the heme pocket dominated the reaction, that is, entropic events greatly influenced the activation barrier for ligand escape. The model explains in a qualitative way the breaks in the Arrhenius plot for ligand binding parameters measured in glycerol/water mixtures at temperatures below 273°C. At low temperature, contraction of the heme pocket allows the ligand less entropic freedom, and the kinetic barrier to escape is dominated by the opening of the gate. At higher temperatures, the protein is more fluid and the barrier is dominated by the frequency at which the ligand can find the open gate.

A more recent study used the locally enhanced sampling technique in which a “swarm” of 60 non-interacting CO molecules diffuse through one myoglobin molecule (Elber and Karplus, 1990). Most of the pathways taken by these ligands involved hydrophobic pockets in the protein interior. These pockets have been shown to bind Xe when equilibrated with high pressures of the pure gas (Tilton *et al.*, 1984). No ligands escaped between the side chains of His-E7 and Val-E11 unless the temperature of these residues was increased well above room temperature. All three of these studies performed the simulations without water at the protein surface.

1.4.2. Mutagenesis studies of distal residues in myoglobin and hemoglobin.

In recent years, the genes for pig (Dodson *et al.*, 1988), cow (Shimada *et al.*, 1989), horse (Tang *et al.*, 1992), sperm whale (Springer and Sligar, 1987) and human myoglobin (Lambright *et al.*, 1989) and human hemoglobin (Nagai *et al.*, 1987) have been cloned. Site-directed mutants of Mb at the distal positions His-E7 (Olson *et al.*, 1988; Springer *et al.*, 1989; Rohlfis *et al.*, 1990; Ikeda-Saito *et al.*, 1991; Petrich *et al.*, 1991), Val-E11 (Egeberg *et al.*, 1990; Smerdon *et al.*, 1991), Leu-B10 (Adachi *et al.*, 1992; Carver *et al.*, 1992) and Arg-CD3 (Lambright *et al.*, 1989; Carver *et al.*, 1991) and double mutants at His-E7/Val-E11 (Egeberg *et al.*, 1991) have been produced and the kinetics of ligand binding to these proteins were studied. His-E7 and Val-E11 mutants in both the α and β subunits have also been produced and studied (Nagai *et al.*, 1987; Mathews *et al.*, 1989, 1991). These studies have been highly productive; however, unresolved problems remain. For example, the His-E7→Gly mutation in myoglobin and α subunits causes marked increases in the association and dissociation rate constants for the binding

of most ligands. This result suggests either that His-E7 is a major part of the kinetic barrier or that a new pathway has been created when the imidazole side chain is removed. An independent approach is needed to examine the structural and function of properties of the E7 and E11 residues in native myoglobin and human hemoglobin. The use of the large alkyl isocyanides to study geometrical constraints in the distal pocket of these heme proteins represents a classical substrate analogue approach and is complementary to the mutant studies.

1.5. Alkyl isocyanide binding studies.

The alkyl isocyanides ($R-N\equiv C$) have long been used to probe the effect of protein structure on ligand binding to heme proteins. A rather elegant study in 1951 used alkyl isocyanides to demonstrate that binding to the iron atom in the heme group of sheep hemoglobin is restricted by the protein structure (St. George and Pauling, 1951). Association equilibrium constants for methyl, isopropyl and *t*-butyl isocyanides (MNC, *i*-PNC and *t*-BNC) binding to free heme and to hemoglobin were compared. The affinities for free heme were roughly equivalent, but the affinities for the protein decreased 500-fold in an inverse correlation with the size of the alkyl side chains of the isocyanides.

A more sophisticated and quantitative study of mammalian myoglobins and hemoglobins showed that the equilibrium barrier to alkyl isocyanide binding is primarily steric in nature (Mims *et al.*, 1983b; Fig. 1.5). The first methyl(ene) group adds about two kcal/mole to the free energy for association when compared to the equilibrium constants for isocyanide binding to chelated model heme in soap micelles. The next alkyl carbon atom is costless in terms of free energy changes. The third and fourth cost ~ 0.8 kcal/mole apiece and then each additional

Figure 1.5. Estimate of the free energy cost of unfavorable steric interactions between distal amino acid side chains and bound alkyl isocyanides in mammalian myoglobins and hemoglobins (Fig. 5. from Mims *et al.*, 1983a)

methyl(ene) groups increases the free energy for isocyanide binding by only ~ 0.1 kcal/mole each. This is consistent with a picture of a rigid first layer of amino acid side chains surrounding the bound ligand which gives way to less rigid protein structures farther away from the heme iron (Fig. 1.5). Myoglobins from invertebrates which have a smaller or apolar residue at the E7 helical position (Leu or Val) have much higher association rate constants for isocyanide binding. From these data, Mims *et al.* (1983b) estimated a value of 1-2 kcal/mole for the distal histidine's contribution to the overall kinetic barrier to the binding of these ligands.

Molecular dynamics calculation of large ligands moving through the protein matrix have not been done. Kinetic studies of alkyl isocyanide binding to myoglobin have shown that the possible pathways for these large ligands to approach the heme are more limited than for the diatomic gases. The association rate constants for alkyl isocyanides binding to myoglobin and hemoglobin correlate, in general, inversely with increasing size of the alkyl moiety; this is more true in constricted heme pockets like those of α -chains and myoglobin (Table 1.1). Alkyl isocyanides react very quickly with sterically unhindered pentacoordinate hemes, and in organic solvents the observed rate and equilibrium constants are independent of alkyl chain length. The association rate constants for alkyl isocyanides binding to model hemes in benzene are $\sim 1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. This is about five times greater than the association rate constant for oxygen binding and more than 20 times greater than for carbon monoxide binding to these model compounds (Olson *et al.*, 1983). In contrast, the association rate constant for isocyanide binding to myoglobin is $\sim 1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. Thus, the rate-determining step is probably not bond formation with the iron atom but rather diffusion up to and away from the binding site (Mims *et al.*, 1983b).

Model heme in:											
Benzene		2% soap		Mb		α Hb		β Hb	Mb	α Hb	β Hb
k'	K _A	k'	K _A	k'	K _A	k'	K _A	k'	K _A		
O ₂	35	.014	26	.55	15	1.50	28	2.30	100	4.50	
CO	8.2	330	2.7	300	0.50	33.3	2.9	630.	7.1	990.	
MNC	200	210	11	12	.105	.023	0.12	0.06	0.62	0.11	Q
ENC	190	160	25	32	.054	.174	0.14	1.54	0.30	0.73	Q
n-PNC	210	240	59	59	.032	.078	0.05	1.04	0.08	0.16	.15
n-BNC	200	260	150	200	.028	.037	0.03	0.19	0.29	0.13	.11
n-ANC	200	290	180	240	.028	.067	0.09	0.35	0.36	0.16	.14
n-HNC	170	240	150	230	.025	.166	0.14	0.56	0.65	0.46	.10

Table 1.1 Association rate (k' , $\times 10^{-6} \text{M}^{-1} \text{s}^{-1}$) and equilibrium ($K_A, \times 10^{-6} \text{M}^{-1}$) constants and observed overall quantum yields (Q) for O_2 , CO and n-alkyl isocyanide binding to model heme in benzene, model heme in 2% soap, sperm whale myoglobin and human hemoglobin. The parameters for ligand binding to model heme are taken from Olson *et al.*, 1983, for ligand binding to myoglobin from Mims *et al.*, 1983a, for O_2 , CO and MNC binding to α and β subunits within intact tetramers from Mathews *et al.*, 1989, for ENC and the larger isocyanides binding to α and β subunits within intact tetramers come from Reisberg and Olson, 1980. Overall quantum yields for myoglobin are from Gibson *et al.*, 1986, and for isolated α and β chains are from Olson *et al.*, 1987.

Methyl isocyanide binds poorly to myoglobin and hemoglobin because of large dissociation rate constants when compared to the longer chain isocyanides which probably form more favorable hydrophobic contacts through the additional methyl(ene) groups. As the chain lengthens, however, these favorable contacts can be lessened by unfavorable steric interactions (Mims *et al.*, 1983a, 1983b). Ethyl isocyanide binds the most tightly to myoglobin and hemoglobin due to high association and low dissociation rate constants.

1.6. X-ray studies of hemoglobin- and myoglobin-alkyl isocyanide complexes.

The very first X-ray structures of horse hemoglobin (Fe III with bound water) (Perutz *et al.*, 1960) and sperm whale myoglobin (Fe III with bound water) (Kendrew *et al.*, 1958), gave a picture of protein-ligand interaction which had been only hinted at by Pauling's 1950 isocyanide binding experiments: the protein structure formed a pocket which sequestered heme and hindered ligand approach to the heme iron. Perutz solved the structure of unliganded horse hemoglobin (Fe II) (H.F. Muirhead and M.F. Perutz, 1963) and proposed a stereochemical explanation for cooperative ligand binding in hemoglobin (M.F. Perutz, 1970). Yet, despite the leap in knowledge about the structures of hemoglobin and myoglobin obtained by the first crystal structures, fundamental questions of how these proteins regulate their affinity for ligands remained to be answered. My work is a part of this process of compiling a compendium of detailed information about the relationship of structure and function in hemoglobin and myoglobin. High resolution X-ray structures of the methyl, ethyl, n-propyl, and n-butyl isocyanides complexes of sperm whale myoglobin and human hemoglobin will help to explain the

equilibrium constants for the binding of these compounds to hemoglobin and myoglobin. The structures will explore each protein's potential for tertiary rearrangements induced by the binding of large ligands. In myoglobin, the restrictions to tertiary structure rearrangements are probably necessary to limit the binding of ligands other than oxygen. In hemoglobin this is also true, but in addition, in this protein the tertiary rearrangements somehow cause the quaternary conformational change which ultimately results in cooperative ligand binding. The isocyanide structures explore the size and flexibility of the heme pockets in myoglobin and α - and β - subunits of hemoglobin.

The high resolution structure of *t*-BNC bound to tetraphenylporphyrin gave the starting parameters for modeling the alkylisocyanide ligand structure when bound to heme iron (Jameson and Ibers, 1979). The observed geometry was essentially linear but even in this unhindered structure the alkyl moiety was seen to have some conformational flexibility. The Fe-C-N angle averages 169° , and the Fe-C vectors are about 6° off normal to the porphyrin plane. This non-linearity is in contrast to that expected for either of two valence-bond structures ($\text{Fe} \leftarrow \text{:C}=\text{NR}$ and $\text{Fe}=\text{C}=\text{N:}^{\text{R}}$) and was attributed to crystal packing forces. The Fe-C bond length was $1.9 \text{ \AA}$ and the C1-N2 isocyanide bond length was $1.15 \text{ \AA}$ which is very close to Pauling's predicted value (Pauling, 1951).

Many liganded forms of both ferric and ferrous myoglobin and hemoglobin have been determined by X-ray crystallography. Most have been of complexes with small ligands, either diatomic gases (O_2 , CO, and NO) bound to ferrous proteins or small anions (H_2O and F^{-1}) bound to ferric proteins. A few structures with large ligands bound to ferric Mb and ferric Hb have been determined. These include the imidazole complex of sperm whale metmyoglobin, (Bolognesi *et al.* 1982;

Lionetti *et al.*, 1991), the irreversible phenyl adduct of sperm whale myoglobin (Ringe *et al.*, 1984), and the azide (Deatherage *et al.*, 1979), isothiocyanate (Korszun and Moffat, 1981) and partially bound imidazole (Bell *et al.*, 1981) complexes of horse methemoglobin. In the case of horse methemoglobin, only difference Fourier maps were presented.

One unexpected result from these X-ray studies was that information concerning a possible pathway for ligand binding was obtained. The shortest route from solvent to the distal heme pocket is one through the position occupied by the distal histidine. When imidazole binds to ferric sperm whale myoglobin, His-E7 swings up and away from the iron atom creating a pathway for ligand binding (Bolognesi *et al.* 1982; Lionetti *et al.*, 1991). This conformation for the distal histidine was also observed in the structure of the phenyl adduct of metmyoglobin and was described as a 'molecular doorstep' (Ringe *et al.*, 1984). Frauenfelder proposed that the large, linear alkyl isocyanide ligands would trail like 'Ariadne's thread' along the pathway from iron to the solvent (personal communication to J.S. Olson). The methyl, ethyl, n-propyl and n-butyl isocyanide isocyanide ligands provide a systematic means of testing this idea and examining the role of the distal histidine in controlling access of large ligands to the distal heme pockets in sperm whale myoglobin and human hemoglobin.

Chapter 2.

MATERIALS AND METHODS

2.1. Crystallization of sperm whale myoglobin and human hemoglobin.

Sperm whale myoglobin and human hemoglobin were crystallized according to well-established protocols (Perutz, 1968 for hemoglobin; Takano, 1977a for myoglobin) as follows.

Sperm whale myoglobin (Fe III; Sigma) was used as purchased with no further purification. Sixty milligrams of myoglobin were dissolved in deionized water to give 1 ml of solution. Saturated $(\text{NH}_4)_2\text{SO}_4$ in water ($\sim 4\text{M}$) was added to aliquots of the filtered protein solution to give a range of conditions centered on a final concentration of 75% saturated $(\text{NH}_4)_2\text{SO}_4$. Crystals grew within one or two weeks to >0.5 mm in longest dimension. Harvested crystals were stored in $\geq 75\%$ saturated $(\text{NH}_4)_2\text{SO}_4$.

Human hemoglobin was obtained from short or outdated units of blood by washing the cells with isotonic saline, lysing the cells with water, and pelleting the membranes after addition of 3.2% w/v NaCl. The deep red supernatant was dialyzed against a solution of 1.6 M mixed (Na,K)PO₄, pH 6.7 prepared by adding 1.6M K₂HPO₄ to 1.6M NaH₂PO₄ until a value of pH 6.7 was reached. After dialysis, the hemoglobin was diluted to a 4% solution (g Hb subunit/v) by addition of the 1.6M mixed (Na,K) PO₄ solution. For crystallization, H₂O and 4M mixed phosphate, pH 6.7, were added to aliquots of the 4% protein solution to give a range of phosphate concentrations centered around 2.7 M. A drop or two of toluene or mixed xylenes was added to the crystallization tubes. Crystals usually grew in two

weeks to a month to a size of 0.5 mm or greater. Harvested crystals were stored in 3.5 M mixed phosphate, pH 6.7. The protein in the crystals were a mixture of oxy(Fe II) and aquomethemoglobin (Fe III).

2.2. Preparation of isocyanide derivatives.

Crystals of Mb(Fe II) and Hb(Fe II) with bound alkyl isocyanides were obtained in a method similar to that used by Kuryian *et al.* (1986) to obtain MbCO crystals. The procedure for the hemoglobin crystals was a bit more complex due to the tendency of R-state hemoglobin crystals to crack if forced into an unliganded state (see below).

The myoglobin-alkyl isocyanide crystals were produced by the following procedure. Two or three Mb (Fe III) crystals in 75% saturated $(\text{NH}_4)_2\text{SO}_4$, 0.1 M $(\text{Na})\text{P}_i$, pH 7.0, were put into a small cuvette or flask which was then fitted with an airtight rubber septum. The crystals are more dense than the solution and lie on the bottom of the container, thus nitrogen could be bubbled into the top half of the solution for ten minutes or more to obtain a mostly anaerobic solution. The appropriate isocyanide was added anaerobically to give $\geq 2 \mu\text{l}$ isocyanide/ml solution and allowed to equilibrate with the crystals for a few minutes. Finally, a concentrated, anaerobic solution of $\text{Na}_2\text{S}_2\text{O}_4$ in the same solution as the crystals was added anaerobically to reach a concentration of 20mg/ml of solution. This reduced the myoglobin crystals to the Fe(II) form with bound isocyanide. The container with crystals was then transferred to a oxygen-free nitrogen-filled glove box (Vacuum Atmospheres Co., model HE-63-P). The extent of bound isocyanide was determined by taking a spectrum of a crystal smashed

between a cover slip and glass slide sealed with wax. Successful derivatization of myoglobin crystals gave very clear Fe(II)-RNC spectra. Finally, a crystal from the same batch was mounted near the end of a quartz capillary with inside diameter of 1.0-1.5 mm. A column of anaerobic mineral oil containing the same alkyl isocyanide was placed in the capillary and the capillary was sealed with wax. On many occasions, the myoglobin crystals derivatized well but did not diffract beyond 4 Å or so due to extensive cracking which occurred after addition of the reductant. To avoid the problem, the reductant was usually added in three or four aliquots over a period of minutes. The first MbENC structure was done in a crystal solution buffered only by 10mM NaP_i, pH 7.0, and, in retrospect, appears to be at a pH much lower than neutral, perhaps about pH 5.6. In one trial experiment, when the crystal suspensions in weak buffer (0.01M) were reduced with 20mg/ml dithionite, the solution was found to be at pH 5.6. This could not be determined accurately because the pH was measured after exposure to air and a drop in pH due to the reaction of Na₂S₂O₄ with O₂ occurs rapidly under these conditions. These side reactions should have been avoided by the anaerobic technique used to derivatize the crystals. None-the-less, the structure of myoglobin with bound ethyl isocyanide in strong phosphate buffer, 0.1M, at pH 7.0 differs from the structure determined in the weaker phosphate buffer, 0.01M, pH 7.0, in a way which can be easily ascribed to a lower pH in the presence of the weaker buffer. This was in some ways serendipitous because the two structures give interesting details concerning the chemical nature of the distal histidine in the protonated state and its role in ligand binding. The distal histidine is likely to be partially protonated in

the low pH structure and thus can more favorably interact with polar solvent molecules at the exterior of the heme pocket than it can with apolar side chains of amino acid residues within the heme pocket.

The hemoglobin-alkyl isocyanide crystals were prepared in the following way. First, a solution of 3.5 M mixed (Na,K)P_i, pH 6.7 in a small cuvette or flask was bubbled with oxygen-free nitrogen for ten minutes. Then a rubber septum was affixed and the alkyl isocyanide was added anaerobically to achieve a concentration of $\geq 1 \mu\text{l/ml}$ of solution. The septum was removed, two or more hemoglobin crystals were quickly added to the container and the septum was replaced. The crystals were allowed to equilibrate with the solution for thirty minutes. This equilibration of the crystals with the ligand before reducing the iron fully to the ferrous state was intended to prevent cracking of the crystals. After equilibration, the crystals were reduced by anaerobic addition of a concentrated solution of Na₂S₂O₄ to reach a final concentration of 10 mg/ml and then transferred to an oxygen-free nitrogen-filled glove box. Again, spectra were taken of a crystal smear to determine if the hemoglobin was fully saturated with isocyanide. A crystal was then mounted for X-ray analysis in the same way as the myoglobin crystals. Several attempts were made to make a hemoglobin-*t*-BNC crystal but the crystals cracked even at low concentrations of this isocyanide. There is an interplay between the association rate of the ligand and the rate of reduction of the iron and residual oxygen by Na₂S₂O₄ which determines whether the crystals crack because of a conformational transition from liganded to unliganded state. It might be possible to slowly add this bulky

ligand and the reductant into the crystal using a flow capillary system, but that will have to wait for future work.

2.3. Data collection and processing.

Data for the nine alkyl isocyanide structures were collected on several different X-ray diffractometers. (Table 2.1) Data from the Xuong-Hamlin detector were reduced using Chris Nielsen's machine-specific software package. Data from the Siemens area detector were reduced using the more general program XDS(Kabsch). Data collected on the Nicolet P2₁ diffractometer were reduced using the program PROCESS (Saper and Quioco, 1983). Data collected on the Siemens P4 were reduced by Siemens-supplied software extensively debugged by George Phillips. The complete data set for the low pH MbENC structure was originally merged and manipulated using the PROTEIN program package (Steigemann, 1974; Remington *et al.*, 1982). Subsequently it and the other eight data sets were merged (separately) using XSCALE (Kabsch). The myoglobin and hemoglobin crystals were monitored after data collection for the presence of Fe(III) by dissolving them in CO-equilibrated 10mM NaP_i, pH 7.0 and recording their Soret and visible spectra. Data from crystals which were found to have been significantly oxidized to the met form were discarded.

The data sets and unit cell parameters are shown in Tables 2.2a,b. The most precise unit cell parameters were those determined for the diffractometer data sets because the diffractometer refines the collection angles for a particular reflection within 0.01 degrees. The Xuong-Hamlin detector came very close to matching this precision. The Siemens detector

Protein-ligand	Detector	Resolution of data	Measured Reflections	Unique Reflections	Percent Complete	$R_{\text{sym}}(\%)$
MbMNC(pH7.0)	1	5-1.9	16405	8135	80.5	5.9
MbENC(lowpH)	2	5-1.7	19213	13912	95.9	6.5
MbENC(pH7.0)	3	5-2.0	15956	7655	88.9	18.5
MbnPNC(pH7.0)	1	5-1.8	20240	10111	84.5	7.1
MbnBNC(pH7.0)	1	5-1.8	21850	10132	84.7	8.9
HbMNC	1	5-2.2	39529	12335	88.2	8.2
HbENC	2,4	5-2.1	31183	14375	88.8	7.4
HbnPNC	4	5-2.2	31145	13753	98.4	5.9
HbnBNC	4	5-2.0	27899	14056	74.3	8.9

Table 2.1. Data collection statistics for alkyl-isocyanide myoglobin and hemoglobin structures. The data were collected on four diffractometers:

1. Siemens multi-wire area detector,
2. Nicolet P2, serial diffractometer,
3. Siemens P3 serial diffractometer and
4. Xuong-Hamlin multi-wire area detector.

R_{sym} is a measure of internal agreement of symmetry related reflections in a data set and is given by $R_{\text{sym}} = \sum(I - \langle I \rangle) / \sum \langle I \rangle$ where I is the intensity of one reflection in a group of crystallographically symmetry related reflection and $\langle I \rangle$ is the average intensity of all reflections in that group.

Protein/ligand	Data set #	a(Å)	b(Å)	c(Å)	$\beta(^{\circ})$
MbMNC	31	64.89	31.03	35.13	105.54
1 crystal, 4 orientations	32	64.76	30.94	35.10	105.43
Siemens area detector	33	64.75	30.95	35.09	105.44
	34	64.67	31.15	34.97	105.41
average (range)		64.77 (0.3%)	31.02 (0.7%)	35.07 (0.5%)	
MbENC(low pH)	11	64.63	30.98	34.89	106.08
	13	64.68	30.93	34.87	106.08
12 crystals	14	64.69	30.86	34.91	106.18
P21 diffractometer	16	64.67	30.94	34.89	106.15
	17	64.73	30.86	34.93	106.16
	18	64.66	30.95	34.88	106.08
	19	64.68	30.95	34.89	106.08
	20	64.76	30.89	34.93	106.14
	21	64.66	30.95	34.88	106.07
	22	64.70	30.94	34.88	106.09
	24	64.71	30.86	34.86	106.15
	25	64.75	30.91	34.91	106.08
average (range)		64.69 (0.2%)	30.92 (0.4%)	34.89 (0.2%)	
MbENC(pH 7.0)	2				
2 crystals	5	64.58	30.91	34.82	105.83
P3 diffractometer					
MbnPNC	11	64.99	30.93	34.95	105.75
2 crystals, 3 orientations	21	65.02	30.86	35.09	105.50
Siemens area detector	22	64.92	31.17	34.89	105.92
average (range)		64.98 (0.2%)	30.99 (1.0%)	34.98 (0.6%)	
MbnBNC	31	64.56	30.99	34.83	106.06
	32	65.08	31.15	34.78	105.79
2 crystals, 7 orientations	33	65.10	30.94	34.84	106.01
Siemens area detector	41	64.60	30.91	35.13	105.72
	42	64.85	30.92	34.83	105.83
	43	64.95	31.08	34.87	105.74
	44	64.99	31.07	34.91	105.77
average (range)		64.88 (0.8%)	31.01 (0.8%)	34.88 (1.0%)	

Table 2.2a. Data sets and unit cell constants for the alkyl isocyanide-myoglobin structures. Sperm whale myoglobin crystallizes in space group $P2_1$ with $a \neq b \neq c$ and $\beta \neq 90^{\circ}$.

Protein/ligand	Data sets	a(Å)	c(Å)
HbMNC	31	53.37	192.87
2 crystals	41	53.97	192.99
4 orientations	42	53.84	193.75
Siemens area detector	43	53.71	195.11
	44	53.75	193.43
average (range)		53.73 (1.1%)	193.63 (1.2%)
HbENC	11	54.09	193.95
2 crystals	21	54.17	194.24
X.-H. area detector	31	54.08	193.67
2crystals	41	54.14	193.17
P21 diffractometer			
average (range)		54.12 (0.2%)	193.76 (0.6%)
HbnPNC	11	54.18	194.15
2 crystals	21	54.10	194.07
X.-H. area detector			
average (range)		54.14 (0.1%)	194.11 (0.0%)
HbnBNC	11	54.17	193.90
2 crystals	21	54.01	194.84
X.-H. area detector			
average (range)		54.09 (0.3%)	194.84 (0.5%)

Figure 2.2b. Data sets and unit cell constants for alkyl isocyanide-hemoglobin structures. R-state human hemoglobin crystallizes in spacegroup $P4_12_12$ with $a=b$ and $\alpha=\beta=\gamma=90^\circ$.

produced values which had ranges of up to 1.2% of the average value. These large errors (compared to the diffractometer-determined values) might be due to inexperience in determining the midpoint of the detector and the crystal to detector distance because the Siemens machine was relatively new when I began using it. The average unit cell parameters for the different myoglobin- and hemoglobin-alkyl isocyanide crystals vary little from crystal to crystal. Thus, the unit cell parameters appears to be accurate within ~0.5% or less. This accuracy gives an estimate for the accuracy of measured distances within the various protein structures. However, the precise connection between unit cell parameters of the crystal and the distance from one protein atom to another is not clear. Variations in unit cells could be caused by changes in solvent structure between protein molecules in the crystal as well as changes in protein volume. Kuryian *et al.* (1986; unpublished data) suggested that a protein structure refined against data from many crystals might be less accurate than one refined against data from a single crystal. However, one could argue that a single-crystal structure could be flawed by an artifact of that particular crystal and that one should determine at least two single-crystal structures to guard against artifacts. In any case, the accuracy of distance measurements in the hemoglobin and myoglobin structures presented here appears to be $<0.05 \text{ \AA}$ for $10. \text{ \AA}$ of distance.

2.4. Structure refinement.

The low pH MbENC was the first structure completed. The starting structure was oxymyoglobin (Phillips, 1984) with the bound oxygen and partially occupied water molecule removed. The structure was refined first using the program PROFFT (Hendrickson and Konnert, 1980; Finzel, 1987) along with the program PROTEIN (Steigemann, 1974; Remington *et al.*, 1982) for making omit maps, $2F_o-F_c$ maps and difference maps of electron density. The positions of side chains were adjusted and 26 water molecules were inserted based on inspection of the maps. The programs XSCALE for merging data and XPLOR for refining structures running on IRIS workstations then became available. The low pH MbENC and the pH 7.0 MbENC structures were further refined using an XPLOR parameters file very similar to the Hendrickson and Konnert file used by PROFFT (Brunger, XPLOR 2.3). A simulated annealing step which consisted of a 1000 step molecular dynamics (total time = 1ps) stage and 250 step fast cooling stage was performed. This was followed by alternating cycles of position and temperature factor refinement until R-factors converged. Low occupancy waters ($\leq 10\%$) were removed by a water filter program written by George Phillips and potential new waters were added by a program written by Michael Quillin which automatically searched difference maps. New waters were kept if they occupied peaks in difference maps (4σ) and in $2F_o-F_c$ maps and were within hydrogen bonding distance of polar atoms. The position of amino acid side chains and ligands were adjusted, if necessary, by inspection of XPLOR-generated $2F_o-F_c$ and difference maps. The occupancies of distal histidine and ligand positions were determined by a grouped occupancy

factor and adjusted if necessary. If manual adjustments had been made to the structure, the preceding steps were iterated until R-factors converged.

The hemoglobin-alkyl isocyanide structures were refined in much the same way. The starting structure was oxyhemoglobin (Shaanan, 1983) with the bound oxygen removed and the appropriate alkyl isocyanide modelled into the density according to initial difference and $2F_o - F_c$ maps. The XPLOR program running on a Vaxstation was used to perform an initial rigid rotation of the $\alpha\beta$ -dimer followed by conventional positional and temperature factor refinement. The coordinates were then transferred to the XPLOR program running on Silicon Graphics IRIS workstation. The three other hemoglobin-alkyl isocyanide structures were initially modeled using the initial coordinates for hemoglobin-ethyl isocyanide but modeling in the appropriate alkyl isocyanide ligand after inspection of initial difference and $2F_o - F_c$ maps. The four structures were further refined in an equivalent manner as the myoglobin structures. A simulated annealing step was performed and followed by position and temperature factor refinement. Manual adjustments to the structures were made, if necessary, and a new round of position and temperature factor refinement was done.

Chapter 3.

X-RAY STRUCTURES OF THE ALKYL ISOCYANIDE COMPLEXES OF MYOGLOBIN

3.1. General description of the refined structures.

The final refinement statistics for the five alkyl isocyanide derivatives of sperm whale myoglobin are shown in Table 3.1. The agreement between the model structures and the respective data are good as shown by final R-factors $\leq 16\%$. The structures were forced to have rms deviations for bond lengths of ~ 0.02 Å which is similar to the bond sigmas observed for crystal structures of small peptides which diffract to < 1.0 Å and have rms values for atomic positions of 0.01 to 0.02 Å. The desired distributions for bond lengths and angles were obtained by adjusting the relative weight of the X-ray data and geometric constraints. The restrained angles have a root mean square deviation of about 2.3° . In all five structures, large deviations for bond angles from the restrained target ($> 10^\circ$ in some cases) involving the N_γ of histidine residues were observed. This must be due to a problem with the model used for the histidyl ring because the histidines in these myoglobin structures fit well to the observed electron density.

A good estimate of the root mean square error in atomic coordinates is necessary for a critical analysis of protein structures. A low estimate of this error is given by the bond sigmas and is approximately 0.02 Å for the structures listed in table 3.1. A higher estimate of the errors can be obtained from a plot of R-factor vs inverse resolution which is compared to theoretical plots of coordinate error (Fig. 3.1) (V. Luzatti, 1952). The errors for the myoglobin structures fall between 0.1 Å and 0.25 Å on the Luzatti plot. An additional estimate was obtained from a

Protein-ligand	R(%)	$\sigma_{\text{bond}}(\text{\AA})$	$\sigma_{\text{angle}}(^{\circ})$	$\sigma_{\text{dihedrals}}(^{\circ})$	$\sigma_{\text{impropers}}(\text{\AA})$	$Q_{\text{His-E7}}$				Q_{RNC}	
						'down'	'up'	'in'	'out'		
MbMNC (pH 7.0)	13.6	0.020	2.38	22.13	5.08	0.94	—	1.00	—		
MbENC (low pH)	16.0	0.021	2.46	21.77	4.83	—	0.50	0.95	—		
MbENC (pH 7.0)	15.9	0.018	2.24	22.76	5.49	0.86	—	0.96	—		
MbnPNC (pH 7.0)	13.3	0.019	2.25	22.04	4.96	0.18	0.82	0.16	0.84		
MbnBNC (pH 7.0)	14.9	0.019	2.32	22.68	5.49	—	0.87	—	1.00		

Table 3.1. Refinement statistics for Mb-RNC structures. R-factors are standard and calculated by the program XPLOR as $\sum(|F_o| - |F_c|)/\sum|F_o|$ for all reflections. The σ 's for bonds, angles, dihedral angles and improper contacts are rms deviations from restrained parameters as output by XPLOR. The refined occupancy factors (Q's) for His-E7 and bound alkyl isocyanides (RNC) were determined after many rounds of position and temperature factor refinement.

Figure 3.1. Luzatti plot for MbMNC compared to theoretical Luzatti plots of coordinate error (Luzatti, 1952). The coordinate errors fall between 0.1 and 0.25 $\text{\AA}$ for the MbMNC structure.

comparison of two MbENC (low pH) structures refined from the same data but using a different random seed to generate initial velocities in the simulated annealing step. The average root mean square error for the least squares fit of C_{α} coordinates of these two structures is an intermediate value of 0.04 Å (Table 3.2). A comparison of rms differences in C_{α} coordinates for the various high resolution myoglobin structures is shown in Table 3.3. The rms differences between the five alkyl isocyanide structures have a value closer to 0.1 Å than those between the oxy, carbonmonoxide and isocyanide structures which show values closer to 0.2 Å. The latter values are probably significant but could be due to systematic differences in the processes used to obtain the oxy- and carbonmonoxide structures. The differences among the five alkyl isocyanide structures are two to three times the rms error seen in the fitted C_{α} coordinates of the two low pH MbENC structures. Thus, even though quite similar, the isocyanide-myoglobin structures exhibit measurable differences and justify a detailed comparison.

3.2. Structure of the bound alkyl isocyanides.

The final observed electron density and modeled geometry for the five alkyl isocyanide complexes are shown in Figure 3.2.a-e. For all of these complexes, the observed electron density in the heme pocket is sufficient to define the polar isocyano group and the apolar alkyl moiety. However, it is not of high enough resolution to position precisely each atom of the ligand, and the exact geometry of the bound ligands is not well-defined. However, the Fe-C-N angle is certainly less than 180° by an amount similar to that observed in the *t*-BNC-model heme structure (Jameson and Ibers, 1979). In the myoglobin structures, the deviations from linearity are not due to crystal packing forces but rather to steric hindrance by

Ligands→	CO pH 7.0	MNC pH 7.0	ENC low pH	ENC pH 7.0	nPNC pH 7.0	nBNC pH 7.0
↓						
O ₂ pH 8.4	.18 Å	.16 Å	.21 Å	.18 Å	.16 Å	.18 Å
CO pH 7.0		.19 Å	.22 Å	.20 Å	.21 Å	.22 Å
MNC pH 7.0			.14 Å	.12 Å	.11 Å	.12 Å
ENC low pH			.04 Å ¹	.17 Å	.14 Å	.16 Å
ENC pH 7.0					.13 Å	.15 Å
nPNC pH 7.0						.10 Å

Table 3.2. The rms residuals for least squares superimposition of C_α coordinates for MbO₂, MbCO and five MbRNC X-ray structures. The fits used residues 2-151.

¹The two low pH MbENC structures were independently refined from the same data but used different random seeds for the initial velocity distribution in the simulated annealing step of refinement.

Figure 3.2a. Final electron density, $(2F_o - F_c)\alpha_c$, and refined structure of MbMNC complex. Shown is the heme pocket viewed from the side chain of Val-E11. The distal histidine is disordered but modeled only in the 'down' conformation. Density in the top left corner indicates a second site for this residue. The methyl isocyanide ligand is modeled in the 'in' conformation, however the continuous density from the ligand to the distal histidine indicates that the ligand is disordered also.

Figure 3.2b. Final electron density, $(2F_o - F_c)\alpha_c$, and refined structure of MbMNC complex. Shown is the heme pocket viewed from the side chain of Leu-B10. The methyl isocyanide ligand lies in the apolar interior of the heme pocket.

Figure 3.2b. Final electron density, $(2F_o - F_c)\alpha_c$, and refined structure of MbMNC complex. Shown is the heme pocket viewed from the side chain of Leu-B10. The methyl isocyanide ligand lies in the apolar interior of the heme pocket.

Figure 3.2c. Final electron density, $(2F_o - F_c)\alpha_c$, and refined structure of the pH 7.0 MbENC complex. Shown is the heme pocket viewed from the side chain of Val-E11. The distal histidine is disordered but modeled only in the 'down' conformation. The ethyl isocyanide ligand is modeled in the 'in' conformation and is more ordered than the methyl isocyanide ligand shown in panel a.

Figure 3.2d. Final electron density, $(2F_o - F_c)\alpha_c$, and refined structure of pH 7.0 MbENC complex. Shown is the heme pocket viewed from the side chain of Leu-B10. The ethyl isocyanide ligand lies in the apolar interior of the heme pocket over the C pyrrole ring.

Figure 3.2e. Final electron density, $(2F_o - F_c)\alpha_c$, and refined structure of the low pH MbENC complex. Shown is the heme pocket viewed from the side chain of Val-E11. The distal histidine is disordered and predominantly occupies the 'up' conformation. The ethyl isocyanide ligand is modeled in the 'in' conformation. One water molecule is modeled in density near the ligand which is more likely associated with a partially occupied position for the ligand or distal histidine.

Figure 3.2f. Final electron density, $(2F_o - F_c)\alpha_c$, and refined structure of the low pH MbENC complex. Shown is the heme pocket viewed from the side chain of Leu-B10. The ethyl isocyanide ligand lies in the apolar interior of the heme pocket over the C pyrrole ring but is less ordered than in the the pH 7.0 MbENC complex..

Figure 3.2g. Final electron density, $(2F_o - F_c)\alpha_c$, and refined structure of the pH 7.0 MbnPNC complex. Shown is the heme pocket viewed from the side chain of Val-E11. The distal histidine appears to be well-ordered and occupies the 'up' conformation. A second histidine was modeled in the 'down' position but has poor geometry and little associated density. The n-propyl isocyanide ligand is modeled both in the 'out' and 'in' conformations. The 'out' conformation for the ligand is more well-defined by the associated electron density.

Figure 3.2h. Final electron density, $(2F_o - F_c)\alpha_c$, and refined structure of the pH 7.0 MbnPNC complex. Shown is the heme pocket viewed from the side chain of Leu-B10. The 'out' position of the n-propyl isocyanide ligand and the 'up' position of the distal histidine are well-observed. The terminal carbon of the ligand in the 'in' conformation makes unfavorable contacts with residues Leu-B13 and Ile-G8.

Figure 3.2i. Final electron density, $(2F_o - F_c)\alpha_c$, and refined structure of the pH 7.0 MbnBNC complex. Shown is the heme pocket viewed from the side chain of Val-E11. The distal histidine is very well-ordered and occupies the 'up' conformation. The n-butyl isocyanide ligand is also well-ordered and is modeled only in the 'out' conformation. The electron density located between the ligand and residue Leu-B13 is probably associated with a very poorly occupied 'in' conformation for the ligand.

Figure 3.2j. Final electron density, $(2F_o - F_c)\alpha_c$, and refined structure of the pH 7.0 MbnBNC complex. Shown is the heme pocket viewed from the side chain of Leu-B10. The 'out' position of the n-butyl isocyanide ligand and the 'up' position of the distal histidine are more well-observed than in the pH 7.0 n-propyl isocyanide complex.

amino acid side chains in the heme pocket. The Fe-C bond length was restrained to be 1.9 Å but tended to lengthen to 2.0 Å or more in the refinement process. The C-N bond length remained at the restrained value of 1.15 Å. The alkyl carbons were modeled in a normal sp^3 geometry and retained that geometry during the refinement.

The temperature factors assigned to the isocyanide atoms indicate that thermal motion increases outward from the bound isocyano carbon atom, C_1 , to the terminal methyl group (Table 3.3). All the C_1 ligand atoms have B-factors comparable to those for protein C_α and C_β atoms. At pH 7.0, the C_1 atom of bound ethyl isocyanide has a very low B-factor which is comparable to that of the heme iron. The isocyano nitrogen, N_2 , and two alkyl carbons, C_3 and C_4 , also have lower B-factors than their counterparts in the low pH MbENC structure. The isocyano nitrogen atoms and alkyl carbon atoms of the pH 7.0 methyl, ethyl and n-propyl isocyanide complexes have similar B-factors, whereas the B-factors for the ligand atoms in the pH 7.0 n-butyl isocyanide-myoglobin structure are significantly greater and comparable to those in the low pH MbENC complex.

In the *bis*-liganded *t*-butyl isocyanide model heme complex reported by Jameson and Ibers (1979), the projection of the slightly bent ligand side chain onto the heme plane fell between two pyrrole nitrogens. In contrast, the second oxygen atom of the ligand in oxymyoglobin lies over a pyrrole nitrogen and is presumably constrained in this unfavorable orientation by distal amino acid residues (S.E.V. Phillips, 1980). This effect is also seen in the isocyanide structures where the third and subsequent alkyl carbons are forced to lie over the N_c pyrrole nitrogen of the heme group and point towards the side chain of Leu-B13 (Fig. 3.2b,d,f,h). The alkyl groups are steered in this inward direction by $C_{\delta 2}$ atom of Leu-B10 and C_δ of

Ligand ↓	Irons	Ligand atoms						Protein atoms	
		O ₁	O ₂					C _α	C _β
O ₂ (pH 8.4)	8.7	20.7	19.6					10.7	12.6
		C ₁	O ₂						
CO(pH 7.0)	6.0	5.9	6.1					8.4	8.8
		C ₁	N ₂	C ₃	C ₄	C ₅	C ₆		
MNC(pH 7.0)	11.9	14.7	17.1	20.3				14.7	15.4
ENC(low pH)	11.4	13.3	24.1	23.7	26.6			12.6	13.8
ENC(pH 7.0)	7.0	5.2	18.1	21.8	22.4			10.5	11.4
nPNC(pH 7.0)	15.7	17.0	21.4	22.0	21.2	22.0		17.0	17.6
nBNC(pH 7.0)	16.4	16.0	26.5	24.8	28.4	30.1	30.4	17.7	18.4

Table 3.3. Temperature factors of iron, ligand and protein atoms in oxy and carbon monoxide myoglobin and the five alkyl isocyanide-myoglobin structures. Units are in Å² which are related to the rms unidirectional displacement (Δr) by $(\Delta r^2) = (B/8\pi^2)$.

Ile-G8. The alkyl groups in the second orientation of n-propyl and that of bound n-butyl isocyanide lie over the heme on a line between pyrrole nitrogens N_A and N_D of the heme group pointing toward the solvent (Fig. 3.2h,j). These ligand atoms displace the His-E7 side chain from its position in oxy- and deoxymyoglobin. The C_β atom of His-E7 and the side chains of Phe-CD1 and Thr-E10 appear to force the carbon atoms of n-butyl isocyanide into this outward, bent conformation.

3.3. Conformations of distal pocket residues.

The most dramatic result seen in the isocyanide-myoglobin structures is the rotation of the distal histidine side chain about its C_α - C_β bond to make room for the bound n-propyl and n-butyl isocyanides (Table 3.1, Fig. 3.2g-j). Both ligand size and pH affect this histidine motion. In the crystal structures of myoglobin containing bound diatomic and triatomic ligands, the His-E7 side chain lies in what can be termed the ‘down’ position where the N_ϵ proton hydrogen bonds with bound oxygen. The imidazole ring swings slightly away from this position when CO binds (Fig. 3.3). Methyl isocyanide destabilizes the ‘down’ position of the E7 side chain to an even greater extent and in the MbMNC structure there is observed electron density corresponding to two histidine conformations (Fig. 3.2.a — top left). In both the low and neutral pH MbENC structures, the distal histidine appears to be markedly disordered. The ‘down’ position is still favored at pH 7.0. The ‘up’ position is characterized by rotation of the imidazole side chain $\sim 100^\circ$ about its C_α - C_β bond in a direction away from the iron atom toward the solvent and is more dominant at low pH. In the nPNC structure at pH 7.0, the ‘up’ position is also dominant though not 100% occupied. Only in the case of the nBNC complex is

Figure 3.3a. Heme pockets of oxy (dark) and carbonmonoxide(light) myoglobin after superposition of the hemes by the method of Kabsch (1978).

Figure 3.3b. Heme pockets of oxy (dark) and pH 7.0 ethyl isocyanide (light) myoglobin after superposition of the hemes by the method of Kabsch (1978).

Figure 3.3c. Heme pockets of oxy (dark) and low pH ethyl isocyanide (light) myoglobin after superposition of the hemes by the method of Kabsch (1978).

the ligand large enough to force the distal histidine side chain to the up position at 100% occupancy.

This movement of the distal histidine in response to the binding of large ligands can be viewed as a direct consequence of the limited size and steric rigidity of the heme pocket (Fig. 3.2a-j.). The amino acids Leu B-10 and His-E7 overlie the bound ligand, but in myoglobin the side chain of Leu-B10 moves very little to accommodate large bound ligands. Phe-CD1 forms a wall on one side and Val-E11 on the other side of the distal heme pocket. Ile-G8 and Leu-B13 limit the length of alkyl isocyanide which can fit in the interior of the distal heme pocket. The effect of steric hindrance can be seen by the distance from the nearest protein atom (Table 3.4) to the terminal ligand atom. The C₃ atom of the methyl isocyanide ligand is close to Val-E11, His-E7, Leu-B10 and Phe-CD1. The C₄ atom of pH 7.0 ENC is much closer to side chains of residues at the interior of the heme pocket. The terminal C₅ atom in the low occupancy conformation of nPNC is sterically hindered by the side chains of Leu-B13 and Ile-G8. The 'out' conformation of this ligand is much less hindered. The C₆ atom of nBNC ligand simply would not fit into the back of the heme pocket without substantial rearrangements of protein structure. As a result, the 'out' ligand conformation is the only possible orientation for bound nBNC without substantial expansion of the distal pocket.

When the side chain of His-E7 swings away from the alkyl isocyanides it appears to relieve partially the steric hindrance experienced by the ligand. This is especially evident when comparing the low and neutral pH structures of MbENC (Fig. 3.3b,c). The terminal C₄ atom of ENC is 0.5 Å closer to the heme and 0.4 Å closer to the side chains of Leu-B13 and Ile-G8 in the neutral pH structure than in

Ligand(atom)→		MNC(C ₃)		ENC(C ₄)		ENC(C ₄)		nPNC(C ₅)		nPNC(C ₅)		nBNC(C ₆)	
		Atom	Dist.(Å)	Atom	Dist.(Å)	low pH	Atom	Dist.(Å)	Atom	Dist.(Å)	'in' Q=16%	Atom	Dist.(Å)
		ph 7.0											
		Atom	Dist.(Å)	Atom	Dist.(Å)	Atom	Dist.(Å)	Atom	Dist.(Å)	Atom	Dist.(Å)	'out' Q=84%	Atom
Heme atoms													
	N _C		3.62	C ₁ C	3.41	C ₁ C	3.98	C ₂ C	3.34	C ₁ A	3.67	C ₁ HA	3.56
	C ₁ C		3.81	N _C	3.49	C ₂ C	3.98	C ₁ MC	3.56	C ₁ HA	3.75	C ₁ A	3.70
	Fe		3.85	C ₂ C	3.57	N _C	4.04	C ₁ C	3.61	C ₂ A	4.04	C ₂ A	3.74
	C ₄ C		3.98	C ₄ C	3.77	C ₃ C	4.09	C ₃ C	3.85	N _A	4.22	C ₄ AA	3.79
	N _B		4.06	C ₃ C	3.78	C ₄ C	4.15	C ₁ HC	4.07	C ₄ D	4.27	C ₁ BD	4.29
				C ₁ HC	3.98								
				C ₁ MC	4.11								
Protein atoms													
	68C _{γ2}		3.30	43C _ζ	3.54	29C _{δ2}	3.14	107C _δ	2.98	64C _{δ2}	3.59	64C _{δ2}	3.53
	64C _{δ2}		3.63	29C _{δ2}	3.63	43C _{ε1}	3.56	32C _{δ2}	3.19	64C _β	3.76	67C _{γ2}	3.66
	29C _{δ2}		3.69	107C _δ	3.72	107C _{γ1}	4.11	29C _{δ2}	3.51	64O	3.93	67C _γ	4.09
	43C _ζ		3.70	32C _{δ2}	4.18	68C _{γ2}	4.17	107C _{γ1}	4.02	67C _{γ2}	3.94	64C _β	4.32
	64C _{ε2}		3.77	43C _{ε1}	4.32	43C _ζ	4.30	43C _ζ	4.42	68C _{γ2}	3.96	64C _β	4.41
	43C _{ε1}		4.24							64C _γ	4.03		
										67C _β	4.11		
C _α 's													
	68		5.70	29	6.10	29	5.87	107	5.71	64	4.21	64	4.96
	29		6.61	68	6.77	68	6.51	29	5.72	67	5.32	67	5.53
	65		6.63	107	6.94	65	6.87	32	6.87	68	5.48	68	6.49
	64		6.82	65	7.54	64	7.32	39	7.47	65	6.50	65	7.61
	107		7.94	32	7.81	107	7.40	68	7.48	66	7.86	63	8.36
								104	7.51	63	7.86		

Table 3.4. Nearest atoms to terminal ligand carbon in the alkyl isocyanide complexes of myoglobin. Residue numbers correspond with helical positions as shown in figure 1.2.

the low pH structure. Thus, the outward movement of the His-E7 side chain relieves steric pressure and allows the ENC ligand to assume a more upright conformation similar to that observed for *t*-butyl isocyanide bound to a model heme (Jameson and Ibers, 1978). Even in the 'up' orientation, the C_β atom of His-E7 appears to hinder sterically the C₅ atoms of bound nPNC and nBNC. This hindrance, in addition to the free energy required to open the histidine gate, serves to explain why nPNC has a significantly lower equilibrium association constant than ENC (Table 1.1; Fig. 3.9). The C₆ atom of bound nBNC causes further disruption of the hydrogen bonding interactions between Thr-E10, the heme propionates, Arg-CD3 and His-E7, and this unfavorable interaction probably accounts for the further decrease in isocyanide affinity. Further elongation of the ligand alkyl moiety would place the C₇ and C₈ alkyl atoms either into the solvent or along the protein surface with little or no steric hindrance. Mims *et al.* (1983b) observed increases in the association equilibrium constant in going from nBNC to n-pentyl isocyanide to n-hexyl isocyanide when examining the binding of these ligands to myoglobin. They interpreted this increase in affinity in terms of increasing the favorable hydrophobic effect with increasing ligand size which was not compensated unfavorably by steric interactions.

Plots of absolute distance from the center-of-position of the amino acid side chains to the iron atom for seven myoglobin structures are shown in Fig. 3.4.a. The extreme jaggedness of the plot is due to the angles at which the various side chains protrude from the helices. The disorder at the C-terminus is also apparent in this plot. The side chain closest to the iron atom on the proximal side is that of His-F8. On the distal side, the closest side chains are those for Val-E11 and His-E7.

Figure 3.4a. Distances from the iron atom to the side chain center of position in various liganded myoglobin structures. For the glycine residues, the coordinates for the corresponding alpha carbons were used. Note that the seven plots show little differences except at the C-termini.

Figure 3.4b. Differences in distances from the iron atom to the side chain centers of position in carbonmonoxide and oxymyoglobin. The distances for MbO₂ were subtracted from those for MbCO. For the glycine residues, the coordinates for the corresponding α carbons were used.

Figure 3.4c. Differences in distances from the iron atom to the side chain centers of position in methyl isocyanide and oxymyoglobin. The distances for MbO₂ were subtracted from those for MbMNC. For the glycine residues, the coordinates for the corresponding α carbons were used.

Figure 3.4d. Differences in distances from the iron atom to the side chain centers of position in pH 7.0 ethyl isocyanide- and oxymyoglobin. The distances for MbO₂ were subtracted from those for pH 7.0 MbENC. For the glycine residues, the coordinates for the corresponding α carbons were used.

Figure 3.4e. Differences in distances from the iron atom to the side chain centers of position in low pH ethyl isocyanide- and oxymyoglobin. The distances for MbO₂ were subtracted from those for low pH MbENC. For the glycine residues, the coordinates for the corresponding α carbons were used.

Figure 3.4f. Differences in distances from the iron atom to the side chain centers of position in n-propyl isocyanide- and oxymyoglobin. The distances for MbO₂ were subtracted from those for MbPNC. For the glycine residues, the coordinates for the corresponding α carbons were used.

Figure 3.4g. Differences in distances from the iron atom to the side chain centers of position in n-butyl isocyanide- and oxymyoglobin. The distances for MbO₂ were subtracted from those for Mb nBNC. For the glycine residues, the coordinates for the corresponding α carbons were used.

Difference distance plots for side chain positions (Fig.'s 3.4.b-g) pick out both random motion of mobile, usually surface, residues as well as the concerted motion of side chains in response to ligand binding. Random placement of a side chain is exemplified by that of Gln-H5 which is disordered in electron density maps. This residue occupies variable positions when comparing the five MbRNC structures to MbO₂ and its location is not dependent on ligand size. In contrast, the center-of-position of His-E7 moves progressively away from the iron atom with increasing ligand size. As described previously, this was evident from the electron density maps (Fig.'s 3.2.a-d), and is quantitated in the $\Delta\text{Fe-SC}$ plots. In the MbMNC structure, the histidyl ring is about the same distance from the iron atom as in oxymyoglobin, although there is perhaps a poorly occupied alternate position seen in the electron density maps (Fig. 3.2.a) which would be almost 3.0 Å from its position in MbO₂. In the pH 7.0 MbENC structure, the side chain has moved ~0.3 Å away from the iron atom. In the low pH MbENC structure, the His-E7 side chain moved to a position which is ~2.8 Å from that in MbO₂. In MbnPNC, the His-E7 side chain moved to ~3.3 Å and in MbnBNC ~3.5 Å from its position in MbO₂. All these changes occurs by rotation about the His-E7 C_α-C_β bond. In the structures of the alkyl isocyanide myoglobin complexes, there appear to be two distinct positions for the histidine side chain. The 'down' position is observed when small ligands are bound and the 'up' position is preferred for large ligands. This result suggests that there is a significant free energy barrier which restricts occupancy of intermediate positions.

The other amino acid side chains which line the distal heme pocket of myoglobin are displaced to a much lesser extent than His-E7 in response to binding of alkyl isocyanides. The side chain of Phe-CD1 appears to be immobile; its

distance from the iron atom varies from the position in MbO₂ by < 0.05 Å in the five MbRNC structures and the MbCO structure. This amino acid is highly conserved in myoglobins and hemoglobins and is important in stabilizing bound heme (Dickerson and Geis, 1983). The rigidity of Phe-CD1 shown in these structures is likely to be an important manifestation of its functionality, since maintaining its position is probably necessary to prevent heme dissociation. Similarly, the side chain of Val-E11 appears to be immobile and moved ≤ 0.1 Å in response to isocyanide binding.

The side chain of Leu-B10 moves 0.1 to 0.2 Å away from the iron atom in the five MbRNC structures when compared to the position of this residue in oxymyoglobin. Its position is farthest from the heme iron in MbnBNC and closest in the MNC complex. The Leu-B10 residue is farther from the iron atom in the low pH MbENC structure than in the pH 7.0 MbENC structure. The latter observation correlates well with the ligand position in the bound state. The alkyl moiety of ENC at pH 7.0 lies more parallel to the heme than at low pH, and the terminal carbon at low pH is closer to the side chain of Leu-B10 than at pH 7.0. The positions of the side chains of Ile-G8 and Phe-B14 exhibit a pattern similar to that of Leu-B10. The position of the side chain of Leu-B13 shows no correlation with the size of the bound ligand. However, all of these displacements are small, particularly compared to the disorder in the side chain of His-E7.

3.4. Changes in the position of the heme group.

Heme geometry and iron reactivity appear to play an important role in determining ligand affinity for myoglobin. Large motions are required to allow heme to enter and leave the protein interior. A phenomenon termed heme rotational

disorder (La Mar *et al.*, 1986) occurs when apomyoglobin is reconstituted with heme. Apomyoglobin binds heme in two orientations related by a 180° rotation about a line drawn from the C_α to C_χ meso atoms of the porphyrin ring. At equilibrium, the conformation seen in X-ray structures is favored and appears to be 90% occupied as determined by $^1\text{H-NMR}$. A kinetic study showed no effect of this rotational disorder on the binding of diatomic gases to myoglobin (Light *et al.*, 1987). The noise level in myoglobin electron density maps would probably obscure any alternative conformation with less than about 25% occupancy (Kuriyan *et al.*, 1986). Smaller changes in the position of the heme are observed in X-ray structures of different liganded form of myoglobin. These include the deoxy to oxy transition which involves lifting of the AD edge, flattening of the heme plane, movement of the C pyrrole corner towards the F-G corner and a pivoting motion in which the heme twists in a clockwise manner (ADCB) with the C pyrrole corner relatively fixed (S.E.V. Phillips, 1980).

It is difficult to compare heme positions in a number of different X-ray structures because of a lack of a definitive reference point. One approach is to superpose the protein structure by means of a least squares fit of C_α coordinates and then compare the relative heme positions (Fig. 3.5). Furthermore, the porphyrin in oxymyoglobin is ruffled. In particular, the N_A atom of the A pyrrole ring rises about $0.3 \text{ \AA}$ above the heme plane. However, the heme group is more planar in the isocyanide complexes. The greatest change in heme position in comparison to oxymyoglobin was observed for the n-butyl isocyanide complex. In the Mb_nBNC structure, the porphyrin ring appears to move back towards the deoxymyoglobin position but retains the planar conformation typical of hexa-coordinate heme (Fig. 3.5). The heme slides $\sim 0.2 \text{ \AA}$ away from the centroid of the

Figure 3.5. The heme pocket of oxymyoglobin (darker) and the heme of n-butyl isocyanide myoglobin (lighter) after superposition of the two C_{α} backbones. The distances after the superposition between the labeled heme atoms in A are: C2A(0.29), C3A(0.37), C2B(0.24), C3B(0.20), C2C(0.30), C3C(0.09), C2D(0.39) and C3D(0.47). The hemes vary greatly in length and width in various liganded myoglobin structures (A):

ligand	CO	O ₂	MNC	ENC pH7	ENC low pH	nPNC	nBNC
from/to							
CA-CC	6.71	6.96	6.90	6.88	6.82	6.85	7.00
CB-CD	6.95	6.76	6.69	7.04	6.97	6.84	6.73.

molecules, with the C pyrrole showing least change in position. The solvent edge of the heme drops about 0.3 - 0.4 Å from its position in MbO₂. These changes in position are readily explainable in terms of the steric forces created when this large ligand is trapped between the heme and the E-helix and imidazole ring of His-E7 in its 'up' conformation (Fig. 3.2i,j).

The hemes in MbMNC and MbENC (low pH) move in a direction similar to that observed for the nBNC complex, but the changes relative to the oxymyoglobin positions are much smaller. In addition, the hemes in MbENC (pH 7.0) and MbnPNC structures appear to show about a 0.2 Å movement in the direction of the DC edge of the heme. However, significant variation was observed for the structures within the heme ring as shown by deviations in the absolute and relative distance between C_A-C_C and C_B-C_D pyrrole atoms (i.e., from 6.71 to 7.00 Å in length and from 7.04 to 6.69 Å in width, respectively) (Fig. 3.4). Because of this variability, only in the MbnBNC structure can the change in heme position be considered significant compared to the position of the heme in the oxymyoglobin structure.

3.5. Changes in overall protein structure caused by isocyanide binding.

Changes in protein structure more distant from the heme are more difficult to analyze. Thermal disorder of heme protein atoms generally increases with distance from the heme group (Evans and Brayer, 1988), and a direct comparison of protein structures far from the heme group in different myoglobin derivatives is difficult. Instead, two other types of analysis are presented. The first is a comparison of relative distances of α carbons from the iron atom (Fig. 3.6). The heme groups were constrained to be planar and remained that way during the refinement, thus

movement of protein C_{α} atoms relative to the iron atom corresponds with movement of the protein in relationship to the position of the porphyrin ring. This analysis examines global perturbations caused by the binding of large isocyanide ligands.

The second analysis examines the average of the temperature factors of the backbone atoms (N, C_{α} and C) and compares the thermal properties of helices and nonhelical segments (Fig. 3.7). This analysis is less exact because the temperature factors incorporate many effects into one number. These values are used to attempt to account for isotropic thermal motion of individual atoms, and, inherently, to scale weak intensities of high resolution data to the strong intensities of low resolution data. Temperature factors or B-factors are influenced by both the temperature of data collection and crystal packing forces. These packing forces tended to reduce the thermal mobility of protein segments in close contact with neighboring protein molecules. When the B-factors for native sperm whale myoglobin in the P2₁ crystal form and those for recombinant wild-type myoglobin in the P6 form were combined into one set by choosing the larger values, it was found that the thermal factors more closely matched the mobilities predicted in molecular dynamics simulations than either of the two individual sets of B-factors (Phillips, 1990). Lastly, the B-factors were constrained to vary from one atom to the next by a fixed amount ($2.0 \text{ \AA}^2$ for main chain atoms) and, thus, tend to underestimate the thermal fluctuation of highly mobile segments.

A simple plot of Fe- C_{α} distances reveals few differences among the seven compared myoglobin structures (Fig. 3.6a). There are large discrepancies ($> 1 \text{ \AA}$) at the C-terminus which are due to a choice of torsion angle about the C_{α} -C bond of Tyr-151. This choice is made to position either the disordered glutaminy side

chain of Gln-HC3 or the terminal α -carboxyl group in the weak electron density found at the C-terminus. Although differences between the liganded structures are masked, Fig. 3.6a does give a nice picture of the arrangements of helices about the iron atom. The two closest approaches of the C_α backbone to the heme iron are at Val-E11 and His-F8; the two furthest points lie on the start of the A-helix and the G-H corner.

Differences in the $Fe-C_\alpha$ distances between the isocyanide structures and that for oxymyoglobin ($\Delta Fe-C_\alpha$) reveal more clearly the perturbations caused by the binding of the larger ligands (Fig. 3.6b-h). Positive $\Delta Fe-C_\alpha$ values represent movement of the C_α away from the heme iron relative to the oxy structure; negative values represent movement toward the heme. Determining which changes in these plots are significant is problematic. The rms error for C_α coordinates in two independently refined MbENC (low pH) structures was 0.04 Å. These errors could lead to an 1σ value of 0.08 Å for any one $\Delta Fe-C_\alpha$ distance. A significant effect for an individual amino acid residue is surely one which exceeds 0.2 Å (i.e., more than quadruple the rms difference seen in the two independently refined low pH MbENC structures). A significant value for helical averages of $\Delta Fe-C_\alpha$ distances could be much less depending on the length of the helix (Fig 3.6h).

The carbonmonoxide myoglobin structure was determined at -14°C, pH 7.0 (Kuriyan, 1986) and shows a large number of negative values in the $\Delta Fe-C_\alpha$ plot (Fig. 3.6b) caused by an overall contraction of protein structure relative to that of oxymyoglobin determined at -12°C, pH 8.4 (S.E.V. Phillips, 1981). A summary of average helical movements (Fig. 3.6h) indicates that only the F-helix and EF corner of MbCO are farther from the iron atom than in MbO₂ but this difference is very small. The F-helix in all the isocyanide structures is also further from the heme iron

Figure 3.6a. Distances from the iron atom to α carbons in seven liganded myoglobin structures. Note that the plot shows little differences except at the C-termini.

Figure 3.6b. Differences between distances from the iron atom to α carbons in carbonmonoxide and oxymyoglobin. The distances for MbO₂ were subtracted from those for MbCO. For the glycine residues, the coordinates for the corresponding α carbons were used.

Figure 3.6c. Differences between distances from the iron atom to α carbons in methyl isocyanide- and oxymyoglobin. The distances for MbO₂ were subtracted from those for MbMNC. For the glycine residues, the coordinates for the corresponding α carbons were used.

Figure 3.6d. Differences between distances from the iron atom to α carbons in pH 7.0 ethyl isocyanide- and oxymyoglobin. The distances for MbO₂ were subtracted from those for pH 7.0 MbENC. For the glycine residues, the coordinates for the corresponding α carbons were used.

Figure 3.6e. Differences between distances from the iron atom to α carbons in low pH ethyl isocyanide- and oxymyoglobin. The distances for MbO₂ were subtracted from those for low pH MbENC. For the glycine residues, the coordinates for the corresponding α carbons were used.

Figure 3.6f. Differences between distances from the iron atom to α carbons in *n*-propyl isocyanide- and oxymyoglobin. The distances for MbO₂ were subtracted from those for MbPNC. For the glycine residues, the coordinates for the corresponding α carbons were used.

Figure 3.6g. Differences between distances from the iron atom to α carbons in n-butyl isocyanide- and oxymyoglobin. The distances for MbO2 were subtracted from those for Mb nBNC. For the glycine residues, the coordinates for the corresponding α carbons were used.

Figure 3.6h. Helical and helical corner averages of the differences between the distances from the iron atoms to α carbons in various liganded myoglobin structures and oxymyoglobin.

than in MbO₂ (Fig. 3.5.h), however, the extent is only ~ 0.1 Å. Unfortunately, the MbO₂ structure shows some evidence of aquomet character because there is a water atom (35% occupancy) in the heme pocket which lies over the N_C heme atom and which is 1.45 Å from the second oxygen atom of the ligand. This is the same position occupied by a water atom in the aquomet structure (Phillips, 1989).

The temperature factors for the main chain atoms of all the myoglobin structures (Fig. 3.7) exhibit an overall pattern of relatively rigid helices separated by more mobile helical corners. The B-factors for the latter segments tend to be higher than the average B-factor for the corresponding structure whereas those for the helices tend to be lower than the average value. The centers of the G and H helices are the least thermally mobile, but the temperature factors for these segments rise rapidly from their centers out to the GH corner and C-terminus. The D and E helices also have lower than average B-factors; however, there is a peak in mobility at or near the E7 (64) position which is the most noticeable in the MbnBNC structure. The F-helix appears to be the least fixed of the 8 helices. In deoxymyoglobin (Takano, 1977), the histidyl ring lies off normal to the plane of the heme group. Upon ligand binding, the histidyl ring is pulled to lie normal to the heme plane and also closer by means of a shortening of the iron-N_E bond and movement of the iron into the plane of the heme.

An examination of the residuals of a least squares fit of C_α coordinates of the various myoglobin structures shows a similar pattern of accuracy in defining the atomic positions as the B-factor analyses. These comparisons can suffer from the weighting process which gives unit weights to residues 2 through 151. A perhaps better, but more subjective method would have been to weight well-fit segments of the protein more heavily than poorly fit ones. The most valid comparison using

Figure 3.7. Temperature factors of main chain atoms of six myoglobin structures. The B-factor for each residue was obtained by averaging the B-factors for the three backbone atoms and then subtracting the average B-factor for residues A1 through H26 of the corresponding structure.

equal weights for all C_{α} coordinates is between the two low pH structures of MbENC which were refined independently (Fig. 3.8a). In the two low pH MbENC structures, the EF corner is the least defined internal segment of protein backbone. This flexibility of the EF corner extends into much of the F-helix making it less well-determined than other 7 helices when the two low pH structures are superimposed. This result parallels that observed for the temperature factors of the F-helix and further underscores its flexibility.

Comparisons of differently liganded structures of myoglobin are more difficult because of varying temperatures of data collections and differing refinement strategies. The MbO₂ vs MbCO plot does show a similar pattern as the MbENC(low pH) self plot (Fig. 3.8b). The EF corner and all of the F-helix comprise the longest poorly fitted segment when the C_{α} coordinates of oxy and carbonmonoxide myoglobin are superimposed. A 2.5σ peak in residuals is located at the GH corner. The CD corners are fitted much better than in the superimposition of the two low pH MbENC structures. This is probably due to the smaller steric interactions between the diatomic oxygen and carbon monoxide and the His-E7 side chain which cause less disorder at residue CD3-Arg and the rest of the CD corner. Despite the limitations in the $\Delta Fe-C_{\alpha}$, B-factor and superimposition plots, the basic conclusion remains that the tertiary structure of myoglobin makes only a small global expansion to accommodate large ligands. Instead, the ligand is distorted and the side chain of His-E7 becomes disordered.

Figure 3.8a. Errors in coordinates of α carbons obtained from a least squares fit (Kabsch, 1978) of the α carbon coordinates of two independently refined low pH MbENC structures.

Figure 3.8b. Differences in coordinates of α carbons obtained from a least squares fit (Kabsch, 1978) of the α carbon coordinates of carbonmonoxide- and oxymyoglobin.

3.6. Discussion.

The five new myoglobin-alkyl isocyanide structures presented in this chapter allow a detailed stereochemical interpretation of the dependence of affinity on ligand size. The first work on the equilibrium binding of alkyl isocyanides to heme proteins demonstrated that the protein structure surrounding the heme group inhibited binding in general when compared to free heme in solution, and that this inhibition became greater as the size of the isocyanide was increased (St. George and Pauling, 1950; Lein and Pauling, 1956). These results were analyzed more quantitatively by Mims *et al.* (1983b) who measured systematically the binding of a complete series of alkyl isocyanides to myoglobin and to a chelated, pentacoordinate model heme system (Table 1.1; Fig. 1.5; Fig. 3.9). The model heme had a n-propyl arm attached to an imidazole group which bound to the iron atom on one side and mimicked spectral and structural features of the active site in hemoglobin and myoglobin.

Two competing effects determined the equilibrium association constants, K_{RNC} , for alkyl isocyanide binding to myoglobin. First, as the size of the alkyl group of the isocyanide is increased, its tendency to be extruded from water into the protein matrix should increase in proportion to the surface area of the ligand (Mims *et al.*, 1983; Reisberg and Olson, 1980a). This hydrophobic effect is observed directly when isocyanide binding to chelated model heme in soap micelles is examined and K_{RNC} increases by a factor of 2-3 for each additional alkyl carbon atom (Table 1.1; Fig. 3.9). Second, distal pocket residues in the more rigid protein structure tend to sterically hinder the binding of all ligand molecules, and this effect is related in a complicated manner to the size and stereochemistry of the ligand molecule. In contrast, the affinities and rate constants for RNC binding to the

Figure 3.9. Comparison of equilibrium constants for alkyl isocyanide binding to model heme and native and mutant myoglobins. The references are the same as in Table 3.5.

unhindered model heme in benzene is independent of ligand size (Table 1.1; Fig. 3.9).

The results in Fig. 3.9 show that the binding of all the isocyanides is sterically hindered in native myoglobin by at least 3- 4 kcal mol⁻¹ compared to the model heme/soap system (i.e., $\Delta G_{\text{hindrance}} = RT \ln(K_{\text{model heme}}/K_{\text{myoglobin}})$). The increase in affinity in going from methyl to ethyl isocyanide suggests that there is no extra hindrance in myoglobin due to the fourth ligand atom and that favorable hydrophobic contacts between the terminal methyl group and the protein occur. Similarly, the increase in K_{RNC} in going from nBNC to nANC to nHNC is also probably due to the hydrophobic effect and indicates that the seventh and eighth ligand atoms are not sterically hindered by the protein (Fig. 1.5; Table 1.1).

The general hindrance to binding in myoglobin for all the alkyl isocyanides and carbon monoxide is due to the steric restrictions by His-E7 and Val-E11 which prevent a linear Fe-X-Y geometry. In the MbCO structure, bound carbon monoxide is bent, or tilted off normal to the heme plane (Kuryian *et al*, 1986) (Fig. 3.9). Similarly, bound methyl isocyanide cannot adopt a linear Fe=C=N- geometry and is highly distorted (Fig. 3.2a). Thus, the steric hindrance which reduces the association equilibrium constants is manifested in myoglobin more as distortion of the bound ligand than a disruption of heme pocket structure. There is some disorder of the imidazole side chain of His-E7 observed for MbMNC structure, but the effect is small compared to the distortion of the bound ligand.

The addition of a fourth ligand atom at neutral pH produces somewhat greater disorder in the position of the side chain of His-E7. However, the terminal methyl group does fit well into the pocket between residues B10, CD1, E11 and G8 making favorable van der Waals contacts with most of these residues. The

geometry of the $F=C=N$ - bonding system for bound ethyl isocyanide is roughly the same as that for bound MNC. Thus, the simplest interpretation of the increased K_{ENC} is that the hindrance exerted by the protein on the first three ligand atoms is the same as that for MNC and that the fourth ligand atom does not cause additional hindrance, allowing full expression of the favorable hydrophobic effect due to increased ligand size.

3.6.1. Roles of distal pocket residues in regulating isocyanide affinity.

The disorder in His-E7 observed in the isocyanide myoglobin complexes suggests that this residue contacts the bound ligand. This idea is supported by mutagenesis experiments (Table 3.5). When His-E7 is replaced by Gly, the affinities of myoglobin for all 4 isocyanides are increased dramatically. For example, the affinity for nBNC is ~2800 times greater than that for native or wild type protein, and only 2-3 fold less than that for the chelated model heme (Table 1.1, Table 3.5, Fig. 3.9). This result indicates that there is a strong unfavorable steric interaction of nBNC with His-E7 which corresponds to an energetic cost of ~4.6 kcal/mole for the complete outward movement of the imidazole ring of His-E7 and the general expansion of the protein in the MbnBNC structure. Evidence that this interaction between His-E7 is due to the size and not the polarity of the imidazole side chain comes from the results for His-E7→Phe mutation (Table 3.5; Fig. 3.9). Phe-E7 myoglobin exhibits association equilibrium constants which are only 2-3 fold greater than those for the native protein. Affinity constants intermediate between those observed for native and His-E7→Gly myoglobin were observed when His-E7 was replaced with Val and Leu (Table 3.5).

Heme complex	Point mutations			
	k' ratio(K_A ratio)	k' ratio(K_A ratio)	k' ratio(K_A ratio)	k' ratio(K_A ratio)
His-E7→	Gly	Val	Leu	Phe
MNC	83 (55.3)	6 (2.1)	15 (30)	1.5 (2.5)
ENC	220 (32.8)	32 (2.5)	15 (30)	1.4 (2.3)
nPNC	640 (640.)	18 (3.5)	9.8 (49)	0.7 (2.3)
nBNC	570 (2850)	6 (2.6)	8.3 (28)	0.8 (2.0)
Val-E11→	Ala	Ile	Phe	
MNC	3.2 (17.8)	0.4 (0.08)	0.1 (10)	
ENC	2.6 (11.3)	0.3 (0.03)	0.1 (10)	
nPNC	2.6 (43.3)	0.4 (0.11)	0.1 (20)	
nBNC	4.7 (235.)	0.6 (0.27)	0.2 (20)	
Leu-B10→	Ala	Val	Phe	
MNC	4.8 (2.7)	2.0 (1.8)	1.6 (0.1)	
ENC	10.1 (4.8)	3.0 (6.0)	1.3 (0.09)	
Protoheme IX→Protoheme IX dimethyl ester				
MNC	1.6 (2.1)			
ENC	2.5 (1.7)			
nPNC	4.5 (5.5)			
nBNC	4.0 (9.3)			
Chelated model heme→				
	in benzene	in 2% soap		
MNC	1900 (9100)	105 (522)		
ENC	3500 (920)	460 (180)		
nPNC	6600 (8600)	1800 (760)		
nBNC	7100 (7000)	5400 (5400)		

Table 3.5. Ratios of association rate (k' ratio) and equilibrium (K_A ratio; in parentheses) constants of mutant myoglobins, protoporphyrin (IX) dimethyl ester (DME)-substituted myoglobin, and chelated model heme in benzene and 2% soap to the association rate and equilibrium constants of native sperm whale myoglobin. Parameters for native myoglobin are in Table 1.1. Binding parameters for position E7 and E11 mutants are taken from Carver *et al.*, 1990, for the position B10 mutants from Carver *et al.*, 1992 (in press), for the DME-substituted myoglobin from Carver *et al.*, 1991, and for model heme from Olson *et al.*, 1983.

The X-ray structures of the alkyl isocyanide myoglobin complexes do not show significant displacement of the side chains of Val-E11 and Leu-B10 from their positions in oxymyoglobin. However, work with E11 and B10 mutants suggest that these residues do sterically hinder the bound isocyanide ligands and, along with the side chain of His-E7 are responsible for the distorted $\text{Fe}=\text{C}=\text{N}-\text{R}$ geometry. When the native amino acids at E11 and B10 are replaced with Ala, 3-5 fold increases in affinity for MNC and ENC binding are observed (Table 3.5, Fig. 3.9). In general, when larger residues are substituted at these positions, the affinities tend to decrease. At the E11 position, the stereochemistry of the side chain is important. For example, the affinity of myoglobin for isocyanides increases when Val-E11 is replaced with Phe even though the side chain volume has increased markedly. In contrast, when Val-E11 is replaced with Ile, K_{RNC} decreases 2 to 7-fold.

The E11 and B10 residues are not very mobile in myoglobin, and as a result, these residues distort the stereochemistry of the bound ligand. The free energies of this distortion can be estimated by comparing the affinity of the native protein with the affinities of the Val-E11→Ala and Leu-B10→Ala mutants:

$\Delta G_{\text{hindrance}} = RT \ln(K_{\text{Mutant}}/K_{\text{native}})$. For the E11 position, the $\Delta G_{\text{hindrance}}$ is 1.7 kcal mol⁻¹ and 1.4 kcal mol⁻¹ for MNC and ENC binding respectively. For the B10 position, the $\Delta G_{\text{hindrance}}$ is 0.6 kcal mol⁻¹ and 0.9 kcal mol⁻¹ for MNC and ENC binding, respectively. These values are small compared to that calculated for distortion by His-E7 from data for the His-E7→Gly mutation in which $\Delta G_{\text{hindrance}} = 2.3$ kcal mol⁻¹ and 2.0 kcal mol⁻¹ for MNC and ENC binding, respectively. This conclusion is supported by results for the double mutant, His-E7→Ala/Val-E11→Ala, which shows a $K_{\text{MNC}} = 1 \times 10^8 \text{ M}^{-1}$ and a $\Delta G_{\text{hindrance}} = 3.0$

kcal mol⁻¹ (Egeberg *et al.*, 1991). The latter value is roughly the sum of the hindrance values for the single mutations and suggests that the effects of the Val-E11 and His-E7 residues are additive.

A ¹H-NMR study by Mims *et al.* (1983a) investigated the ring-current shifted resonances of the C_{γ2} protons of Val-E11 when various ligands were bound. The basis for the study was that large bound ligands should displace the C_{γ2} protons away from the heme center and thus decrease the ring current's effect on the chemical shifts of these resonances. No differences were observed for the Val-E11 resonances in the CO, MNC and ENC complexes of myoglobin. This fits well with the lack of gross distortion seen in the distal heme pocket. Shifts of 0.3ppm and 0.7ppm were observed for nPNC and nBNC, respectively, and are consistent with a general, but small, expansion of the protein with concomitant movement of C_{γ2} away from the heme center.

In sperm whale myoglobin, the guanidino group of Arg-CD3 forms hydrogen bonds with Asp-E3 and the heme-6-propionate. This triad of electrostatic interactions has been suggested to stabilize the 'down' position of His-E7 (Lecomte and LaMar, 1985). In addition, a water molecule appears to interconnect Arg-CD3, His-E7 and Thr-E10 and further stabilizes the 'down' position. The X-ray structures of MbnPNC and MbnBNC show, respectively, partial and complete disruption of this lattice of hydrogen bonding interaction at the heme-solvent interface. The free energy cost of disrupting this network of hydrogen bonds and salt bridges can be estimated from ligand binding studies with native myoglobin reconstituted with protoporphyrin IX dimethyl ester (Table 3.5). A ≤ 2-fold increase in the affinity for MNC and ENC was observed when the heme-6-propionate group was esterified. This small effect on MNC and ENC binding fits

with the structural observations that the side chain of His-E7 is primarily in the 'down' position when ENC and MNC are bound at pH 7.0. Thus, the binding of these ligands is not greatly facilitated by disruption of the His-E7, Arg-CD3, and heme-6-propionate interactions.

In contrast, the affinity of myoglobin for nPNC and nBNC increases 9 fold when the heme-6-propionate group is esterified. The corresponding change in binding free energy ($1.3 \text{ kcal mol}^{-1}$) provides an estimate for the energy needed to disrupt the Arg-CD3, His-E7, and heme-6-propionate interactions since these interactions must be broken to accommodate bound nPNC and nBNC in the native protein. Thus, the X-ray results coupled with chemical modification of the heme group suggest strongly that the lattice of hydrogen and salt bridge interactions at the heme periphery holds the E-helix and CD-corner near the heme group and inhibits the binding of large ligands. A similar conclusion was obtained by Carver *et al.* (1991) who mutated Lys-CD3 in pig myoglobin.

3.6.2. The histidine gate theory and ligand binding kinetics.

A number of studies have suggested that the distal histidine acts as a gate to the entry and exit of ligands from the distal portion of the heme pocket in myoglobin. Previous crystallographic studies and those in this work have shown that His-E7 is the most mobile residue near the binding site and occupies different positions in different liganded structures. In MbO₂, it is locked tightly in position by a hydrogen bond with bound oxygen (Phillips & Schoenborn, 1981). In MbCO, it swings slightly away from bound carbon monoxide. In ferric sperm whale, seal and horse heart myoglobin, the distal histidine occupies a position similar to that found in sperm whale oxymyoglobin, near the water molecule coordinated to the

oxidized iron atom. When imidazole is bound to ferric sperm whale myoglobin, the side chain of His-E7 swung away from the iron atom toward the protein surface (Bolognesi *et al*, 1982; Lionetti *et al.*, 1991). A similar position for His-E7 was observed when phenyl hydrazine was reacted with ferric sperm whale myoglobin to generate an aryl adduct with the iron atom (Ringe *et al*, 1984). These latter E7 conformations are almost identical to the His-E7 position observed in MbnPNC and MbnBNC. As described in Chapter 1, section 1.4.1, Case, Karplus and coworkers have suggested that the major trajectory for ligand entrance into and exit from the heme pocket is a pathway leading from the solvent between Thr-E10, Val-E11 and His-E7 to the heme iron atom, and that access to this pathway occurs when the imidazole side chain rotates about its C $_{\alpha}$ -C $_{\beta}$ bond into the 'up' position seen in the large ligand complexes.

Kinetics studies have confirmed that the distal histidine is a large part of the steric barrier to ligand binding. Mims *et al.* (1983b) systematically compared the O $_2$, CO and RNC binding properties of a wide variety of heme proteins and pentacoordinate model heme compounds. Based on their work and that of Stetzkowski *et al* (1979), they suggested that the distal histidine in myoglobin raises the kinetic barrier to the binding of triatomic or larger ligands by $\geq 2\text{kcal mol}^{-1}$.

The more recent kinetic data for E7-position mutants confirms this view (Table 3.5). Removal of the large imidazole side chain by the His-E7 $\rightarrow$ Gly mutation increases the association rate constants 80-, 220-, 640-, and 570-fold, respectively, for MNC, ENC, nPNC and nBNC binding. When the imidazole ring is replaced with a large, neutral side chain as in the His-E7 $\rightarrow$ Phe mutant, the association rate constants are little affected. When the salt bridges and hydrogen bonding between His-E7, Arg-CD3 and the heme-6-propionate are broken by

esterification of the heme propionates, the isocyanide association rate constant increases 2- to 4-fold, with the largest effect occurring for n-butyl isocyanide binding.

3.6.3. Quantum yields and geminate recombination rates for isocyanides.

Flash photolysis experiments are used to determine quantum yields (Q) and geminate recombination rates, both of which reflect intramolecular ligand rebinding within the protein matrix. These processes are most simply described using a simple, two step consecutive reaction scheme (Carver *et al*, 1990):

In the case of isocyanide liganded complex, the ground state A absorbs one photon, the iron-ligand bond breaks with a photophysical quantum yield, Q_{ns} , of ~ 1.0 , and state C is formed. From state C the ligand can escape to solvent to form Mb + X at a rate equal to k_{CX} , or rebind geminately on a nanosecond time scale to reform the ground state, A, at a rate k_{CA} . The overall quantum yield (Q_{overall}) is the fraction of ligand molecules which escape to solvent (F_{esc}) and is given by:

$$Q_{\text{overall}} = Q_{\text{ns}} (F_{\text{esc}}) \text{ where } F_{\text{esc}} = (k_{\text{CX}})/(k_{\text{CA}} + k_{\text{CX}}).$$

In the case of CO and the alkyl isocyanides, $Q_{ns} \cong 1.0$ so $Q_{overall} \cong F_{esc}$, the fraction of escape from state C.

From these relationships, it can be seen that a high $Q_{overall}$ indicates that the fraction of geminate recombination is small, whereas low values indicate that the ligand is 'trapped' in the protein and the fraction of escape is small ($k_{CA} \gg k_{CX}$).

As shown in Table 3.6, methyl isocyanide is 'trapped' to a greater extent inside the protein, and as a result, the fraction of escape of MNC ($F_{esc} \cong 0.15$) is significantly less than that observed for O_2 and CO ($F_{esc} \cong 0.95 - 0.50$). Similar results were observed for ethyl isocyanide and n-propyl isocyanide myoglobin complexes which all showed low quantum yields for photodissociation, presumably because these ligands are kept in the protein by the 'down' conformation of His-E7. In contrast, the quantum yield for nBNC is unusually high, $\cong 0.4$, and this result correlates with the structural observation that the bound n-butyl moiety has completely opened the histidine gate allowing rapid escape following photodissociation. Studies with mutant myoglobins support this view that residue E7 regulates the overall quantum yield and extent of isocyanide escape. For MNC and ENC there are 4 to 5-fold increases in quantum yields when Gly replaces His-E7, whereas the value of Q decreases when His-E7 is replaced by Phe (Carver *et al.*, 1990). This indicates that the extent of escape from state C depends greatly on the size of the residue at the E7 position. Unfortunately, the Phe-E7 mutant has not been crystallized; however the photochemical results indicate that the phenyl ring occupies the 'down' position and acts as a strong barrier to ligand escape.

Chapter 4.

X-RAY STRUCTURES OF THE ALKYL ISOCYANIDE COMPLEXES OF HEMOGLOBIN

4.1. General description of the refined structures

The final refinement statistics for the four alkyl isocyanide derivatives of human hemoglobin are shown in Table 4.1. The refined structures model the respective data well as shown by final R-factors that are $< 19\%$. The structures have good geometry as shown by the rms deviations in bond lengths and angles of ~ 0.02 Å and 2.4° , respectively. These targeted values were reached by adjusting the relative weighting of data and geometric constraints in the last few refinement cycles.

Estimates of the uncertainty of atomic coordinates for these alkyl isocyanide hemoglobin complexes were obtained by three methods. A low estimate is given by the bond σ 's, ~ 0.02 Å (Table 4.1). A higher estimate is obtained from a plot of R-factor vs inverse resolution which is compared to theoretical plots of coordinate error (Fig. 4.1) (V. Luzatti, 1952). The errors for the hemoglobin structures fall mostly between 0.20 Å and 0.25 Å on the Luzatti plot. An additional estimate was obtained from a comparison of two HbENC structures refined from the same data but using a different random seed to generate initial velocities in the simulated annealing step. The rms residual for the least square fit of C_α coordinates of these two structures is 0.13 Å for α -chains and 0.14 Å for β -chains (Table 4.2). Superimpositions of C_α coordinates for the various high resolution hemoglobin structures (Table 4.2) shows that the rms residual between each of the four HbRNC

Ligand	R(%)	$\sigma_{\text{bond}}(\text{\AA})$	$\sigma_{\text{angle}}(^{\circ})$	$\sigma_{\text{dihedral}}(^{\circ})$	$\sigma_{\text{improper}}(\text{\AA})$	$Q_{\text{His-E7}}$			Q_{RNC}		
						α	β		α	β	
HbMNC	17.5	0.020	2.39	24.9	5.63	1.01	1.01		1.03	1.03	
HbENC	17.1	0.021	2.44	24.6	6.13	1.00	1.00		1.03	0.97	
HbnPNC	16.9	0.019	2.69	24.3	6.22	0.98	0.95		1.02	0.97	
HbnBNC	17.9	0.020	2.30	25.1	5.95	0.98	0.96		1.09	0.97	

Table 4.1. Refinement statistics for HbRNC structures. R-factors are standard and calculated by the program XPLOR as $\sum(|F_o| - |F_c|)/\sum|F_o|$ for all reflections. The σ 's for bonds, angles, dihedral angles and improper contacts are rms deviations from restrained parameters as reported by XPLOR. The refined occupancy factors (Q's) for His-E7 and bound alkyl isocyanides (RNC) were determined after many rounds of position and temperature factor refinement.

Figure 4.1. Luzatti plot for HbMNC compared to theoretical Luzatti plots of coordinate error (Luzatti, 1952). The coordinate errors fall between 0.2 and 0.25 $\text{\AA}$ for the HbMNC structure.

Ligands→ ↓		MNC	ENC	nPNC	nBNC
O ₂	α	.32 Å	.35 Å	.36 Å	.34 Å
	β	.37 Å	.37 Å	.40 Å	.39 Å
MNC	α		.21 Å	.20 Å	.24 Å
	β		.24 Å	.23 Å	.27 Å
ENC	α		.13 Å ¹	.20 Å	.21 Å
	β		.14 Å ¹	.20 Å	.25 Å
nPNC	α				.23 Å
	β				.23 Å

Table 4.2 The rms residuals for least squares superimposition of C_α coordinates for HbO₂ and four HbRNC X-ray structures. The α subunit fits used residues 1-139 and the β subunit fits used residues 3-144.

¹The two Hb-ENC structures were independently refined from the same data but used different random seeds for the initial velocity distribution in the simulated annealing step of refinement.

structures and HbO_2 is 2-3 times that for the two HbENC structures. The pairwise rms residuals for the HbRNC structures are < 2 times that for the two independently refined HbENC structures. Thus, each of the isocyanide hemoglobin complexes is significantly different from the oxyhemoglobin structure but is much less different from each of the other HbRNC structures.

4.2. Structure of the bound alkyl isocyanides in the α and β subunits.

The final observed electron densities and modeled geometries for the four Hb-alkyl isocyanides structures are shown in Fig. 4.2.a-p. Each of the eight ligand-Fe complexes, with the exception of βnBNC , was well-modeled into electron density that was sufficient to contain the polar isocyano group and the apolar alkyl moiety. However, the electron density was not of high enough resolution to position precisely each ligand atom. The Fe-C-N angle is much less than 180° in all eight complexes. In α subunits, this distortion is pronounced, and even in β subunits, where less hindrance is observed, bound MNC adopts a nonlinear Fe-C-N geometry. The Fe-C bond length was restrained to be 1.9 Å and varied as much as 0.1 Å from this target value. In α subunits, the Fe-C bond length decreases from 1.94 Å in the MNC and ENC complexes to 1.89 Å in αnPNC and 1.80 Å in αnBNC . In β subunits, the Fe-C bond lengths are greater than in α subunits and are 2.00 Å, 1.98 Å, 1.95 Å and 2.02 Å for MNC, ENC, βnPNC and βnBNC , respectively.

The alkyl groups of the isocyanide ligands lie in the interior apolar portion of the heme pocket in both subunits (Fig. 4.2a-p). The projection of the αRNC alkyl groups onto the heme plane lies on the N_C pyrrole (Fig. 4.2a-h). The projection of βRNC alkyl groups lies between the N_C and N_B pyrroles for βMNC and βENC ,

Figure 4.2a. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the methyl isocyanide α subunit complex. Shown is a view of the heme pocket looking from the distal residue Val-E11. The terminal carbon atom of the methyl isocyanide ligand makes a favorable contact with residue Leu-B10. The density which trails from the distal histidine to the top left corner of the figure indicates that this residue is disordered.

Figure 4.2b. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the methyl isocyanide α subunit complex. Shown is a view of the α heme pocket looking from the distal residue Leu-B10.

Figure 4.2c. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the ethyl isocyanide α subunit complex. Shown is a view of the α heme pocket looking from the distal residue Val-E11.

Figure 4.2d. Final electron density ($2F_o - F_c$) α_c and refined structure for the ethyl isocyanide α subunit complex. Shown is a view of the α heme pocket looking from the distal residue Leu-B10.

Figure 4.2e. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the n-propyl isocyanide α subunit complex. Shown is a view of the α heme pocket looking from the distal residue Val-E11.

Figure 4.2f. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the n-propyl isocyanide α subunit complex. Shown is a view of the α heme pocket looking from the distal residue Leu-B10.

Figure 4.2g. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the n-butyl isocyanide α subunit complex. Shown is a view of the α heme pocket looking from the distal residue Val-E11.

Figure 4.2h. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the n-butyl isocyanide α subunit complex. Shown is a view of the α heme pocket looking from the distal residue Leu-B10. The alkyl moiety of the n-butyl isocyanide ligand avoids unfavorable contacts with residue Leu-G8 by occupying a site between Phe-CD1 and Met-B13.

Figure 4.2i. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the methyl isocyanide β subunit complex. Shown is a view of the β heme pocket looking from the distal residue Val-E11. The terminal carbon of the methyl isocyanide ligand is much farther from the residue Leu-B10 than in the α subunit complex.

Figure 4.2j. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the methyl isocyanide β subunit complex. Shown is a view of the β heme pocket looking from the distal residue Leu-B10.

Figure 4.2k. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the ethyl isocyanide β subunit complex. Shown is a view of the β heme pocket looking from the distal residue Val-E11. The ethyl isocyanide ligand is more normal to the heme plane than in the α subunit complex.

Figure 4.21. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the ethyl isocyanide β subunit complex. Shown is a view of the β heme pocket looking from the distal residue Leu-B10. The ethyl isocyanide ligand is almost normal to the heme plane and makes only favorable contacts with surrounding distal residues.

Figure 4.2m. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the n-propyl isocyanide β subunit complex. Shown is a view of the β heme pocket looking from the distal residue Val-E11. The terminal carbon of the n-propyl isocyanide ligand is disordered and makes unfavorable contacts with residue Leu-G8.

Figure 4.2n. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the n-propyl isocyanide β subunit complex. Shown is a view of the β heme pocket looking from the distal residue Leu-B10.

Figure 4.2o. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the n-butyl isocyanide β subunit complex. Shown is a view of the β heme pocket looking from the distal residue Val-E11. The n-butyl isocyanide ligand is disordered as shown by the poorly observed density for the fifth and sixth ligand atom.

Figure 4.2p. Final electron density $(2F_o - F_c)\alpha_c$ and refined structure for the n-butyl isocyanide β subunit complex. Shown is a view of the β heme pocket looking from the distal residue Leu-B10. The continuous density between the n-butyl isocyanide ligand and residue Val-E11 is indicative of the greater disorder of the ligand when compared to the α subunit complex.

closer to the N_C pyrrole for $\beta nPNC$ and closer to the N_B pyrrole for $\beta nBNC$ (Fig. 4.2i-p).

The αRNC alkyl groups in general lie closer and more parallel to the heme ring than do the βRNC alkyl groups (Fig. 4.2a-h). This is especially evident when comparing the αENC to the βENC complex, and the $\alpha nBNC$ to the $\beta nBNC$ structure. The terminal carbon in βENC is 4.59 Å from the nearest heme atom whereas that of αENC is 3.43 Å (Table 4.3a-b). Likewise, the terminal carbon of $\beta nBNC$ is >5. Å from the nearest heme atom whereas the corresponding distance in $\alpha nBNC$ is 3.03 Å. In apparent contrast to this trend, the terminal carbon atom for $\beta nPNC$ is 0.53 Å closer to a heme atom than is the same atom in $\alpha nPNC$; however, this terminal atom is disordered in the $\beta nPNC$ structure (Fig. 4.2.m,n).

In α subunits, the side chain of Leu-B10 interacts sterically with the alkyl ligand atoms in all four RNC complexes. The electron density surrounding the alkyl carbon atom of boundMNC is not resolved from that of the $C_{\delta 2}$ atom of $\alpha Leu-B10$ and the interatomic distance is 3.03 Å which is indicative of unfavorable van der Waals contacts. Similar unfavorable contacts with Leu-B10 occur in the ENC and nPNC complexes of α subunits. For $\alpha nBNC$, the ligand alkyl group is farther away from the B10 residue and lies closer to the heme in order to accommodate the 6th ligand atom near Met-B13. Even in this conformation, the terminal ligand atom still makes a short Van der Waals contact with the C_ϵ atom of Met-B13.

In β subunits, the side chain of Val-E11 makes short van der Waals contacts with the second and third ligand atom in all four alkyl-isocyanide structures. In βMNC , the $C_{\gamma 2}$ atom of Val-E11 is 0.73 Å closer to the terminal ligand atom than in the αMNC complex. In αMNC , the $C_{\gamma 2}$ atom makes a favorable contact of 3.55

Ligand(atom)→		MNC(C ₃)		ENC(C ₄)		nPNC(C ₅)		nBNC(C ₆)	
	Atom	Dist.(Å)	Atom	Dist.(Å)	Atom	Dist.(Å)	Atom	Dist.(Å)	Atom
Heme atoms	N _C	3.62	N _C	3.43	C _{2C}	3.79	C _{BC}	3.03	
	C _{4C}	3.80	C _{1C}	3.44	C _{3C}	3.84	C _{3C}	3.32	
	C _{1C}	3.92	C _{2C}	3.56	C _{1C}	3.85	C _{AC}	3.65	
	Fe	3.97	C _{4C}	3.71	C _{HC}	3.97	C _{2C}	3.73	
			C _{3C}	3.77	N _C	4.00	C _{4C}	3.84	
Protein atoms			C _{HC}	3.84					
	29C _{δ2}	3.04	101C _{δ2}	3.32	29C _{δ2}	3.56	32C _E	2.76	
	43C _ζ	3.80	29C _{δ2}	3.63	32C _E	3.59	43C _{E1}	3.04	
	101C _{δ2}	3.95	32C _E	4.09	101C _{δ2}	3.79	43C _ζ	3.59	
	58C _{δ2}	4.02	101C _γ	4.41	101C _{δ1}	4.03	33C _{E1}	3.89	
C _α 's	62C _{γ2}	4.28	101C _{δ1}	4.41	101C _γ	4.35	32S _δ	3.93	
	29	6.05	29	6.18	29	5.91	39	5.78	
	62	6.59	101	6.22	101	5.98	29	6.37	
	58	7.01	62	7.02	62	7.25	40	6.86	
	59	7.26	98	7.74	98	7.41	42	6.96	
	101	7.28	59	7.98	32	7.69	101	7.01	

Table 4.3a. Nearest atoms to terminal ligand carbon in the alkyl isocyanide complexes of α subunits. Residue numbers correspond with helical positions as follows:
 29 - Leu-B10, 32 - Met-B13, 43 - Phe-CD1, 58 - His-E7, 62 - Val-E11, and 101 - Leu-G8.

Ligand(atom)→	MNC(C ₃)		ENC(C ₄)		nPNC(C ₅)		nBNC(C ₆)	
	Atom	Dist.(Å)	Atom	Dist.(Å)	Atom	Dist.(Å)	Atom	Dist.(Å)
Heme atoms	N _C	3.52	C _{1C}	4.59	C _{HC}	3.26		
	C _{1C}	3.73	N _C	4.66	C _{1C}	3.52		
	Fe	3.77	C _{HC}	4.68	C _{4B}	3.70		
	C _{4C}	4.11	Fe	4.78	C _{2C}	3.96		
Protein atoms			C _{4B}	4.82	N _C	4.11		
	67C _{γ2}	3.55	28C _γ	3.29	106C _{δ2}	2.95	106C _{δ1}	3.11
	28C _γ	3.99	106C _{δ2}	3.39	106C _{δ1}	3.22	106C _{δ2}	3.22
	106C _{δ2}	4.00	67C _{γ2}	3.43	106C _γ	3.55	28C _γ	3.33
C α 's	42C _ζ	4.13	28C _{δ2}	3.63	31C _{δ2}	3.90	28C _α	3.45
	28C _{δ1}	4.31	67C _{γ1}	4.10	106C _β	4.13	28C _β	3.45
	28	5.74	28	4.77	28	5.39	28	3.43
	67	6.03	64	5.50	106	5.43	27	5.01
	64	6.35	67	5.73	67	6.51	63	5.20
	63	6.84	63	6.60	103	7.06	106	5.77
	106	7.34	106	6.79	31	7.08	67	6.11

Table 4.3b. Nearest atoms to terminal ligand carbon in the alkyl isocyanide complexes of β subunits. No heme atoms are within 5 Å of the C₆ ligand atom of the β nBNC complex. Residue numbers correspond with helical positions as follows:
 27 - Ala-B9, 28 - Leu-B10, 31 - Met-B13, 42 - Phe-CD1, 63 - His-E7, 64 - Gly-E8, 67 - Val-E11, and 106 - Leu-G8.

Å with the ligand methyl group (Table 4.3b). Also, the electron density surrounding the ligand is not resolved from that of β Val-E11 in the β MNC, nPNC and nBNC complexes (Fig. 4.2j,n,p). As in the α subunits, the position of the side chain of β Leu-B10 appears to force a non-linear Fe=C=N-R geometry; however in β subunits it is the C_γ atom of Leu-B10, not the $C_{\delta 2}$ atom, which is closest to the ligand. The distance from the C_γ atom of β Leu-B10 is 0.95 Å farther from the ligand methyl carbon in the β MNC complex than the $C_{\delta 2}$ atom of α Leu-B10 is from the ligand methyl carbon in the α MNC complex (Table 4.3a,b). This allows a less distorted ligand geometry in β subunits. Unfavorable steric interactions with the side chain of β Leu-G9 appear to cause the terminal carbon in β nPNC to be disordered (Table 4.3b; Fig. 4.2n). This same side chain causes the bound nBNC ligand in β subunits to adopt a much different orientation with respect to the heme than that for nPNC in order to fit the 6th ligand atom into the interior of the distal pocket (Fig. 4.2h).

In Table 4.4, the temperature factors assigned to ligand atoms are compared with those assigned to the iron atoms and C_α and C_β atoms of the entire protein. The B-factors increase from the bound C_1 atom out to the terminal methyl carbon atom for all eight ligand complexes indicating that the alkyl groups are significantly more disordered than the isocyano head group. The alkyl moieties of β nPNC, α nBNC and β nBNC have significantly higher B-factors than those for the alkyl groups of the five smaller ligand complexes and the B-factors of the C_α and C_β atoms in the protein. However, the first ligand atoms of all eight protein-ligand complexes have B-factors lower than the average for main chain C_α atoms implying that complete saturation of the iron atoms with ligand occurred in the crystals. This, in turn, suggests strongly that poor definition of the terminal alkyl

Ligand ↓	subunit ↓	Irons	Ligand atoms							Protein atoms	
			O ₁	O ₂						C _α	C _β
O ₂	α	27.4	17.8	33.8						32.1	36.3
	β	36.7	42.1	37.4						37.3	40.0
			C ₁	N ₂	C ₃	C ₄	C ₅	C ₆			
MNC	α	25.0	18.5	24.1	25.9				28.1	28.1	
	β	32.8	23.1	32.3	36.1				33.6	34.3	
ENC	α	18.6	20.3	23.3	25.7	30.2				25.4	25.7
	β	21.2	23.6	33.5	33.7	38.6				30.5	31.2
nPNC	α	24.0	24.5	28.6	27.1	31.9	30.9			28.7	29.3
	β	28.5	27.7	36.7	43.1	50.1	49.5			34.2	34.5
nBNC	α	32.4	27.2	39.3	40.3	46.6	49.8	48.3	30.7	31.0	
	β	35.6	32.4	43.5	47.3	54.6	60.7	62.1	37.3	40.0	

Table 4.4. Temperature factors of iron, ligand and protein atoms in oxyhemoglobin and the four alkyl isocyanide-hemoglobin structures. Units are in Å² which are related to the rms unidirectional displacement (Δ) by $(\Delta r^2) = (B/8\pi^2)$.

carbon atoms in the nPNC and nBNC complexes is due to disorder and not the absence of bound ligand.

In all four β subunits complexes, the B-factors of the ligands are greater than those for the corresponding α subunit complexes. This difference in ligand disorder between the subunits was also observed in the structures of oxyhemoglobin (Shaanan, 1983). In fact, the atoms of bound dioxygen in β subunits have such high B-factors that one wonders whether the sample was partly oxidized or the structure needs additional refinement or redetermination. However, Derewenda *et al* (1990) also observed poorly defined electron density for the bound ligand in β subunits of carbon monoxide human hemoglobin. This latter result combined with the isocyanide data suggests strongly that ligand disorder is an inherent property of ferrous β subunits.

4.3. Conformation of distal pocket residues: His-E7, Val-E11, Leu-B10 and Leu-G8.

The positional center of the side chain of the distal histidine is 5.83 Å from the iron atom in αO_2 subunits and 5.72 Å in βO_2 subunits. The imidazole group moves little in response to the binding of large alkyl isocyanides in either subunit (Fig. 4.3b-h). The position of $\alpha\text{His-E7}$ does not vary in distance from the heme iron in the αO_2 , αMNC and αENC complexes. This residue does move 0.32 Å farther away from the heme iron in the αnPNC complex. However, in the αnBNC complex, the position of His-E7 is only 0.15 Å farther from the heme iron than in αO_2 subunits. In β subunits, the side chain of His-E7 moves 0.23 Å away from the heme iron in the MNC complex compared to its position in oxy subunits. This is due to a twisting of the imidazole ring away from the ligand by a rotation

Figure 4.3a. Distances from the iron atom to side chain centers of position in the alkyl isocyanide and oxy α subunit complexes. For the Gly residues, the coordinates for the corresponding α carbons were used.

Figure 4.3b. Differences between distances from the iron atom to side chain center of position in the methyl isocyanide and oxy α subunit complexes. For the Gly residues, the coordinates for the corresponding α carbons were used.

Figure 4.3c. Differences between distances from the iron atom to side chain center of position in the ethyl isocyanide and oxy α subunit complexes. For the Gly residues, the coordinates for the corresponding α carbons were used.

Figure 4.3d. Differences between distances from the iron atom to side chain center of position in the n-propyl isocyanide and oxy α subunit complexes. For the Gly residues, the coordinates for the corresponding α carbons were used.

Figure 4.3e. Differences between distances from the iron atom to side chain center of position in the n-butyl isocyanide and oxy α subunit complexes. For the Gly residues, the coordinates for the corresponding α carbons were used.

Figure 4.4a. Distances from the iron atom to side chain centers of position in the alkyl isocyanide and oxy β subunit complexes. For the Gly residues, the coordinates for the corresponding α carbons were used.

Figure 4.4b. Differences between distances from the iron atom to side chain center of position in the methyl isocyanide and oxy β subunit complexes. For the Gly residues, the coordinates for the corresponding α carbons were used.

Figure 4.4c. Differences between distances from the iron atom to side chain center of position in the ethyl isocyanide and oxy β subunit complexes. For the Gly residues, the coordinates for the corresponding α carbons were used.

Figure 4.4d. Differences between distances from the iron atom to side chain center of position in the n-propyl isocyanide and oxy β subunit complexes. For the Gly residues, the coordinates for the corresponding α carbons were used.

Figure 4.4e. Differences between distances from the iron atom to side chain center of position in the n-butyl isocyanide and oxy β subunit complexes. For the Gly residues, the coordinates for the corresponding α carbons were used.

about the $C_\gamma-C_\delta$ bond. The imidazole side chain remains at the same distance from the heme in the β ENC complex, but does move an additional 0.25 Å away from the heme iron in β nPNC and nBNC complexes. However, the total displacement from its position in β O₂ subunit is only 0.48 Å for these larger isocyanides, which is significantly less than the large outward movement seen in the nPNC and nBNC complexes of sperm whale myoglobin.

Based on the positional center of the side chain, Val-E11 is the closest of the distal residues to the heme iron in both the α and β subunits of liganded hemoglobin (Fig. 4.3a). In oxyhemoglobin, the center of the Val-E11 side chain is 5.58 Å from the heme iron in α subunits and 5.08 Å in β subunits (Table 4.5). The isopropyl side chain appears to be more immobile in α subunits and moves less to accommodate the large isocyanide ligands than in β subunits. In the α MNC complex, the Val-E11 side chain is displaced 0.21 Å from its position in α O₂ and moves less than 0.2 Å farther from the heme iron in ENC, nPNC and nBNC complexes. In contrast, the side chain of Val-E11 in the β MNC complex is pushed 0.72 Å away from the heme iron when compared to its position in the β O₂ complexes. However, its position in the β ENC, β nPNC and β nBNC complexes is not significantly different from that in the β MNC complex (Fig. 4.4b-d). Thus, the third atom in the isocyanide ligands appears to cause greatest shift in position of the Val-E11 residue in both subunits, with the greatest displacement occurring in β subunits.

The bound MNC, ENC and nPNC ligands in α subunits are constrained in a hydrophobic pocket capped by the relatively immobile side chain of α Leu-B10 (Fig. 4.2a,c,e). As described in section 4.2, the side chain of Leu-B10 occupies a position which prevents the preferred linear Fe-C-N geometry for bound alkyl

isocyanides. The positional center of this side chain is 7.88 Å from the heme iron in αO_2 subunits and 7.23 Å in βO_2 subunits (Table 4.5). Like that of $\alpha\text{Val-E11}$, the side chain of $\alpha\text{Leu-B10}$ moves very little to accommodate the large alkyl isocyanides. The third ligand atom in the αMNC complex causes no significant change in position of the $\alpha\text{Leu-B10}$ side chain when compared to its position in the αO_2 complex. In the αENC and αnPNC complexes, the center of Leu-B10 is ~ 0.3 Å farther from the heme iron, and in αnBNC , this side chain is displaced only 0.17 Å from its position in αMNC . The 6th ligand atom of nBNC bound to the α subunits is located between the side chain of $\alpha\text{Phe-CD1}$ and $\alpha\text{Met-B13}$ (Fig. 4.2h). This latter conformation reduces the unfavorable van der Waals contacts between the bound ligand and the $\alpha\text{Leu-B10}$ side chain compared to those seen in the three smaller αRNC complexes.

In β subunits, the positional center of Leu-B10 is displaced 0.70 Å farther from the heme iron by the third ligand atom in the βMNC complex than in the corresponding βO_2 structure (Table 4.5). This places the $\text{C}_{\delta 1}$, $\text{C}_{\delta 2}$ and C_{γ} carbons of $\beta\text{Leu-B10}$ approximately equidistant from the heme plane creating significantly more room for the alkyl carbons of the bound isocyanide (Table 4.3a,b). There is no significant difference in the position of Leu-B10 in the βMNC , βENC and βnBNC structures. There is an additional 0.25 Å movement of Leu- βB10 away from the heme iron in the βnPNC complex. This places Leu- βB10 about 1.0 Å farther from the heme iron than in the βO_2 structure.

Thus, the extent and type of movement observed for Leu-B10 differ in α and β subunits when comparing the isocyanide structures to oxyhemoglobin. In α subunits, isocyanide binding causes little movement of Leu-B10, ≤ 0.2 Å to 0.3 Å, and the $\text{C}_{\gamma}\text{-C}_{\delta 2}$ bond still points toward the heme plane. In β subunits, the side

chain of Leu- β B10 is displaced as much as 1.0 Å away from the heme iron, and the side chain has rotated about the C_{β} - C_{γ} bond so that the C_{γ} - $C_{\delta 2}$ and C_{γ} - $C_{\delta 1}$ bonds are parallel to the heme plane.

The side chain of Leu-G8 appears to restrict the conformational freedom of the alkyl groups of the RNC ligands in the interior of both the α and β heme pockets. The positional center of this side chain is 7.31 Å from the heme iron in αO_2 and 7.05 Å in βO_2 . There is no significant change in position of this side chain in response to the binding of MNC, ENC, nPNC or nBNC in the α heme pocket. In β subunits, the side chain of Leu-G8 is pushed ~ 0.30 Å away from the heme iron in response to the binding of nPNC and nBNC, but is not affected by MNC or ENC binding. This 0.30 Å movement is roughly equivalent to the difference in distance of the G8 residue from the heme iron in αO_2 compared to βO_2 . In both subunits, the position of $C_{\delta 2}$ of Leu-G8 appears to orient the terminal alkyl carbon atoms of bound nPNC and nBNC toward the heme-3-vinyl group in the space surrounded by the CD1, B13 and B10 residues.

4.4. Changes in overall protein structure caused by isocyanide binding.

An examination of Fe to C_{α} distances within each hemoglobin subunit reveals a strikingly similar arrangement of tertiary structure about the heme (Fig. 4.5a,d). The major distinctions between the subunits are the lack of a D-helix in α subunits and the much greater distance (>2 Å) of the A helix and the first few C_{α} 's of the B helix from the iron atom in β subunits when compared to α subunits. The remainder of the B helix is ~ 0.5 Å farther from the heme iron in α subunits than in β subunits.

Figure 4.5a. Distances from the iron atom to α carbons in the alkyl isocyanide and oxy α subunit complexes. Note that there are few differences.

Figure 4.5b. Differences between distances from the iron atom to alpha carbons in the RNC and O2 alpha subunit complexes.

Change in distances from the iron atom to helices and corners (Å)

Figure 4.5c. Differences between the distances from the iron atom to the helices and helical corners in alkyl isocyanide and oxy complexes of α subunits.

Figure 4.5d. Distances from the iron atom to α carbons in the alkyl isocyanide and oxy β subunit complexes. Note that there are few differences.

Figure 4.5e. Differences between distances from the iron atom to α carbons in the alkyl isocyanide and oxy β subunit complexes.

Figure 4.5f. Differences between the distances from the iron atom to the helices and helical corners in alkyl isocyanide and oxy complexes of β subunits.

Despite the great similarity in tertiary structure of the α and β subunits, significant differences in the mobility of structural elements in the two subunits can be seen from an examination of the differences in iron to C_α distances between the four Hb-RNC structures and HbO₂, (Fig. 4.5b,c,e,f). Both α and β subunits show an average overall expansion of protein structure about the heme irons when isocyanides are bound, with the β subunits showing the greatest increase in interatomic distances. The H helix in α subunits moves about 0.32 Å away from the heme iron in the RNC structures when compared to its position in the α O₂ subunits. The EF corner moves a similar amount. This contrasts with the FG corner which remains in the same position in the α RNC structures as in α O₂. The F helix is displaced between 0.1 and 0.2 Å farther from the heme iron when the larger ligands are bound. The C and E helices also show displacement from the iron atom in the α RNC structures in comparison to the α O₂ structure, and these movements seem to correlate with ligand size (Fig. 4.5c). In α MNC, the C-helix is the same distance and the E helix is 0.1 Å farther from the iron atom than in α O₂. In the α nBNC complex, both of these helices are displaced an additional ~ 0.12 Å farther away from the iron atom than in the α MNC complex. In the α ENC and nPNC complexes, the C and E helices occupy intermediate positions.

The changes in heme iron to α carbon distances ($\Delta\text{Fe-C}_\alpha$) for β subunits show a much different pattern. The overall expansion of the β subunits is 2 to 3 times greater. The B and D helices and the CD corner in the β RNC complexes show the greatest changes in Fe- C_α distances relative to those in β O₂ and the magnitude of the effect correlates with ligand size. The B helix is displaced 0.35, 0.45, 0.55 and 0.60 Å away from the heme iron as the size of the bound isocyanide is increased from MNC to ENC to nPNC to nBNC, respectively. The D helix

moves $> 0.6 \text{ \AA}$ in both the βnBNC and βnPNC structures when compared to βO_2 . The A and C helices move $\sim 0.1 \text{ \AA}$ and $0.15 \text{ \AA}$, respectively, in all four βRNC complexes whereas the F and H helices have $< 0.1 \text{ \AA}$ movements relative to the heme iron. The most striking feature in the $\Delta\text{Fe-C}_\alpha$ plot for β subunits is the movement of the C_α atoms of Asp-B3 away from the iron atom (Fig. 4.5d). The displacement from the B3 position in the βO_2 complex is about $0.2 \text{ \AA}$ in βMNC , $\sim 0.8 \text{ \AA}$ in βENC and βnPNC , and $\sim 1.3 \text{ \AA}$ in βnBNC . Thus, large isocyanides cause a large expansion of the β subunit which causes substantial movements of a portion of the B helix, the D helix and the CD corner away from the heme group.

As was seen for myoglobin, the temperature factors for HbO_2 and the four HbRNC structures exhibit a general pattern of thermally less mobile helices separated by more mobile helical corners (Fig 4.6a-h). Despite the generally similar pattern, there are differences in the distribution and extent of thermal disorder between the α and β tertiary structures. The average B-factor for main chain atoms, excluding the N- and C-terminal regions, is $\sim 5 \text{ \AA}^2$ ($> 0.25 \text{ \AA}$ in average unidirectional displacement) larger for β subunits (Figure 4.6) in the O_2 and four RNC structures. The average B-factor for both α and β main chain atoms in HbENC is lower than that for the other three HbRNC structures. The HbO_2 structure is relatively poorly ordered compared to the ENC structure. In addition, the temperature factors for the main chain atoms in HbO_2 do not vary smoothly from residue to residue and have much more extreme values. This is probably not due an intrinsic property of HbO_2 , but may reflect the unfinished state of refinement of the structure. However, the HbO_2 structure is nearly as well-ordered as that of HbnBNC and is adequate for detailed comparison with the isocyanide complexes.

Figure 4.6a. Temperature factors of main chain atoms of α subunits of oxyhemoglobin minus the average main chain B-factor ($30.66 \text{\AA}^2$). The B-factor for each residue was obtained by averaging the B-factors of the three backbone atoms.

Figure 4.6b. Temperature factors of main chain atoms of β subunits of oxyhemoglobin minus the average main chain B-factor (37.27 $\text{\AA}^2$). The B-factor for each residue was obtained by averaging the B-factors of the three backbone atoms.

Figure 4.6c. Temperature factors of main chain atoms of the α subunits of isocyanide-hemoglobin complexes minus the average main chain B-factors (30.0, 26.0, 29.3, and 31.1 $\text{\AA}^2$ for MNC, ENC, nPNC and nBNC, respectively) The B-factor for each residue was obtained by averaging the B-factors for the three backbone atoms.

Figure 4.6d. Temperature factors of main chain atoms of the β subunits of isocyanide-hemoglobin complexes minus the average main chain B-factors (34.4 , 31.0 , 34.8 , and $36.7 \text{ \AA}^2$ for MNC, ENC, nPNC and nBNC, respectively) The B-factor for each residue was obtained by averaging the B-factors for the three backbone atoms.

In α subunits, the average temperature factor for each helix, except for that of the F helix, is below the average main chain B-factor. The B-factors for the A helix are near average. The average B-factor for each of the helical corners, including the very short AB corner, is greater than the mean. The most ordered internal section of the α subunit is the center of the G helix which contains the distal pocket residue Leu-G8. The B-factors then rise very sharply in going from the center of the G helix to the least ordered FG corner.

In β -chains, the average B-factor for the D, E and F helices is near or above the main chain average. The most disordered internal section of this subunit is a very sharp region centered near residue Asp-B3 (Fig. 4.6a-d). This correlates with the peak seen in the $\Delta\text{Fe-C}_\alpha$ plots for RNC binding to β subunits. The most ordered internal segment includes the G and H helices. The GH helical corner is at or below the average temperature factor. The B-factors of the E helix increase with increasing size of the alkyl moiety of the bound alkyl isocyanide. The E helix has an internal peak of B-factors in ENC and nPNC complexes which is centered at Lys-E10. In βnBNC , this disorder in the E helix continues from Lys-E10 to a peak at the EF corner near residue Asp-EF3.

Uncertainties in the refined coordinates for the C_α atoms can also be estimated from a comparison of the two independently refined HbENC structures (Fig. 4.7a,b). These errors are not independent of temperature factors and exhibit a similar dependence on residue number. In α subunits, two regions have residuals for the least squares fit of C_α coordinates which are at least twice the rms residual. A 2σ peak is located near the end of the CE corner centered at residue $\alpha\text{His-D1}$ and a 3σ peak is centered at residue $\alpha\text{His-F10}$. These match peaks in plots of temperature factors. In β subunits, the C-terminus is relatively well-positioned

Figure 4.7a. Errors in C_{α} coordinates obtained from a least squares fit of the C_{α} coordinates of two independently refined α ENC complexes. The fit was done according to Kabsch (1978).

Figure 4.7b. Errors in C_α coordinates obtained from a least squares fit of the C_α coordinates of two independently refined β ENC complexes. The fit was done according to Kabsch (1978).

compared to α subunits. A 2.5σ peak is located at the beginning of the B helix centered at position β Gly-B6. This is near the very sharp peak in temperature factors at residue β Asp-B3. A 4.5σ peak occurs at residue β Gly-CD7 which also matches the disorder seen in temperature factors of the CD corner.

4.5. Tertiary structure and cooperativity.

A comparison of the tertiary structures of T-state deoxyhemoglobin with the tertiary structure of R-state oxyhemoglobin and the four alkyl isocyanide complexes (Fig. 4.8a-d) reveals differing conformational flexibility within the R-state α and β subunits. The tertiary structural differences between T- and R-state subunits result from proximal and distal effects which are hard to differentiate. In addition, the importance of these effects must differ between the oxy and alkyl isocyanide complexes because of hydrogen bond formation between bound dioxygen and the distal histidine and because of the greater size of the bound alkyl isocyanides. Overall, the structures of the R-state isocyanide complexes are very similar to one another but quite distinct from either the R-state oxy complex or the T-state deoxy structure. The R-state isocyanide complexes appear to have a tertiary structure that is intermediate between that for R-state oxyhemoglobin and that for T-state deoxyhemoglobin.

An overall contraction of the protein occurs in the transition from T-state deoxy to R-state oxy and alkyl isocyanide tertiary conformations. In α subunits, the C_{α} atoms of residues A1 to H21 move an average of $0.27 \text{ \AA}$ closer to the heme iron during oxygenation (Fig. 4.8a,b). The overall contractions in α MNC, α ENC, α nPNC and α nBNC are much smaller, $0.08 \text{ \AA}$, $0.07 \text{ \AA}$, $0.05 \text{ \AA}$ and $0.04 \text{ \AA}$, respectively. This decrease in the extent of contraction relative to deoxy T-state α

Figure 4.8a. Differences between distances from the iron atom to α carbons in the liganded R-state and deoxy T-state α subunits.

Figure 4.8b. Differences between the distances from the iron atom to the helices and helical corners in alkyl isocyanide R-state and deoxy T-state α subunits.

Figure 4.8c. Differences between distances from the iron atom to α carbons in the liganded R-state and deoxy T-state β subunits.

Figure 4.8d. Differences between the distances from the iron atom to the helices and helical corners in alkyl isocyanide R-state and deoxy T-state β subunits.

subunits correlates with the displacement of the C and E helices away from the α heme iron as the size of the bound isocyanide increases. The C_{α} atoms of residues A1 to H21 in β subunits move an average of 0.35 Å closer to the beta heme iron when O_2 binds. The inward movements for β MNC, ENC, nPNC and nBNC are 0.17 Å, 0.19 Å, 0.12 Å and 0.13 Å, respectively.

The greater contraction of tertiary structure in both subunits of R-state oxyhemoglobin is due to two factors. First, the putative hydrogen bond between bound dioxygen and the distal residue His-E7 pulls the E helix closer to the iron atoms, and second, the in-plane movement of the iron atoms to form a 6-coordinate complex pulls the F helix closer. The steric interactions between the alkyl isocyanides and distal side chains would push the B-E helices farther from the iron atoms and oppose the contractions caused by formation of the 6-coordinate iron complex and hydrogen bonding interaction with His-E7. Experimentally, isocyanide binding to hemoglobin exhibits less cooperativity than O_2 binding. The Hill coefficient for isocyanide binding decreases from 2.3 to 1.6 in going from methyl to n-hexyl isocyanide whereas the value for O_2 binding is 2.7 to 3.0. The smaller changes in tertiary structure between T-state deoxyhemoglobin and the R-state isocyanide complex provide a structural explanation of this phenomenon (Fig. 4.8b,d).

4.6. Discussion.

The four hemoglobin-alkyl isocyanide structures present an opportunity to examine the structure-function relationships in the α and β subunits of human hemoglobin on two levels. The first is the way in which heme pocket structure accommodates bound ligands of different size. The second involves the

cooperative mechanism and the way in which a range of tertiary structures can occur in the R quaternary state. Because X-ray structures are time-averaged, they can be only used rigorously to interpret equilibrium binding parameters. In the case of hemoglobin, interpretations are made more difficult because the equilibrium constants for the individual α and β subunits within the intact native tetramer must be determined accurately. Fortunately, the subunits usually bind ligands with similar affinities. In addition, the α and β subunits can be separated and studied independently. Although the equilibrium constants for the isolated chains are similar to those for the R-state subunits, there are measurable differences in the rate constants for ligand binding, implying some differences in tertiary structure (Reisberg and Olson, 1980b). It is quite likely that the segments of protein involved in intersubunit contacts have reduced mobility in the tetramer compared to those in the isolated chains. Thus, only equilibrium constants derived from studies with intact tetrameric R-state hemoglobin were examined for correlations with the X-ray structures.

The R-state α and β subunits appear to have similar affinities for all four of the alkyl isocyanides used in this study (Table 1.1). The α and β equilibrium constants show a similar dependence on ligand size. The affinity constant for ethyl isocyanide binding to R-state hemoglobin is 4 to 8-fold greater than that for MNC. The affinity constant then drops for nPNC and even further for nBNC which has a K_{RNC} value similar to that for MNC binding. The absolute values of the R-state equilibrium constants for hemoglobin are 3 to 8 times greater than those observed for myoglobin.

Although the equilibrium association constants for alkyl isocyanide binding to the α and β subunits are somewhat similar, there are significant structural and

functional differences. In α subunits, the distal residues Leu-B10, His-E7 and Val-E11 move little to accommodate the large alkyl isocyanide ligands. Because of this inflexibility, the ligand atoms in α subunits make unfavorable van der Waals contacts with all three of these residues and are greatly distorted from a linear $\text{Fe}=\text{C}=\text{N}-\text{R}$ geometry. In β subunits, the side chains of Val-E11 and Leu-B10 move $\sim 0.7 \text{ \AA}$ to accommodate the third ligand atom in MNC and can be further displaced to accommodate the larger alkyl isocyanides. As a result, the isocyanide-Fe complex in β subunits is less distorted than in α subunits. Thus, hindrance of alkyl isocyanide binding is expressed differently in the two tertiary structures as a more distorted ligand in α subunits and as an expansion of the distal heme pocket in β subunits. As shown in Table 4.6, the equilibrium association constants for MNC binding to α subunits is about half that for binding to R-state β subunits. However, as the size of the ligand increases $K_{\alpha\text{RNC}}$ becomes 2 to 3-fold greater than $K_{\beta\text{RNC}}$. This result suggests that the expansion of the β subunits when larger ligands bind is energetically more costly than the distortion of the preferred ligand geometry seen in the α subunits. However, these differences in equilibrium constants are small compared to the errors in the measurements, $\sim \pm 50$ to 100% (see Mathews et al., 1989).

The α and β subunits exhibit more marked kinetic differences for the binding of isocyanides (Table 1.1). The β subunits association and dissociation rate constants are 2-10 times greater than the corresponding α subunit parameters. Thus, the kinetic barrier to ligand entry and exit from the β distal pocket must be significantly less than that in α subunits. This result correlates well with the greater thermal mobility and displacement of distal residues observed in the βRNC

structures. In contrast, the α subunit appears to be more rigid and this structural feature is correlated with overall kinetic barrier to isocyanide association.

The X-ray structures of the isocyanide complexes also help to explain previous studies of methyl isocyanide binding to mutant hemoglobins containing substitutions at the E11 and E7 positions (Mathews *et al.*, 1989; Table 4.5). Replacing Val-E11 with Ala had little effect on the equilibrium constant for MNC binding to R-state α subunits but caused a 30-fold increase in the affinity of β subunits for this ligand. Substituting Ile for Val-E11 also had little effect in α subunits but decreased the affinity of β subunits for MNC 30-fold. The importance of Val-E11 in regulating ligand affinity in β subunits is also seen in the X-ray structures. This residue is ~ 0.5 Å closer to the iron atom in βO_2 than in αO_2 and is displaced more than this amount when MNC binds in the β heme pocket.

In contrast to the E11 mutations, the His-E7 $\rightarrow$ Gly mutation had little effect on the affinity of β subunits for MNC but increased the affinity of α subunits for this ligand 10-fold. The X-ray structures show that α His-E7, α Leu-B10, and α Val-E11 move very little to accommodate the third ligand atom of MNC. In β subunits, the distal histidine moves a little, but Leu-B10 and Val-E11 move ~ 0.7 Å away from bound MNC compared to their position in βO_2 . Thus, reduction in the size of the E7 residue does remove a significant amount of steric hindrance of bound MNC in the α subunit but has little effect in β subunits presumably because the side chains of β Leu-B10 and β Val-E11 must still move to accommodate bound methyl isocyanide even when Gly-E7 is present.

Quantum yields for RNC photodissociation from isolated α chains are ≤ 0.1 and $\approx 0.10 - 0.15$ for isolated β chains and similar values appear to apply to the subunits within intact R-state hemoglobin tetramers (Olson *et al.*, 1987) (Table

Mutation	α subunit K_A ($\times 10^{-6} \text{ M}^{-1}$)	β subunit K_A ($\times 10^{-6} \text{ M}^{-1}$)
His-E7/Val-E11 (native)	0.063	0.11
Gly-E7/Val-E11	0.68	0.17
His-E7/Ala-E11	0.092	2.9
His-E7/Ile-E11	0.033	0.0026

Table 4.5. Association equilibrium constants (K_A) for methyl isocyanide binding to position E7 and E11 mutants of α and β subunits within tetrameric R-state hemoglobin (Mathews *et al.*, 1989).

1.1). For both subunits, the quantum yields are low and correlate with the lack of an open pathway for ligand escape to solvent. The time courses for geminate MNC and ENC rebinding to isolated α chains are monophasic and exhibited rate constants that are 2 to 3-fold greater than those for β chains and 10-fold greater than those for sperm whale myoglobin. This indicates that even after photolysis, the ligands are held in place in α chains and thus rebind more often and more quickly. This correlates well with the restricted nature of the α heme pocket and with the lower temperature factors observed in the X-ray structures of the α RNC complexes. In contrast, geminate recombination in isolated β MNC and β ENC chains exhibits multiple slow phases. This also correlates well with the observed β RNC structures which show a heme pocket with much more flexibility allowing more orientations for the photolyzed ligands. This greater mobility leads to more and slower rebinding processes (see Olson *et al.*, 1987).

Chapter 5.

A COMPARISON OF THE ALKYL ISOCYANIDE COMPLEXES OF MYOGLOBIN AND HEMOGLOBIN

Studies of structure-function relationships in myoglobin and hemoglobin have been closely linked ever since Perutz and Kendrew published the first X-ray structures of these proteins (Perutz *et al*, 1960; Kendrew *et al*, 1958). Monomeric myoglobin is often used as a simpler model for understanding the tertiary structure of the subunits in the more complex cooperative hemoglobin tetramers. However, a detailed examination of distal pocket architecture reveals significant differences between the roles that individual amino acids play in Mb, α and β subunits. The malleability of the underlying tertiary structure also differs in myoglobin and the subunits of hemoglobin.

A detailed comparison of the structures of myoglobin and hemoglobin requires confidence that the different crystal environments are not the sole reasons for the observed differences. An analysis of the residuals in least squares fits of C_{α} coordinates for two structures independently refined against the same data gives an empirical estimate of coordinate error in X-ray structures (Fig.'s 3.8a;4.7a,b). For low pH MbENC, the rms coordinate error obtained in this way was ~ 0.04 Å. For α ENC the rms coordinate error was 0.13 Å and for β ENC 0.14 Å. The increase in coordinate rms error appears to indicate an overall increase in mobility of the atoms in the hemoglobin subunits. This increase is also reflected in the temperature factor plots for the myoglobin and hemoglobin structures. The average B-factor for internal main chain atoms of MbMNC, α MNC and β MNC is 14.2 Å^2 , 27.5 Å^2 and 33.4 Å^2 , respectively. The α and β subunits also have a wider range of

B-factors for internal residues (9.8 to 50.1 Å² and 12.7 to 62.9 Å², respectively) than does Mb (7.6 to 26.8 Å²). Thus, the general lattice order of the hemoglobin crystals, as shown by the lowest B-factors for internal residues, is only ~2 to 5 Å² greater than that for the myoglobin crystals. This similar lattice order for the myoglobin and hemoglobin crystals appears to indicate that the much higher thermal mobility of the hemoglobin structures is not an artifact of the crystal environment. The cooperative mechanism in hemoglobin requires a more flexible tertiary structure in the α and β subunits. In least squares fits of the C $_{\alpha}$ backbone, the rms residual for oxymyoglobin vs deoxymyoglobin is 0.09 Å, while those for α O₂ and β O₂ vs the respective deoxy T-state subunits are 0.50 Å and 0.71 Å, respectively. Thus, the cooperative nature of hemoglobin requires that α and β subunits be able to assume a much wider range of tertiary conformations than the myoglobin monomer.

When the distances from C $_{\alpha}$ atoms to the heme iron in deoxy T-state α and β subunits were compared to the corresponding distances in R-state oxyhemoglobin, an average contraction of 0.28 Å and 0.35 Å was observed in the tertiary structures of the α and β subunits, respectively (Fig. 4.8). In contrast, the myoglobin monomer contracts on average a distance of only 0.02 Å when oxygen binds (Fig. 5.1). In α O₂ each of the seven helices is closer to the iron atom than in the deoxy protein (Fig. 4.8b). In β O₂, the B, C, D, G and H helices move closer to iron atom (Fig. 4.8d), whereas in Mb, only the B, C and D helices appear to contract toward the heme (Fig. 5.1b). An average expansion of 0.14 Å, 0.23 Å and 0.22 Å is seen in the nBNC complexes of Mb, α and β subunits, respectively, compared to the corresponding oxy complexes (Fig. 5.2). Expansion to accommodate this larger ligand molecule is small in myoglobin, more substantial

Figure 5.1. Differences between the distances from the iron atom to the helices and helical corners in various liganded myoglobin structures and deoxymyoglobin.

Figure 5.2. Differences between the distances from the iron atom to the helices and helical corners in nBNC and oxy complexes of myoglobin and hemoglobin.

and global in the α subunit involving movements of all C_α atoms, and greatest in the β subunit where it occurs primarily by substantial outward movements of the B, D and E helices and the CD corner.

5.1. The roles of specific distal residues in regulating isocyanide binding.

A 1-2 kcal mol⁻¹ barrier is observed for the placement of a third ligand atom in the active site in myoglobin and the α and β subunits of R-state hemoglobin. This steric hindrance is manifested in the isocyanide complexes by distortion of Fe=C=N- geometry, by disorder in the side chains of certain distal pocket residues, and by a general expansion of tertiary structure. The distances from the iron atom to the center of position of the side chains of the Val-E11, Leu-B10, and His-E7 provides an indication of how each residue restricts ligand binding in myoglobin and the α and β subunits of hemoglobin (Table 5.1, Fig.'s 5.3a,b).

Val-E11 is the closest residue to the bound ligands and hinders MNC binding in all three proteins. In the oxy complexes, the isopropyl group is closest to the heme iron in β subunits, at an intermediate distance in myoglobin, and farthest in α subunits. The distances of the isopropyl side chain from the heme iron in the native proteins is correlated with the effect of replacing Val-E11 with Ala on the equilibrium constants for binding methyl isocyanide. This substitution leads to a 30-fold increase in the association equilibrium constant for MNC binding in β subunits, a 17-fold increase in myoglobin and only a 1.5-fold increase in α subunits. (Table 3.5; Table 4.5). When MNC replaces O₂, the isopropyl side chains are displaced away from the heme iron and the distance of Val-E11 from the heme iron is the greatest in β subunits, intermediate in α subunits and least in myoglobin. Thus, in α subunits, hindrance by Val-E11 is not dominant and

Myoglobin				α subunits				β subunits			
residue		O ₂	MNC	residue		O ₂	MNC	residue		O ₂	MNC
His-F8	93	3.64	3.70	His-F8	87	3.50	3.70	His-F8	92	3.62	3.48
Val-E11	68	5.36	5.47	Val-E11	62	5.58	5.79	Val-E11	67	5.08	5.84
His-E7	64	5.96	5.93	His-E7	58	5.83	5.88	His-E7	63	5.72	5.95
Phe-CD1	43	6.79	6.82	Phe-CD1	43	7.26	7.03	Phe-CD1	42	7.36	7.27
Ile-G8	107	7.55	7.74	Leu-G8	101	7.31	7.39	Leu-G8	106	7.05	7.08
Leu-B10	29	8.48	8.60	Leu-B10	29	7.88	7.79	Leu-B10	28	7.23	7.93
Leu-B13	32	9.69	9.84	Met-B13	32	9.53	9.51	Leu-B13	31	9.22	9.31

Table 5.1. Summary of distances ($\text{\AA}$) from selected distal heme pocket residues to the iron atom in the oxy and methyl isocyanide complexes of myoglobin and the α and β subunits of hemoglobin.

chain of His-E7 does not change position in Mb and α subunits when comparing the bound MNC structures to that of the corresponding oxy complex but is displaced 0.23 Å farther away from the heme iron in the β MNC complex. Curiously, the His-E7→Gly mutation produces no effect on the binding of O₂ or MNC to R-state β subunits, suggesting that the hydrogen bond with bound O₂ in β subunits is weak or non-existent and that the major hindrance to MNC binding is exerted by β Val-E11 and Leu-B10. In contrast, this same mutation increased the affinities of myoglobin and α subunits for MNC 80-fold and 10-fold, respectively. Thus, His-E7 appears to exert little steric hindrance to the binding of methyl isocyanide in β subunits, marked hindrance in myoglobin and intermediate hindrance in α subunits.

5.2. The histidine gate theory for ligand entry into myoglobin and hemoglobin.

The most dramatic difference between the ligand complexes of myoglobin and the hemoglobin subunits is the large rotation of the imidazole side chain of His-E7 away from the iron atom when n-propyl isocyanide and n-butyl isocyanide are bound to myoglobin. The lack of His-E7 rotation in R-state α and β subunits, regardless of isocyanide size, may be due to the greater thermal mobility of most of the side chains and C _{α} atoms in hemoglobin. The α nBNC and β nBNC structures show a greater average expansion of tertiary structure than the MbnBNC structure. In addition, in myoglobin the imidazole side chain in the 'down' position is a few tenths of an angstrom farther away from the heme plane than in the α and β subunits (Fig. 5.3). In β subunits, the imidazole ring can rotate about its C _{γ} -C _{δ} bond to lessen contact with bound MNC. Rotation of His-E7 about the C _{β} -C _{γ} bond in the hemoglobin subunits requires direct contact with the porphyrin ring

reduction in the size of this residue produces only a small enhancement of isocyanide binding. In β subunits, hindrance by Val-E11 appears to dominate and its relief by the Ala substitution raises the methyl isocyanide affinity to that of chelated model heme in soap micelles (Mathews *et al.*, 1989). A large effect is also exerted by the Val-E11 residue in myoglobin.

Leu-B10 caps the distal pockets and hinders bound isocyanides by not allowing the preferred linear $\text{Fe}=\text{C}=\text{N}$ geometry. The isobutyl side chain projects toward the heme in myoglobin and α subunits whereas in β subunits the γ and δ carbons lie in a plane roughly parallel to the porphyrin ring. The Leu-B10 side chain does not move much in response to the binding of isocyanides in myoglobin and α subunits, but is displaced 0.7 Å farther away from the iron by bound methyl isocyanide in β subunits in comparison to its position in βO_2 . The van der Waal's contacts between bound MNC and Leu-B10 are favorable in the β subunits complex but unfavorable in α subunits and myoglobin. The latter observation is supported by mutagenesis studies in which Leu-B10 was replaced with Val and Ala in sperm whale myoglobin causing the affinity of the protein for MNC to increase 2 to 3-fold.

His-E7 forms a hydrogen bond with bound dioxygen but also sterically hinders all diatomic and larger ligands. The formation of this hydrogen bond is necessary to raise the affinity for oxygen to values suitable for storage and transport and to inhibit autooxidation (Olson *et al.*, 1983). In MbO_2 , the His-E7 side chain is 0.13 Å farther from the heme iron than in αO_2 and 0.24 Å farther than in βO_2 . The Fe-O-O angles are $\sim 155^\circ$ in αO_2 and βO_2 (Shaanan, 1983) but only $\sim 115^\circ$ in the oxymyoglobin complex (S.E.V. Phillips, 1980) and 120° in the oxy complex of myoglobin containing the Leu-B10→Phe mutation (Carver *et al.*, 1991). The side

Figure 5.3a. The heme pockets of oxy complexes of myoglobin (lighter) and α subunits (darker) after superposition of the heme groups (Kabsch, 1978). Shown is a view from the Val-E11 position looking toward the bound dioxygen.

Figure 5.3b. The heme pockets of oxy complexes of myoglobin (lighter) and α subunits (darker) after superposition of the heme groups (Kabsch, 1978). Shown is a view from the Leu-B10 position looking toward the bound dioxygen.

Figure 5.3c. The heme pockets of oxy complexes of myoglobin (lighter) and β subunits (darker) after superposition of the heme groups (Kabsch, 1978). Shown is a view from the Val-E11 position looking toward the bound dioxygen.

Figure 5.3d. The heme pockets of oxy complexes of myoglobin (lighter) and β subunits (darker) after superposition of the heme groups (Kabsch, 1978). Shown is a view from the Leu-B10 position looking toward the bound dioxygen.

Figure 5.3e. The heme pockets of methyl isocyanide complexes of myoglobin (lighter) and α subunits (darker) after superposition of the heme groups (Kabsch, 1978). Shown is a view from the Val-E11 position looking toward the bound MNC.

Figure 5.3f. The heme pockets of methyl isocyanide complexes of myoglobin (lighter) and α subunits (darker) after superposition of the heme groups (Kabsch, 1978). Shown is a view from the Leu-B10 position looking toward the bound MNC.

Figure 5.3g. The heme pockets of methyl isocyanide complexes of myoglobin (lighter) and β subunits (darker) after superposition of the heme groups (Kabsch, 1978). Shown is a view from the Val-E11 position looking toward the bound MNC.

Figure 5.3h. The heme pockets of methyl isocyanide complexes of myoglobin (lighter) and β subunits (darker) after superposition of the heme groups (Kabsch, 1978). Shown is a view from the Leu-B10 position looking toward the bound MNC.

which could potentially raise both the kinetic and equilibrium barrier to adoption of the 'up' conformation by the E7 side chain.

The disorder of His-E7 observed in myoglobin led to the proposal of a swinging histidine gate which regulates ligand entry into and exit from the distal pocket. However, there is mounting evidence against this idea for diatomic ligands. First, recent dynamic simulations have suggested that there are multiple pathways for ligand escape from the distal pocket including trajectories through the hydrophobic interior of the protein (Elber and Karplus, 1991). The latter pathways involve movements in which the ligands jump between relatively stable pockets in the protein. These spaces become occupied by Xe atoms when myoglobin crystals are incubated with high pressures of the pure gas (Tilton *et al.*, 1984). Second, the association rate constants for O₂ and CO depend more on the polarity of the amino acid residue at the E7 position than its size. The barrier to bimolecular association appears to consist, at least in part, of a water molecule located directly above the iron atom and hydrogen bonded to the N_ε of His-E7 in deoxymyoglobin. This restriction will occur regardless of the direction in which a ligand approaches the iron atom. Third, the Val-E11→Phe mutation in Mb causes a 10-fold reduction in the association rate constants for all three diatomic ligands, O₂, NO and CO, and the alkyl isocyanides (Table 3.5). The phenyl side chain is located in the cavity between residue G8 and E15 toward the interior of the distal pocket (Quillin *et al.*, 1992) and blocks the escape pathways proposed by Elber and Karplus (1991). On the other hand, Carver *et al.* (1990) argued that Phe-E11 residue could act to tie down the E helix to the heme group and the G helix, which would in turn inhibit the rotation of the His-E7 side chain, thus it is conceivable that His-E7 plays a key role.

The crystal structures of the isocyanide complexes present additional puzzles regarding the pathways for ligand entry and exit. It seems most logical that the large isocyanide ligands enter the distal pocket from solvent by the shortest possible route, a trajectory between residue E7, E10, and E11. This route requires outward movement of the distal histidine “gate”. Curiously, even though this movement is observed in the equilibrium crystal structures of the large isocyanide-myoglobin complexes, this protein shows the smallest association rate constants for isocyanide binding. In general, the association rate constant for isocyanide binding to myoglobin is ~ 2 -fold smaller than that for α subunits and 4 to 10-fold smaller than that for β subunits. Thus, although His-E7 is in the down position in all the hemoglobin complexes, either its outward motion must be more rapid than the corresponding motions in myoglobin, this step in the ligand entry process is not rate-limiting, or there is an alternate pathway.

5.3. The effect of ligand size on cooperativity in hemoglobin.

The elucidation of intermediate conformations which occur during the T- to R-state tertiary conformational switch has been the subject of intensive research for the past 20 years. The results of these studies are still puzzling and can be divided into those which conclude that the intermediate conformations are more similar to the T-state and those which conclude that the intermediates are more similar to the R-state. One study found that singly liganded tetramers or doubly liganded tetramers in which one $\alpha\beta$ dimer was filled could form a species which was similar to a T-state conformer (Daugherty *et al.*, 1991). In contrast, another study proposed that the thermodynamic properties of transition state species were more

like those of the R-state quaternary conformation than those of the T-state (Eaton *et al.*, 1991).

The X-ray structure of the isocyanide hemoglobin complexes show that there can be great variability in tertiary structure within the R-state tetramer. The tertiary conformations of these complexes are intermediate to those of R-state oxyhemoglobin and T-state deoxyhemoglobin. In α subunits, the relative positions of the F and H helices are more similar to those in the deoxy tertiary structure than those in the than oxy tertiary structure even though the overall orientations of the subunits with respect to each other are still those of the R quaternary state and no crystal cracking was observed. A somewhat similar situation occurs in β subunits which expand to a greater extent in response to isocyanide binding and also adopt a tertiary conformation more similar to that observed in T-state deoxyhemoglobin. These structural observations provide an explanation for the lower n_{Hill} , ~ 2.3 , for isocyanide binding compared to the value of 2.7-3.0 observed for oxygen binding.

Reisberg and Olson (1980c) found that the free energy differences between ligand binding to the R vs T states ($\Delta G_{\text{T} \rightarrow \text{R}}$) decreased 30% in going from MNC to n-hexyl isocyanide in α subunits but stayed the same in β subunits (~ 3 kcal/mole). They proposed that steric effects on the distal side of the α heme group were responsible for this decrease in cooperativity and reasoned that a less open distal pocket in α -chains restricted the binding of large ligands in both quaternary conformations. A comparison of the tertiary structures of the oxy and RNC complexes in the R-state with the corresponding deoxy T structures may explain these observations (Fig. 5.4).

In the α RNC complexes, all eight helices are displaced by isocyanide binding to positions closer to those occupied in the more expanded deoxy T tertiary

Figure 5.4. Differences between the distances from the iron atom to the helices and helical corners in nBNC and deoxy complexes of myoglobin and hemoglobin.

structure. The movement of the C and E helices in α subunits appears to be well correlated with the size of the bound isocyanide. For example, when nBNC is bound, the C and E helices are further from the heme iron and closer to the positions occupied in the deoxy complex than when MNC is bound. As a result, the α nBNC complex exhibits more "T-state-like" structural characteristics which correlates with the lower ratio of equilibrium association constants for nBNC binding than would be expected from the results for MNC binding (i.e.

$K_{R-\alpha MNC}/K_{T-\alpha MNC}$ and $K_{R-\alpha ENC}/K_{T-\alpha ENC}$ are 3-10 fold greater than $K_{R-\alpha nBNC}/K_{T-\alpha nBNC}$).

The β subunits also show displacements of the B,C,D and E helices which correlates with size of the bound isocyanide. For the B,C and D helices, this displacement moves the β RNC complexes closer to the T-state and away from the R-state oxy tertiary structure. However, movement of the E helix in β RNC complexes places it farther from the position that it occupies in the deoxy T tertiary structure. Movement of the E helix in β subunits during the T to R quaternary switch is necessary to prevent the side chains of His-E7 and Val-E11 from blocking ligand binding to the iron atom. In β RNC complexes, the value of $\Delta G_{T \rightarrow R}$ is determined by tertiary rearrangements which make the absolute value of this parameter greater (i.e., movement of the E helix away from the T-state position) and those which decrease the value (large movements of the B,C and D helices towards the T-state position). These positive and negative contributions to $\Delta G_{T \rightarrow R}$ in β subunits appear to compensate each other as the size of the isocyanide ligand is increased, and as a result $K_{R-\beta MNC}/K_{T-\beta MNC} \approx K_{R-\beta nBNC}/K_{T-\beta nBNC}$.

5.4. Epilogue.

The isocyanide binding experiments conducted by Pauling and coworkers in the 1950's (St. George and Pauling, 1951; Lein and Pauling, 1956) can be viewed as a theme for the 40 subsequent years of structure-function studies of myoglobin and hemoglobin. The alkyl isocyanides have proven to be very useful for probing the architecture of the distal heme pocket and for discerning how the size and stereochemistry of the bound ligand affects its binding parameters (Mims *et al.*, 1983b; Reisberg and Olson, 1980a,b). Mutagenesis studies have led to great advances in understanding the roles that individual amino acid side chains play in the hindrance of ligand binding by protein structure which was observed first in the studies of Pauling and coworkers. My X-ray structures have nicely complemented these studies by noting how distal residues in native myoglobin and hemoglobin interact with the bound isocyanides. The most interesting observation from these structures is how different are the ligand binding functions of myoglobin and hemoglobin, that is, how poor a model is myoglobin for understanding the dynamics of ligand binding to hemoglobin. This could also be determined from a close inspection of the high resolution structures of the oxy and carbonmonoxide complexes of these two proteins, however, the isocyanide complexes show much more dramatic differences. These differences are most noticeable in the dynamics of motion of the distal histidine in myoglobin and in the more ready expansion of tertiary structure in hemoglobin in response to isocyanide binding.

To this date, mutagenesis studies have focused mostly on very specific functional aspects of particular amino acids, for example, the steric and polar properties of the distal histidine. One notable exception was the study of Komiyama *et al.*, 1991, in which a D-helix was inserted into α subunits or also removed from β subunits in order to investigate the functional (and evolutionary) role of this short helix. The isocyanide structures (and previous structures) have pointed to more global functional aspects of protein structure which might be well-studied by mutagenesis. The high mobility of the F-helix in both proteins and the overall greater thermal mobility of hemoglobin are two such aspects. An interesting note is that both α and β subunits lack the nine isoleucines found in myoglobin mainly in the G-helix. An interesting mutation might be one that places the sterically stiffer amino acid Ile in the complementary positions in the hemoglobin subunits.

The greatest tertiary structural difference between myoglobin and hemoglobin is the arrangement which brings the H helix and the C-termini much closer to the heme iron in the hemoglobin subunits. Proximal structural changes caused by ligand binding have long been invoked as the effector of cooperativity. The isocyanide structures appear to be direct evidence for distal structural changes also being part of the cooperative mechanism. The hydrogen bond between bound oxygen and the N_ε of His-E7 in hemoglobin may be not only necessary to stabilize bound oxygen but also to alter distal protein structure in such a way as to favor the switch from low affinity T to the high affinity R state.

Crystallography has advanced a great many steps since Perutz's and Kendrew's first protein structures, having been transformed from a laborious, grudging process to an experimental method in which only a few weeks may be necessary to determine a structure. The X-ray structures of the alkyl isocyanide can complexes of myoglobin and hemoglobin have resulted from these advances and have shown how the manipulation of pH and substrates in crystallographical studies lead to discovery of new structural properties in proteins which have been studied intensely for so many years.

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