CHARACTERIZATION OF AN AXIAL LIGAND SUBSTITUTION
IN SPERM WHALE MYOGLOBIN

by

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ABSTRACT

Characterization of an Axial Ligand Substitution in Sperm Whale Myoglobin

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Of central importance to the study of heme proteins are the effects imposed by axial ligand(s) on the heme structure and, therefore, on the overall activity of the protein. In this study, we confirm and extend the spectroscopic characterization of a mutated sperm whale myoglobin in which the proximal Histidine is replaced with a Tyrosine residue (MbH93Y). The MbH93Y, as well as wild-type sperm whale myoglobin and horse erythrocyte catalase (HEC), was purified and characterized by optical absorption and x-ray absorption (XAS) spectroscopies. Optical absorption spectra of HEC and the metmyoglobin, cyanometmyoglobin, reduced, oxy, and carbon-monoxy forms of both sperm whale myoglobin (SWMb) and MbH93Y were identical to previously reported values within the respective errors. Extended x-ray absorption fine structure (EXAFS) studies revealed that the proximal bond length in MbH93Y was 2.13 ± 0.03 Å, compared to 2.14 ± 0.02 Å for sperm whale metmyoglobin and 1.90 ± 0.02 Å for catalase.
Additionally, the sixth coordination site normally occupied in wild type sperm whale metmyoglobin and in catalase at low temperatures was vacant in MbH93Y, a result corroborated by the optical absorption spectra and cyanogen bromide modification of the distal histidine. Measurements were also made on the cyanide complexes of the three proteins as well, among which, (i) the average iron-to-pyrrole nitrogen bond distance for MbH93Y-CN was 1.96 ± 0.015 Å compared to 2.00 ± 0.015 Å for WT SWMb-CN and HEC-CN and (ii) the proximal bond length in MbH93Y-CN was 2.07 ± 0.02 Å, while that of WT SWMb-CN was 2.10 ± 0.02 Å and that of HEC-CN was found to be 2.12 ± 0.02 Å. Further, upon exposure to 2-molar equivalents of hydrogen peroxide, sperm whale myoglobin formed a Compound II-like spectrum, while the Soret absorbance of MbH93Y was rapidly, significantly, and irreversibly decreased. Furthermore, the dissociation constants for CN⁻ binding to MbH93Y were found to be, on average, approximately three orders of magnitude higher than those of wild-type sperm whale myoglobin and are consistent with the many-fold higher cyanide binding kinetics for wild type, relative to the mutant protein. Finally, the pKₐ of the mutant was found to be more than three orders of magnitude higher than that of the native protein. Explanations focusing on probable electronic effects of the phenolate oxygen atom in the sperm whale myoglobin pocket are discussed.
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LIST OF ABBREVIATIONS

Abs, Absorbance
BLC, Bovine liver catalase
CM52, Carboxymethyl cellulose - 52
CN-, Cyanide anion
CNBr, Cyanogen bromide
CO, Carbon monoxide
EPR, Electron paramagnetic resonance
EXAFS, Extended x-ray absorption fine structure
Fe-N\text{e}, Fe-imidazole nitrogen bond length
Fe-N\text{p}, Fe-pyrrole nitrogen bond length
Fe-O, Fe-oxygen bond length
Fe-X, Fe-distal ligand bond length
Gly, Glycine
HEC, Horse erythrocyte catalase
HEC-CN, Cyanide-bound HEC
His, Histidine
H\text{2}O, Water
H\text{2}O\text{2}, Hydrogen peroxide
KCN, Potassium cyanide
K\text{Irr}, Potassium hexachloroirridate
K\text{D}, Dissociation constant
Leu, Leucine
LFIR, Ligand field indicator region
L5, Ligand binding site on the proximal side of the heme
L6, Ligand binding site on the distal side of the heme
Mb, Myoglobin
MbCN, Cyanide-bound myoglobin
MbH93Y, SWMb in which the proximal His is replaced with a Tyr residue
MbH93Y-CN, Cyanide-bound MbH93Y
MES, [2(N-Morpholino)ethanesulfonic acid]
MOPS, [3(N-Morpholino)propanesulfonic acid]
NMR, Nuclear magnetic resonance
NO, Nitric oxide
N\text{p}, Pyrrole nitrogen
N\text{2}, Nitrogen gas
N\text{3}-, Azide
O\text{2}, Oxygen (diatomic) gas
R\text{e}, Reinheitszahl; Abs\text{403nm}/Abs\text{275nm} ratio
SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser, Serine
SWMb, Sperm whale myoglobin
SWMb-CN, Cyanide-bound SWM
Tyr, Tyrosine
UV, Ultra-violet
Val, Valine
WT, Wild-type
XAS, X-ray absorption spectroscopy
CHAPTER I
INTRODUCTION

The study of structure-function relationships has, for a long time, been a dominant theme in heme protein chemistry. Indeed, one of the most obvious approaches to tackle this problem has been through the use of various ligands or substrates to probe the active sites of the respective proteins (e.g., for some of the earlier work on myoglobin and hemoglobin, see Antonini and Brunori, 1971). In the case of myoglobin, virtually every aspect of this protein has been examined in this way, since from a structural point of view, myoglobin is among one of the simplest of heme proteins. The binding and releasing of diatomic molecules (e.g., CO, NO, CN⁻, O₂) to and from myoglobin has physiological relevance and, depending on the pH (Shimada and Caughey, 1982; Ramsden and Spiro, 1989), either O₂ (Potter et al., 1987) or CO (Cheng and Schoenborn, 1991; Makinen et al., 1979; Shimada and Caughey, 1982; Moore et al., 1988; Balasubramanian et al., 1993a, b) binding culminates in discrete geometric conformations or a transient intermediate (Sato et al., 1990) of the ligand-bound myoglobin. Furthermore, Bormett et al. (1992) found one covalent band and one ionic band in the vibrational circular dichroism spectra of N₃⁻-bound elephant myoglobin, suggesting the presence of two conformational substates.

In addition, the reaction pathway that the diatomic molecules follow in the heme pocket has been studied in detail (for a brief review, see Gibson, 1989). That is, the reaction pathway taken by these ligands has been painstakingly dissected using several approaches, including low- (Chance et al., 1990) and room-temperature (Ramsden and
Spiro, 1989) kinetic studies. In addition, there are studies of the specific trajectory taken by the ligand, revealed by discrete geminate (recombining) states (Powers et al., 1987), possibly due to the presence of many discrete conformational substates, each of which depends on the $H^{2}\leftrightarrow H^{1}$ tautomerism and orientation of the distal His (Oldfield et al., 1992) and each of which possesses its own recombination rate constant (Siebrand et al., 1985). The variety of studies also include structural (Sage et al., 1991b; Sage et al., 1992; Tian et al., 1993) and kinetic (Sage et al., 1991a; Sage et al., 1992; Tian et al., 1993) interactions with the Fe-His bond at low pH. Finally, other studies have focused on the introduction of large ligands (e.g., $n$-butyl isocyanide) into the heme pocket (e.g., Gibson et al., 1986); thermodynamics of ligand binding (Sato et al., 1990a), and the effect of pressure (Adachi and Morishima, 1989).

The underlying implication of all these studies is that the protein matrix surrounding the active site is directly responsible for which ligands gain access to the heme pocket, how fast they do it, how tightly they bind, and how fast they dissociate. The structural and kinetic mechanisms of the binding of CN$^{-}$ to heme proteins has lately become a model upon which binding of other ligands such as O$_{2}$ (Yamamoto et al., 1992) and CO (Emerson and LaMar, 1990b; Rajarathnam et al., 1992) are based. To this end, many studies have recently appeared using either $^1$H-1-dimensional (Lecomte et al., 1989; Satterlee and Erman, 1991; Keating et al., 1992; Rajarathnam et al., 1992; Yamamoto et al., 1992; Vyas et al., 1993) or $^1$H-2-dimensional (Emerson and LaMar, 1990a, b; Satterlee and Erman, 1991; Qin et al., 1992; Qin & LaMar, 1992) NMR of cyanomet myoglobin in the buccal muscle of Aplysia mollusc (Qin et
al., 1992; Qin and LaMar, 1992), the stomach of Dolabella mollusc (Yamamoto et al., 1992), skeletal muscle of sperm whale (Emerson and LaMar, 1990a, b), liver fluke Dicrocoelium (Lecomte et al., 1989), heme model compounds (Keating et al., 1992), and elephant (Vyas et al., 1993), as well as in other heme proteins, such as cyanide-bound horseradish peroxidase (Thanabal et al., 1988; Banci et al., 1991; LaMar et al., 1992) and cyanide-bound cytochrome c peroxidase (Banci et al., 1991; Satterlee et al., 1991; Satterlee and Erman, 1991).

At least three of these heme proteins are known to function through a dual mechanism of action. At high concentrations of \( \text{H}_2\text{O}_2 \), catalase catalyzes the dismutation of this substrate to two \( \text{H}_2\text{O} \) and one \( \text{O}_2 \) molecule, while at low concentrations of \( \text{H}_2\text{O}_2 \), the oxygen from one equivalent of this substrate is used to oxidize a small number of organic compounds, such as ethanol, methanol, or guaiacol (Figure 1; e.g., Sichak and Dounce, 1986). Myoglobin and hemoglobin are also known to possess some small degree of catalase activity, although the significance of this particular function has generally been overshadowed by that of their primary physiological function of oxygen storage and transport (Figure 1). Nevertheless, the potential importance that such proteins may have to do with the well-being of the cell should not be overlooked. The mechanism of the catalytic decomposition of \( \text{H}_2\text{O}_2 \) by oxymyoglobin has been studied in detail (Whitburn, 1987 and references cited therein). But metmyoglobin and methemoglobin can also, in the presence of a suitable reducing agent (e.g., ascorbic acid), work in a peroxidatic mode, weakly (King and Winfield,
Figure 1: A schematic representation of the 4 pyrrole nitrogens of the heme provides the backdrop for illustrating (a) the peroxidatic (or catalatic) reactions of peroxidase (or catalase), which are responsible for the breakdown of 2 molecules of $\text{H}_2\text{O}_2$ (peroxide O-O cleavage) with the formation of a transient intermediate known as Compound I. (b) A schematic representation for illustrating the function of the globins, which simply load and unload $\text{O}_2$; note that the met species of the protein can be reduced with dithionite to form the deoxy species.
(a) 

**Peroxidase**

(b) 

**Globin**
1963) eliminating $\text{H}_2\text{O}_2$ (Keilin and Hartree, 1950; King and Winfield, 1963; Harel and Kanner, 1989; Ortiz de Montellano and Catalano, 1985; Romero et al., 1992). In doing so, however, metmyoglobin is irreversibly altered through repeated reaction cycles (King and Winfield, 1963) and probably forms hydroxy radicals (Harel and Kanner, 1988); therefore, it cannot be considered a true enzyme. Because metmyoglobin can form hydroxy radicals when functioning as a catalase, this activity of the protein can be deleterious to the cell. It has been known for some time that metmyoglobin can accelerate lipid peroxidation (Kanner and Harel, 1985; Newman et al., 1991), and that this activity can be modulated by NO (Kanner et al., 1980; Dee et al., 1991).

The investigation of the reactions of myoglobin with $\text{H}_2\text{O}_2$ has a long history, starting with the work of Keilin and Hartree (1950 and earlier). Since then, many studies have appeared which characterize the fine points of the reaction cycle (e.g., George and Irvine, 1952, 1953, 1956; King and Winfield, 1963; Whitburn, 1987; Tajima and Shikama, 1993) using protein analogues of myoglobin such as leghemoglobin (Davies and Puppo, 1992), hemin model compounds (Kremer, 1989), and computer simulations (Wazawa et al., 1992).

Heme proteins have always been the subject of intense interest in the field of protein chemistry, not only because of their varied and well-known physiological importance, but also because of their prosthetic group (Figure 2). Central to the active site of these proteins is the heme prosthetic group (Figure 2), which exists in many different chemically similar forms (Bonnet, 1978). The hemeprotein active site
Figure 2: The heme protein active site. Shown are the active sites of WT SWMb, catalase, cytochrome c peroxidase, and MbH93Y.
contains the heme prosthetic group, which is responsible for the physiological functions of each respective protein. In myoglobin, the heme group is buried in a mostly hydrophobic pocket with only one side of the heme roughly exposed to solvent. In catalase, the heme is deeply buried within the protein and is sandwiched between a hydrophobic side distally and a hydrophilic side proximally. The terms "distal" and "proximal" are common usage in heme protein nomenclature and are abbreviated L6 (Ligand-6) and L5 (Ligand-5) sites, respectively. In heme, the Fe is bound by four pyrrole nitrogen atoms, which are an integral component of the heme molecule. When a ligand, denoted X, gains entry to the heme pocket, it binds the Fe on the distal side of the heme; such a ligand is denoted L6 or distal ligand. On the opposite side of the heme, the proximal side, an amino acid provides the only linkage of the protein to the heme; this amino acid is a His residue in myoglobin and a Tyr residue in catalase.

In an effort to understand how the active site residues structurally and electronically influence the heme part of the active site and reactivity in these proteins, many studies of synthetic heme analogues have appeared over the past 2-3 decades (e.g., Hanson et al., 1981; Fujita et al., 1983; Hansen and Goff, 1984; Chen and Stynes, 1986; Goldsby et al., 1986; Barteri et al., 1986; Cairns et al., 1987; Mitchell et al., 1987; Traylor & Xu, 1987; Brown et al., 1987; Hatfield et al., 1987; Levine and Holten, 1988; Safo et al., 1990; Guilard et al., 1991; Hatano et al., 1991; Garcia et al., 1991; Robert et al., 1991). A central theme in many of these studies has been the effects of the axial ligand(s), since, although many heme proteins have identical prosthetic groups, many of them do not have identical axial ligands. In both sperm
whale myoglobin (SWMb) and in the α and β subunits of hemoglobin, the fifth ligand, which is axially coordinated to the heme Fe, is known to be the imidazole of a His residue. This group provides the only linkage to the protein. However, in many naturally occurring hemoglobin mutants (Nagai et al., 1983; 1989), as well as in catalase (Murthy et al., 1981; Fita and Rossmann, 1985), this axial fifth ligand is the phenolate of a Tyr residue. Sperm whale myoglobin (MW 17.8 kDa) has been the subject of intensive investigations focused on understanding the dynamics of ligand binding through the use of site-directed mutagenesis of the heme distal pocket (Olson et al., 1988; Ikeda-Saito et al., 1991, 1992; Rohlfs et al., 1990; Morikis et al., 1989; Bellelli et al., 1990a,b; Carver et al., 1990; Egeberg et al., 1990a,b; Springer et al., 1989; Rizzi et al., 1993) and/or the proximal side (Morikis et al., 1990; Egeberg et al., 1990a; Adachi et al., 1991, 1993).

The existence of hemoglobin mutants with Tyr as axial ligand led Egeberg et al. (1990a) to replace the proximal His in SWMb with a Tyr residue using site-directed mutagenesis of their synthetic SWMb gene (Springer and Sligar, 1987). Optical UV/visible, electron paramagnetic (EPR), and resonance Raman spectroscopies were used to characterize the spin and coordination states of the MbH93Y. Adachi et al. (1991, 1993) also constructed the mutant, MbH93Y, using a synthetic human myoglobin gene, and characterized it via optical absorption, EPR, and 1H-NMR spectroscopies.

For Egeberg et al. (1990a), the rationale behind placing a Tyr residue in place of the proximal His in SWMb was to gain insight into the electronics and geometry of
the naturally occurring, nonfunctional hemoglobin mutants. The single, point mutation of the axial ligand from a His to a Tyr has resulted in profound adverse physiological consequences in the naturally occurring hemoglobin mutants (Nagai et al., 1983, 1989). Decreased activity was also found in site-directed mutants of cytochromes b₄ and b₅ (Yun et al., 1991), c₁ (Nakai et al., 1990), and P-450₄ (Shimizu et al., 1988).

The other relevant widely studied protein is catalase (Bovine Liver Catalase, BLC; Nicholls and Schonbaum, 1963). This protein is known to be among the fastest reacting enzymes (Fersht, 1985), approaching the diffusion-controlled limit. However, the high degree of catalatic or peroxidatic activity found in catalase (Fersht, 1985) and cytochrome c peroxidase (CcP) is not found in the globins. The latter have been shown to also dismutate H₂O₂, but only through a limited number of reaction cycles (ca. 8, King and Winfield, 1963), at the end of which the structural and/or functional integrity of the protein has been compromised. For this reason, myoglobin cannot be considered an enzyme. Since SWMb, BLC, and CcP all possess the same heme group at their respective active site (Poulos, 1988), the striking functional difference which separates the globins from the peroxidases and catalases must lie with the differences of both the specific residues at homologous sites and their respective spatial orientation; i.e., active site structure. As shown in Figure 2, all three proteins possess some degree of H-bonding network within their respective active site. In BLC (Fita and Rossmann, 1985) and CcP (Finzel et al., 1984), the H-bonding network is shared among several key residues speculated to be largely responsible for the activity of each respective enzyme. Therefore, it is reasonable to propose that, as a result of molecular
dynamics simulations and rational design, certain key residues in the nonpolar heme pocket of SWMb can be mutated to polar residues that are thought to reside in a homologous site and particular orientation, strategically poised in HEC or CcP for performing the chemistry of these enzymes. Although the active site structure of catalase and SWMb differ considerably (e.g., Poulos, 1988), the presence of identical axial ligands and prosthetic groups in catalase and MbH93Y provides us with an excellent opportunity to investigate the structure-function relationship of these active sites (Morikis et al., 1990; Egeberg et al., 1990a; Adachi et al., 1991, 1993). What unique arrangement of amino acids in the active site of catalase is responsible for activity that is so markedly different from that of SWMb? In addition to the heme, as a result of site-directed mutagenesis, the proximal ligand in MbH93Y is identical to that in catalase.

The purpose of this study is 2-fold: Firstly, I will confirm and extend the spectroscopic characterization of the proximal mutation of SWMb started by Egeberg et al. (1990a) using optical absorption and X-ray absorption (XAS) spectrosopies and compare the results to those obtained for recombinant wild type (WT) SWMb and purified HEC. Because cyanide (CN⁻) is the ligand of choice (see below) for characterizing the ligand-binding properties of the mutant, the results of the CN⁻ complexes of each of these three proteins are also compared; secondly, I will characterize the ligand-binding properties and peroxide reactivity properties of MbH93Y.
X-ray absorption spectroscopy is useful for determining the local structure around the active sites of metalloproteins (Lee et al., 1981; Powers, 1982; Strange et al., 1990). However, XAS has several disadvantages such as (i) the inability to distinguish two atom ligands if they differ only slightly in atomic number, (ii) the inability to locate ligands more than ~4 Å from the metal center, and (iii) the requirement of a highly pure and concentrated sample. Nevertheless, despite these limitations, XAS has proven to be a powerful spectroscopic tool to probe the active site structure of a large variety of metalloproteins since the information it yields complements that obtained through other means, such as $^1$H-NMR, EPR, and x-ray crystallography. In addition, XAS can provide structural information within an error of ± 0.015 Å.

Cyanide is an ideal ligand with which to examine the ligand-binding properties of the mutant heme protein, since the cyanide-ligated protein has served as a useful model for the binding of other diatomic ligands. The metcyano-complex is a good model for the oxy form because its large magnetic anisotropy is expected to induce a large downfield hyperfine shift for the resonances arising from the protons located near the ligand-binding site in magnetic resonance experiments. Cyanide also forms a stable hexacoordinate complex that is easier to handle than the carbonmonoxy- or oxy-bound forms of the protein.

To functionally assess the effects of the axial mutation, I performed the following characterizations: (i) the mutant protein was exposed to H$_2$O$_2$ in order to determine if the mutated SWMb can form the ferryl intermediate, Compound I, which occurs in the peroxidatic and catalatic reactions; (ii) the mutant protein was titrated with increasing
concentrations of KCN in order to assess its effectiveness in binding cyanide, relative to the native or WT; (iii) the distal His was modified with CNBr to sterically probe the ligand binding dynamics of the distal pocket; and (iv) the acid←alkaline transition of the mutant myoglobin was characterized.
CHAPTER II
EXPERIMENTAL PROCEDURES

Purification of MbH93Y. Site-directed mutagenesis of the synthetic SWMb gene was performed by Egeberg et al. (1990a) and purification of the mutant myoglobin was executed as previously specified (Springer and Sligar, 1987; Egeberg et al., 1990a). A stab culture of Escherichia coli (E. coli) strain TB-1 transformed with a plasmid, pMb413a, containing the mutated SWMb gene (Egeberg et al., 1990a) was kindly provided by Dr. Stephen Sligar's laboratory (University of Illinois, Urbana). The cells were grown overnight to late stationary phase in Luria Broth (LB; 10 g bactotryptone, 5 g yeast extract, 10 g NaCl per liter) in the presence of 200 mg per liter ampicillin (Sigma), then to exponential phase for 3-4 h, and then again to late stationary phase in 2 liters of LB media for 2-3 days. Essentially, this procedure entailed diluting the cells and regrowing them. Growth was carried out at 37°C in a shaking incubator at 120 rpm (New Brunswick). All cells were collected by centrifugation (Sorvall) at 8,000 x g, 4°C, for 10 minutes. Pellets were stored at -80°C until at least 200 g cells had been accumulated.

For purification, the cells were thawed into ~600 ml ice-cold lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 units DNase I per ml, 3 units RNase A per ml, and 2 mg lysozyme per ml and stirred for 6 h at 4°C. The cell debris was removed by centrifugation at 8,000 x g at 4°C for 20 minutes. The pellets were then resuspended in the lysis buffer and stirred at 4°C overnight while the supernatant was
stored at 4°C overnight. Twelve-16 h later, the lysed pellets and supernatants were combined and stirred for 2-3 h and centrifuged at 8,000 x g at 4°C for 20 minutes. The supernatant was then decanted and maintained at 4°C while the pellet was subjected to French press at 8,000 psi. The pressed pellet was then centrifuged at 8,000 x g at 4°C for 20 minutes several times until the resultant volume of the supernatant was negligible. All supernatants were then combined. Solid ammonium persulfate was added (55% saturation) and the solution was stirred at 4°C for ~45 minutes, then centrifuged at 6,000 x g at 4°C for 45 minutes. The pellets were discarded and the supernatant was stirred overnight or for several hours, after which fractionation with 95% ammonium persulfate was carried out. This last salt fractionation was allowed to stir at 4°C for ~1 h and then centrifuged at 6,000 x g at 4°C, for ~1 h. The supernatant was then discarded and the pellet then dissolved in a minimal volume of gel filtration buffer consisting of 50 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA (pH 8.0). This dissolved pellet was then stirred overnight at 4°C in the presence of hemin (Sigma).

This myoglobin preparation was then applied to a Biogel P-60 (Biorad) size-exclusion, gel filtration column (5 x 90 cm), previously equilibrated at 4°C in the gel filtration buffer. Fractions were collected and the decision of which fractions to pool was determined by optical absorption spectra (Shimadzu UV/visible scanning spectrophotometer--UV 2101PC) and by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli, 1970). The optical spectra and SDS-PAGE were used to determine the extent of purity. The narrow width of the various
absorption bands (Soret, charge transfer, β, α) in the optical spectra would have indicated that the myoglobin was free of impurities and the respective fractions that exhibited one band on the silver-stained polyacrylamide gel that electrophoresed to the position corresponding to ~17.8 kDa would also have indicated a highly pure protein. The fractions that corresponded to the optical absorption peaks and to the 17.8-kDa band on the gel were pooled. These pooled fractions were then dialyzed at 4°C, overnight, in several changes of 20 mM phosphate buffer, pH 6.7, followed by application to a CM-52 (Whatman) cation exchange column (2.6 x 30 cm) previously equilibrated in the dialysis buffer. Once again, fractions were collected and both the optical spectra and SDS-PAGE were used to determine which fractions to pool and concentrate under Ar in an ultrafiltration cell (Amicon).

Purification of Wild-Type Sperm Whale Myoglobin. Wild-type SWMb was purified using the method of Springer and Sligar (1987), which is identical to that used for the purification of MbH93Y and was discussed above. Again, the extent of purity at each stage of purification was determined by its optical spectra and SDS-PAGE.

Native Sperm Whale Myoglobin. Native SWMb was purchased from Sigma. The protein was further purified to remove isozymes, as well as any extraneous debris according to the method of Powers et al. (1987). Briefly, a known amount of native SWMb was weighed and dissolved in a known volume of 0.1 M phosphate buffer at pH 7.0 and dialyzed against this buffer for 12-16 h (overnight), with 2-3 changes at 4°C. The precipitate obtained following centrifugation at 10,000 x g for 30 minutes at 4°C was discarded. The pH of the supernatant containing the myoglobin was
adjusted to 6.4 and the solution applied to a cation exchange column (Whatman, CM-52) previously equilibrated at 4°C with 0.1 M phosphate buffer, pH 6.5. The optical spectra and SDS-PAGE confirmed that the fractions to pool were those of the dark brown band that eluted off the column. The purified native protein was then concentrated under Ar through an ultrafiltration cell (Amicon).

**Horse Erythrocyte Catalase.** Horse erythrocyte catalase was purified according to the method of Nagahisa (1962). Briefly, 1 liter of horse blood was collected in a chilled glass jar containing 5% citrate as anticoagulant and centrifuged at 8,000 g at 4°C for 20 minutes, after which the hematocrit was measured. The supernatant containing the plasma and citrate was discarded and the erythrocytes were gently washed with isotonic saline 3-4 times and centrifuged following each wash. Following the final decant of the isotonic saline, the erythrocytes were lysed in a volume of cold water equal to twice the initial volume of erythrocytes and was left to hemolyze at 4°C overnight. Following complete hemolysis, a volume equal to that of the initial erythrocytes of cold ethanol/chloroform (3:1) was slowly added at 10-20°C under constant stirring to precipitate the hemoglobin. The extremely viscous slurry was then filtered through Whatman #1 filter paper on a large ribbed, conical funnel. The organic phase was then evaporated off as quickly as possible, while the temperature was maintained at 12-15°C. Then, 1 M acetate buffer at pH 4.7 was added to make the concentration of acetate equal to 0.1 M, pH 4.4. The protein/solvent mixture was then centrifuged at 8,000 x g at 4°C for 20 minutes and the pellets were discarded. Acetone was slowly added while stirring at 4°C, until the mixture became cloudy.
(usually ~15-30% by volume), after which, the solution was centrifuged and the pellet was discarded. The preceding step of acetone addition was repeated until the supernatant again became cloudy; the solution was then centrifuged. The resulting supernatant was discarded and the pellet dissolved in a minimal volume of 0.1 M acetate buffer at pH 4.7, centrifuged at 8,000 x g at 8-12°C for 15 minutes and the pellet discarded. The protein was then precipitated by the slow addition of solid ammonium persulfate to 35% saturation while stirring at 4°C for 1 h. The solution was then centrifuged at 8,000 x g at 4°C for 20 minutes and the precipitate discarded. Finally, the protein was dialyzed against 0.01 M phosphate buffer, pH 6.2, at 4°C with several changes of dialysis buffer over a 12-18-h period. To determine the purity of the HEC, optical spectra were recorded and native (non-denaturing) gel electrophoresis was performed. The Rz value (Abs405nm/Abs280nm) was determined to be 1.25 and is in excellent agreement with the previously reported value for HEC (Nicholls and Schonbaum, 1963). The catalase was then concentrated in an ultrafiltration cell (Amicon) under N2.

The HEC was assayed for its ability to decompose H2O2, using the method of Beers and Sizer (1952). Briefly, HEC was added to a final concentration of 10^{-10} M in 10 mM phosphate buffer at pH 6.3 in a reaction volume containing ~48 µM H2O2. For each successive reaction, the concentration of H2O2 was held nearly constant, but the concentration of HEC was increased incrementally for each reaction up to ~10^{-9} M. The kinetics for the dismutation was followed at 240 nm for 2 minutes. Formation of Compound I was followed according to the method of Jones and
Middlemiss (1972) and Palcic and Dunford (1980). Briefly, catalase compound I was made by titration of a known concentration of HEC with an increasing amount of peracetic acid until the molar ratio of peracetic acid to catalase was 1.2 (Jones and Middlemiss, 1972). The ratio of $\text{Abs}_{406\text{nm}}/\text{Abs}_{410\text{nm}}$, which represents the ratio of compound I/native catalase, was calculated to be 0.478 and agrees with the previously published value of Nicholls and Schonbaum (1963).

Heme integrity of HEC as well as of the myoglobins was determined using the pyridine haemochromagen assay according to the method described by Paul et al. (1953). Briefly, to $\sim$20 µM protein in 1 ml H$_2$O, 2 µl pyridine, 70 mM NaOH, $\sim$10 mg sodium dithionite and H$_2$O were added to a final volume of 5 ml. The optical spectra were recorded and the ratio of $\text{Abs}_{557\text{nm}}/\text{Abs}_{527\text{nm}}$ yielded the concentration of the protein sample. If this ratio equals 2.0, then the catalase tetramer has all four hemes intact. If, however, this ratio is less than 2.0 and/or there is absorbance at $\sim$650 nm, then broken hemes are present.

**Cyanide-Bound Horse Erythrocyte Catalase.** For the XAS studies, HEC was mixed with micromolar concentrations of KCN and placed into the EXAFS holder.

**Formation of the Various Liganded Forms (CO, O$_2$, CN) and Oxidation States (Fe$^{3+}$, Fe$^{2+}$) of Myoglobins.** The WT, native, and MbH93Y myoglobins were purified in the met (Fe$^{3+}$) oxidation state. Subsequent formation of the various liganded forms as well as of the various oxidation states was facilitated by use of an air-tight vessel, which was composed of an inlet and an outlet valve, both of which fed into a common reservoir and were connected to a common neck that led to the top of a 3-ml capacity
cuvette. Before each of the different forms was made, the myoglobin solution was purged with Ar through this air-tight vessel. The reduced (Fe$^{2+}$, deoxy) form of each myoglobin was prepared by the addition of a small amount (several crystals) of sodium dithionite in the reservoir of the air-tight vessel. The carbon monoxy form (CO) of each myoglobin was then prepared by bubbling CO into the inlet valve of the vessel leading into the cuvette in semi-darkness containing the reduced form. The oxy form (O$_2$) of each myoglobin was prepared by bubbling O$_2$ into a cuvette containing a freshly prepared reduced form at 4°C. Finally, the cyanomet (CN$^-$) form of each myoglobin was prepared from the met (Fe$^{3+}$) form by the addition of the stoichiometric amount of KCN from a concentrated stock solution. Once made, each form of each myoglobin was sealed in the air-tight vessel and then scanned for its optical absorption.

**Extinction Coefficients.** Millimolar extinction coefficients were determined using the pyridine haemochromagen assay of Paul et al. (1953). Briefly, to ~20 µM protein in 1 ml H$_2$O, 2 µl pyridine, 70 mM NaOH, ~10 mg sodium dithionite and H$_2$O was added to a final volume of 5 ml. The optical spectra were recorded and the ratio of Abs$_{557}$/Abs$_{527}$ yielded the concentration of the protein sample. Using the Lambert-Beer relationship, $\varepsilon_{403}$ = $\text{Abs}/c\ell$, where $c$ = concentration and $\ell$ is the path length of the sample cuvette ($\ell$ = 1), the millimolar extinction coefficient was calculated. The working concentrations of all reactions involving either the native or WT SWMm were determined using the millimolar extinction coefficient of 168 mM$^{-1}$ cm$^{-1}$ (Clark and Gurd, 1967), a value I confirmed. The working concentrations of all
reactions involving MbH93Y were determined using the calculated $\varepsilon_{403} = 169.46$ mM$^{-1}$ cm$^{-1}$.

**Reactions with H$_2$O$_2$.** Reactions with either 2-fold molar excess H$_2$O$_2$ or 4-fold molar fewer equivalents with respect to myoglobin were followed spectrophotometrically at 1-minute intervals. All reactions were maintained at a constant temperature of 23°C by a thermodruated cuvette holder (Brinkman).

**Reactions with KCN.** Recombinant SWMb, native SWMb, and MbH93Y were each titrated with increasing concentrations of KCN (Malinkrodt) in 0.1 M low ionic strength MES/MOPS buffer at pH values of 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. Again, all reactions were maintained at a constant temperature of 23°C. Dissociation constants ($K_D$) were determined by calculation of the differences in absorbances for unbound protein, bound protein, and unbound ligand according to the method used by Schonbaum (1973). From the absorption spectra of each individual titration of myoglobin with increasing concentrations of KCN, the inverse of the total cyanide concentration ([CN$^-$]), [CN$^-$]$^{-1} \times 10^3$ is plotted as a function of the inverse of the change in absorbance, $\Delta A^{-1}$, at 409 nm (native and WT SWMb) or 403 nm (MbH93Y) and at 423 nm (CN$^-$-bound myoglobin, MbCN). The inverse of the slope of the line is equal to the $K_D$. The concentration of MbCN ([MbCN]) was calculated using the relation, $[MbCN] = \Delta A_{obs}/\Delta \varepsilon_T$, where $\Delta A_{obs}$ is the change in the observed absorbance and $\Delta \varepsilon_T$ is the change in the total extinction coefficient and is equal to ($K_D \times$ slope)/[Mb]$^{-1} = \Delta A_{max}/[Mb]$, where $\Delta A_{max}$ is the maximum change in absorbance and
[Mb]_T is the total concentration of myoglobin in all forms (free Mb and CN'-bound Mb).

Kinetics of the forward rate of reaction of CN' binding to the protein were calculated (Vega-Catalan et al., 1986) using the relation \( k_{+1(\text{app})} = K_D \times k_{-1(\text{app})} \), where \( k_{+1(\text{app})} \) and \( k_{-1(\text{app})} \) are the apparent forward and reverse rate constants, respectively, of the reaction \( \text{Mb}^+ + \text{CN}' \leftrightarrow \text{MbCN} \). The same experiments as those used for determining the binding constants were used for the kinetic analyses.

**Cyanogen Bromide Modification of Myoglobins.** All three myoglobins (recombinant, native, and MbH93Y) were reacted with CNBr (Aldrich) according to the procedure first used by Jajczay (1970), except that ∼100-fold excess, rather than stoichiometric amounts (Jajczay, 1970) of CNBr were used relative to each protein. The reaction with CNBr therefore followed pseudo first-order kinetics. The UV/visible spectra were recorded and the rate of decrease of the Soret band was monitored following the addition of molar equivalents of H₂O₂ to myoglobin (native or MbH93Y).

**Acid↔Alkaline Transitions.** The acid↔alkaline transition profiles of native SWMb and MbH93Y were determined in both the forward and reverse directions with increasing concentrations of NaOH and HCl, respectively. Proton dissociation constants (pKₐ) were determined from a plot of the percent change in absorbance as a function of pH (Yamazaki et al., 1974). Hill coefficients were calculated graphically, \( \log[y/(1-y)] \) vs. pH, where \( y \) is the fraction of sites filled and \( 1-y \) is the fraction of sites not filled. The slope of this line is \( n \).
**XAS Data Collection and Analysis.** XAS experimental data were recorded at the Stanford Synchrotron Radiation Laboratory (SSRL), Stanford, CA, on Beamlines VI-2 and IV-3, and at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory, Upton, NY, on Beamline X9, using procedures and experimental parameters identical to those previously described (Powers et al., 1981; Chance et al., 1984; Powers et al., 1984, 1987; Sinclair et al., 1992). The beamline monochromators at both sources were equipped with Si 111 crystals providing 2-3 eV resolution at the Fe K-edge (7,110 eV) (Sinclair et al., 1992). Fluorescence was monitored using a 13-channel Ge detector. Data for all myoglobin samples were collected at both the NSLS and SSRL while that for HEC was collected at only NSLS. The data were recorded by counting the fluorescence photons emitted from the sample at specific energies. Scans ranged from 7,050 to 7,800 eV (Figure 3) and were analyzed by procedures described previously (Powers et al., 1981; Lee et al., 1981; Powers et al., 1984). The fractional modulation of the absorption due to EXAFS, which is deduced from XAS data, is

\[
\chi(k) = \sum A_i(k) \sin\{2[kr_i + \alpha_i(k)]\}
\]

where \(A_i(k) \sim \frac{N_iZ_i \exp[-2\sigma_i^2k^2]}{k^2r_i^2} \exp[-2\tau/\lambda(k)]\) for \(k \geq 4 \text{ Å}^{-1}\)

and \(k \sim [E_{x\text{-ray}} - E_0]^{1/2}\)
Figure 3: Representative scans of WT SWMb (top), MbH93Y (middle), HEC (bottom) and of their cyanide-ligated complexes. Shown for each protein is a typical x-ray absorption spectrum where, above threshold, the extended x-ray absorption fine structure (EXAFS) region occurs.
(a) Ferric State

![Graph showing x-ray energy (eV) vs. F/I₀ for WT SWMb, MbH93Y, HEC, WTSWMb-CN, MbH93Y-CN, and HEC-CN.](image)

(b) CN Derivatives

![Graph showing x-ray energy (eV) vs. F/I₀ for WT SWMb-CN, MbH93Y-CN, and HEC-CN.](image)
where $A_0(k)$ is the amplitude, which describes the magnitude of the interference and depends only on the scattering atoms, the chemical nature of which is determined by a back-scattering factor $|f(k, \pi)| \sim Z/k^2$; an $r^2$ factor is due to the spherical nature of the waves, each possessing an $r^3$ dependence; a Debye-Waller factor $\exp[-2\sigma^2 k^2]$ describes the attenuation of the oscillations due to thermal and lattice disorder producing an uncertainty in $r$; and the finite lifetime of the photoelectron is described by a mean free path $\exp[-2r/\lambda(k)]$. The sine term is the total phase change and is due to the potential of both the absorbing and scattering atoms and the change in distance the photoelectron takes in its round-trip journey. $N$ is the number of neighboring atoms.

Six to eight scans comprised the data from each light source and were analyzed separately to enhance the signal-to-noise ratio (S/N). This was followed by background subtraction using a least squares procedure. Multiplication by $k^3$ followed, where $k$ is the photoelectron wave vector (Figure 4) and is equal to $2\pi/\lambda_{\text{electron}}$ where the denominator is the wavelength of the photo-electron. The EXAFS modulations from each light source were weighted in averaging according to their respective S/N ratio. The modulation covering $k=0-12.5\ \text{Å}^{-1}$ was used for further analysis. Although limiting the modulation below $k=13\ \text{Å}^{-1}$ may result in some loss of resolution of Fourier transform spectra, it reduces the background noise level of the Fourier transform (Figure 5). The last wiggle of the modulation should have a S/N of 2/1. The weighted EXAFS spectrum is Fourier transformed in order to isolate contributions from respective coordination shells (Figure 3). The first peak (major peak) of the
Figure 4: Background-subtracted EXAFS data normalized to one Fe atom and multiplied by $k^3$. (a) Ferric states of WT SWMb (top), MbH93Y (middle), and HEC (bottom). (b) CN$^-$ derivatives of WT SWMb (top), MbH93Y (middle), and HEC (bottom).
(a) Ferric State

\[ k^2 \chi(k) \]

- WT SWMb
- MbH93Y
- HEC

(b) CN Derivatives

\[ k^2 \chi(k) \]

- WT SWMb-CN
- MbH93Y-CN
- HEC-CN
Figure 5: Fourier transformations of the EXAFS data of Figure 4.

(a) Ferric states of WT SWMb (top), MbH93Y (middle), and HEC (bottom).

(b) cyanide derivatives of WT SWMb (top), MbH93Y (middle), and HEC (bottom).
(a) Ferric State

Ferric State

WT SWMb
MbH93Y
HEC

(b) CN Derivatives

CN Derivatives

WT SWMb-CN
MbH93Y-CN
HEC-CN
Fourier transform resulting from the first shell scattering wave signal was filtered and backtransformed into k space (Figure 6) and yields the phase component $\phi = 2kr + \phi(k)$ for a single-distance system and is used to extract the interatomic distance, $r$, if $\phi(k)$ is known for a model compound (Figure 6). Three model compounds, Fe$^{3+}$-bis(imidazole-tetraphenylporphinato)chloride (FeTPP(Im)$_2$), Fe$^{2+}$-acetylacetonate (Fe$^{2+}$-acac), and Fe$^{3+}$-acetylacetonate (Fe$^{3+}$-acac) were treated with the same procedures as described above except that transmission mode was used for data collection. For FeTPP(Im)$_2$, the Fe-N bond length is 1.986 Å (Collins et al., 1972) and for Fe$^{2+}$-acac and Fe$^{3+}$-acac, the Fe-O bond length is 1.990 Å (Iball and Morgan, 1976). The scattering signal derived from the first transform peak of each protein was fitted to that of the model compounds with two types of scattering atoms. Each atom type in the fitting procedure is represented by an average distance ($r$), an amplitude factor containing the number of ligands ($n$), a change in the Debye-Waller factor ($\Delta\sigma^2 = \sigma^2_{\text{model}} - \sigma^2_{\text{protein}}$) and a change in threshold energy ($\Delta E_0 = E_{\text{model}} - E_{\text{protein}}$). Because the number of scatterers is highly correlated with $\Delta\sigma^2$, precautions were taken to avoid erroneous results, which have been described in detail by Powers (1982). In this study, the two-scatterer nonlinear least squares fitting procedure with amplitude ratios was fixed at 4:2 to search parameter space for the scattering from the Fe-porphyrin (Fe-N$_p$) or 5:1 for the scattering from the Fe-axial ligand (Fe-N$_e$ or Fe-O). Several possible solutions were judged to be statistically different only if the $\Sigma R^2 > \Sigma R_{\text{min}}^2(1 + \phi_d^{-1})$, where $\Sigma R_{\text{min}}^2$ is the minimum sum of residuals for a physically reasonable solution, $\phi_d = \phi_d - p$, where $p$ is the number of variables in the fit and $\phi_d$ is the maximum number.
Figure 6:  Comparisons among the first-shell filtered data for each protein. (a) Ferric states of WT SWMb (top), MbH93Y (middle), and HEC (bottom) and (b) Cyanide derivatives of WT SWMb (top), MbH93Y (middle), and HEC (bottom).
(a) Ferric State

Ferric State

(b) CN Derivatives

K^2\chi(k)

\begin{align*}
\text{WT SWMb} \\
\text{MbH93Y} \\
\text{HEC}
\end{align*}

K^2\chi(k)

\begin{align*}
\text{WT SWMb-CN} \\
\text{MbH93Y-CN} \\
\text{HEC-CN}
\end{align*}
of degrees of freedom in the filtered data. The variable $\phi_d$ was estimated by $2\Delta w\Delta k/\pi$, where $\Delta w$ is the width of the filter window used for the Fourier transform peak and $\Delta k$ was the length of the data used. After solutions were found for each contribution to the first shell (Fe-N$_p$, Fe-N$_e$, Fe-O, or Fe-C), a three-atom consistency test was carried out using all three sets of scatterers with $r$ and $n$ held constant for each set of scatterers in order to verify that all three distances were contained in the data. The $\Sigma R^2$ for the consistency test must be smaller than those obtained for the two atom-type solutions. To ensure that the three distances obtained from the above steps constitute a true minimum, each distance was allowed to vary one at a time. Total error for each distance was determined by changing it to a different value while holding all other parameters constant in the fitting procedure until the sum of the $\Sigma R^2$ increased by a factor of two on each side of the minimum. The Hessian matrix, which also determines the errors for each parameter, was used to determine how much the parameters can be adjusted and still lie within the error. Some of the errors were not symmetric around the minimum value, in which case, the larger value of the errors was taken into account in the results. A detailed discussion of error analysis has been previously provided (Powers and Kincaid, 1989).

Inasmuch as various complicated physical processes, such as chemical bonding effects and core relaxation, are involved in the absorption process near the threshold, no simple relationship has been defined for the features and $E_0$, which was used to compute $k$ (Lee et al., 1981). To prevent an ambiguous answer, three arbitrary points
for $E_0$ were initially chosen for the comparison of $X(k)$. It is important to note that $E_0$ is a variable in the fitting procedures.

The ligand field indicator ratio (LFIR) was calculated according to Chance et al. (1984; Figure 7). It was calculated by drawing a perpendicular line from the inflection points on the horizontal baseline of the normalized X-ray spectrum to each of the two peaks, labeled A and B. The LFIR was then calculated as the ratio of the lengths of the perpendicular lines, $A/B$.

![Figure 7: The ligand field indicator region of MbH93Y.](image)
CHAPTER III

RESULTS

UV/Visible Spectroscopy. The electronic absorption spectra of the met-, cyanomet, reduced, CO, and oxy forms of both the MbH93Y and WT· SWMb are displayed in Figure 8 and Table 1; the latter also displays the results obtained by previous investigators for comparison.

In this study, we confirm and complement the spectroscopic studies initiated by Egeberg et al. (1990a) and Morikis et al. (1990) using UV/visible and XAS spectroscopies. We were able to reproduce the optical absorption spectra of three of the liganded forms, O₂, CO, and cyanomet, and the Fe³⁺ (met) and Fe²⁺ (Table 1) reported earlier by four other groups (Table 1; Egeberg et al., 1990a; Adachi et al., 1991; Dawson et al., 1992; Tsai et al., 1993) of a mutant myoglobin in which the native proximal His was mutated to a Tyr residue. The Soret peak (λ = 403 nm) millimolar extinction coefficient (ε_{403nm}) of MbH93Y was determined to be 169.46 mM⁻¹cm⁻¹. Note that the met form of MbH93Y, like that of the WT protein, is high spin. However, unlike the WT protein, the Soret band of the mutant is significantly blue-shifted relative to WT SWMb (Table 1) which suggests pentacoordination.

Horse Erythrocyte Catalase. Because the hemes of purified BLC are often degraded to bile pigments (Nicholls and Schonbaum, 1963; Palcic and Dunford, 1980), we have purified catalase from horse erythrocytes (R₂ = 1.25) and have shown by haemochromagen assay (Paul et al., 1953) that no degraded hemes are present. Both the pseudo first-order rate constant and apparent rate constant for the dismutation of
Figure 8: Optical absorption spectra of the various oxidation and liganded states of Wild-Type Sperm Whale Myoglobin and MbH93Y. All forms were made in 20 mM phosphate buffer, pH 7.0, at 23°C. (a) met WT SWMb and met MbH93Y. (b) Cyanomet WT SWMb and cyanomet MbH93Y. (c) Reduced, oxy and carbonmonoxy forms of Wild-Type Sperm Whale Myoglobin. (d) Reduced, oxy and carbonmonoxy forms of MbH93Y. See Table 1 for the corresponding wavelengths.
### Table 1: Optical Absorption Peak Data of the Met, Cyanomet, Carbon-Monoxy, Reduced and Oxy Forms of Wild-Type Sperm Whale Myoglobin and MbH93Y.

<table>
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<th>Soret</th>
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<tr>
<td></td>
<td>Met (Fe$^{3+}$)</td>
<td>MbH93Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>403 (402)</td>
<td>483 (480)</td>
<td>515 (524)</td>
<td>597 (598)</td>
</tr>
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<td></td>
<td>WTSWMb 408 (408)</td>
<td>504 (502)</td>
<td></td>
<td>632 (632)</td>
</tr>
<tr>
<td>Reduced (Fe$^{2+}$)</td>
<td>MbH93Y 427 (428) [427]</td>
<td>564 (556) [560]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WTSWMb 433 (432) [433]</td>
<td>555 (554) [558]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxy (Fe$^{2+}$)</td>
<td>MbH93Y 420 (~414) [414]</td>
<td>542 (540) [540]</td>
<td>568 (576) [576]</td>
<td></td>
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<tr>
<td></td>
<td>WTSWMb 423 (418) [417]</td>
<td>542 (544) [543]</td>
<td>577 (580) [580]</td>
<td></td>
</tr>
<tr>
<td>Carbon-monoxy (Fe$^{2+}$)</td>
<td>MbH93Y 419 (418) [420]</td>
<td>539 (536) [539]</td>
<td>569 (568) [567]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WTSWMb 422 (422) [422]</td>
<td>542 (542) [541]</td>
<td>578 (578) [579]</td>
<td></td>
</tr>
<tr>
<td>Cyanomet (Fe$^{3+}$)</td>
<td>MbH93Y 419 [421]</td>
<td>550 [550]</td>
<td></td>
<td></td>
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<tr>
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<td>WTSWMb 423 424 423'</td>
<td>542 541 540'</td>
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Specifically, the most significant absorption maxima are shown for the various liganded forms of WT SWMb and MbH93Y. Values for this study are shown in plain text. For comparison, values from Egeberg et al. (1990a) are shown in parentheses ( ), while those of Adachi et al. (1991) are shown in brackets [ ]. For the cyanide-liganded forms, values marked with a superscripted ' refer to those of Tsai et al. (1993) or a superscripted d refer to those of Dawson et al. (1992).
H$_2$O$_2$ were determined to be \( k' = 2.415 \times 10^2 \text{ sec}^{-1} \) and \( k_{\text{app}} = 1.793 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1} \), respectively, which are in agreement with previously reported values (Nicholls and Schonbaum, 1963). Finally, our purified HEC was able to form Compound I in the presence of H$_2$O$_2$ (Palcic and Dunford, 1981).

**XAS Spectroscopy.** The LFIR ratios of WT SWMb, MbH93Y, and HEC, and of their cyanide-ligated complexes, are shown in Figure 9, the calculations from which, are shown in Table 2 with their corresponding Fe-out-of-heme plane distances (Fe-C$_7$) (Chance et al., 1986a). These values are consistent with the average Fe-to-pyrrole nitrogen (Fe-N$_p$) distances (Chance et al., 1984; Chance et al., 1986b) of 2.045 (WT SWMb), 2.04 (MbH93Y), 2.03 (HEC), 2.00 (WT SWMb-CN), 1.96 (MbH93Y-CN), and 2.01 (HEC-CN). However, multiple scattering is responsible for the shape of the LFIR which can also be affected by ruffling of the heme plane.

Table 3 shows the results of the two-atom-type fitting procedure for the met (Fe$^{3+}$) form of WT SWMb at pH 7.0 and these are identical to those previously reported (Chance et al., 1983; Powers et al., 1984). The two-atom-type fitting procedure shows that \( 2.14 \pm 0.02 \text{ Å} \) (Solution A) and \( 1.97 \pm 0.015 \text{ Å} \) (Solution B) are the axial ligand distances. To confirm these distances, we used a three-atom-type consistency test; the results are shown as Solution C at the bottom of Table 3. Since nitrogen has only one fewer electron than oxygen, the analysis cannot distinguish between the scattering amplitude of these two atoms. Thus, the Fe-N and Fe-O models yield the same results. However, the sixth ligand is probably the oxygen atom of a water molecule, since our sample is the metaquo form of SWMb. Therefore, the Fe
Figure 9: The ligand field indicator regions of (a) Wild-Type Sperm Whale Myoglobin (top), MbH93Y (middle), Horse Erythrocyte Catalase (bottom) and of their cyanide-ligated complexes.
(a) Ferric State

F / I_0

0.2

0.1

WT SWMb

MbH93Y

HEC

x-ray energy (eV)

7125 7175 7225

(b) CN Derivatives

F / I_0

0.2

0.1

WT SWMb-CN

MbH93Y-CN

HEC-CN

x-ray energy (eV)

7125 7175 7225
Table 2: The Ligand Field Indicator Region Ratios and Corresponding Fe-Out-of-Heme Plane Distances (Fe-Cr) (Chance et al., 1986a) of Wild-Type Sperm Whale Myoglobin, MbH93Y and Horse Erythrocyte Catalase and Their Cyanide-Ligated Complexes.

<table>
<thead>
<tr>
<th></th>
<th>WT SWMb</th>
<th>MbH93Y</th>
<th>HEC</th>
<th>WT SWMb-CN</th>
<th>MbH93Y-CN</th>
<th>HEC-CN</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFIR</td>
<td>1.20</td>
<td>1.37</td>
<td>1.34</td>
<td>0.81</td>
<td>0.70</td>
<td>1.00</td>
</tr>
<tr>
<td>Fe-Cr</td>
<td>0.40</td>
<td>0.50</td>
<td>0.50</td>
<td>0.15</td>
<td>0.10</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Distances of Fe-Cr are in Å. Each ratio has an error of ± 0.05, which corresponds to an error of ± 0.03 Å for the Fe-Cr distance.

Table 3: Results of the Two-Atom-Type Fitting Procedure and Three-Atom Consistency Test for the First Coordination Shell of Wild-Type Sperm Whale Metmyoglobin at pH 7.0.

<table>
<thead>
<tr>
<th>Model</th>
<th>r</th>
<th>N</th>
<th>(\Delta\sigma^2)</th>
<th>(\Delta E_\theta^*)</th>
<th>(\Sigma R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.03</td>
<td>5</td>
<td>2.9x10^-3</td>
<td>0.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Fe-N</td>
<td>2.14</td>
<td>1</td>
<td>5.1x10^-3</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2.06</td>
<td>5</td>
<td>2.2x10^-3</td>
<td>0.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Fe-N</td>
<td>1.97</td>
<td>1</td>
<td>5.4x10^-3</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2.045±0.015</td>
<td>4</td>
<td>3.8x10^-3</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Fe-N</td>
<td>2.15±0.02</td>
<td>1</td>
<td>4.7x10^-3</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>Fe-O</td>
<td>1.98±0.03</td>
<td>1</td>
<td>1.6x10^-3</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

* Each solution for a set of models is designated by a bold upper-case letter. \(r\), Fe-ligand distance in angstroms. \(N\), number of ligands, held constant. \(\Delta\sigma^2\), difference in Debye-Waller factor (model-protein) in square angstroms ± 45%. \(\Delta E_\theta\), difference in threshold energy (model-protein) in electron volts ± 2.5 eV.
of metSWMb has four pyrrole nitrogen ligands at an average distance of \(2.045 \pm 0.015\, \text{Å}\), one axial ligand at \(2.14 \pm 0.02\, \text{Å}\), and a second axial ligand at \(1.97 \pm 0.03\, \text{Å}\).

The fitting procedure for MbH93Y is shown in Table 4. Inspection of Table 4 reveals our search of parameter space for possible two-atom type solutions. Although many solutions exist (Table 4), elimination of some of these solutions as possibilities was based upon the large \(\Delta E_0\) values (e.g., Solution D). Specifically, the solution is not any better when total \(N = 6\) (Solutions B, F, H), than when total \(N = 5\) (Solutions A, E), as revealed by the \(\Sigma R^2\), strongly indicating that only five ligands are bound to the Fe. That is, even when the attempt was made to fit total \(N = 6\) ligands, the \(\Sigma R^2\) was no different. And when the magnitude of the \(\Delta E_0\) values are taken into account, the amplitude ratios of 4/1 gave better \(\Sigma R^2\) values than those of 5/1. The three-atom-type consistency test (Solution H), therefore, confirms that MbH93Y has only five ligands. The average Fe-pyrrole nitrogen distance is \(2.04 \pm 0.015\, \text{Å}\), while the length of the proximal Fe-O bond is \(2.13 \pm 0.02\, \text{Å}\).

We applied the same fitting procedures described above for HEC (Table 5), where the two-atom-type fitting procedures revealed axial ligand distances of \(1.92 \pm 0.02\, \text{Å}\) (Solution B) and \(2.11 \pm 0.03\, \text{Å}\) (Solution C). A three-atom-type consistency test (Solution E) revealed the average Fe-pyrrole nitrogen ligand distance to be \(2.03 \pm 0.015\, \text{Å}\), proving to be no different from those of either WT metSWMb or MbH93Y. This agreed well with the two-atom-type fits for axial ligand distances (Solutions B and C).

Among these three proteins, the only significant differences in bond lengths
Table 4: Results of the Two-Atom-Type Fitting Procedure and Three-Atom Consistency Test for the First-Coordination Shell of MbH93Y at pH 7.0.

<table>
<thead>
<tr>
<th>Model</th>
<th>$r$</th>
<th>$N^c$</th>
<th>$\Delta\sigma^d$</th>
<th>$\Delta E_0^e$</th>
<th>$\Sigma R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.03</td>
<td>4</td>
<td>5.2x10$^3$</td>
<td>-1.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Fe-N</td>
<td>2.13</td>
<td>1</td>
<td>9.5x10$^3$</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2.01</td>
<td>5</td>
<td>4.3x10$^3$</td>
<td>-1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Fe-N</td>
<td>2.12</td>
<td>1</td>
<td>9.3x10$^3$</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2.04</td>
<td>5</td>
<td>1.3x10$^3$</td>
<td>-2.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Fe-N</td>
<td>1.68</td>
<td>1</td>
<td>-8.3x10$^3$</td>
<td>-13.5</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>2.04</td>
<td>4</td>
<td>1.9x10$^3$</td>
<td>-1.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Fe-N</td>
<td>1.76</td>
<td>1</td>
<td>-1.6x10$^3$</td>
<td>-39.5</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>2.06</td>
<td>4</td>
<td>5.3x10$^3$</td>
<td>-0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Fe-N</td>
<td>1.95</td>
<td>1</td>
<td>9.3x10$^3$</td>
<td>-5.0</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>2.06</td>
<td>5</td>
<td>4.1x10$^3$</td>
<td>-1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Fe-N</td>
<td>1.96</td>
<td>1</td>
<td>8.8x10$^3$</td>
<td>-11.1</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>2.03</td>
<td>5.02</td>
<td>1.5x10$^3$</td>
<td>-1.2</td>
<td>2.8</td>
</tr>
<tr>
<td>H</td>
<td>2.04±0.015</td>
<td>4</td>
<td>4.7x10$^3$</td>
<td>-0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Fe-O</td>
<td>2.11±0.03</td>
<td>1</td>
<td>5.4x10$^3$</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>Fe-N</td>
<td>1.95±0.02</td>
<td>1</td>
<td>4.1x10$^3$</td>
<td>-2.7</td>
<td></td>
</tr>
</tbody>
</table>

* Each solution for a set of models is designated by a bold upper-case letter.  
  $^b$ $r$, Fe-ligand distance in angstroms.  
  $^c$ $N$, number of ligands, held constant.  
  $^d$ $\Delta\sigma^2$, difference in Debye-Waller factor (model-protein) in square angstroms ± 45%.  
  $^e$ $\Delta E_0$, difference in threshold energy (model-protein) in electron volts ± 2.5 eV.
Table 5: Results of the Two-Atom-Type Fitting Procedure and Three-Atom Consistency Test for the First Coordination Shell of Horse Erythrocyte Catalase at pH 7.0.

<table>
<thead>
<tr>
<th>Model</th>
<th>$r^b$</th>
<th>N$^c$</th>
<th>$\Delta \sigma^d$</th>
<th>$\Delta E_0$</th>
<th>$\Sigma R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Fe-N</td>
<td>2.04</td>
<td>5</td>
<td>8.6x10$^4$</td>
<td>-0.7</td>
<td>4.2</td>
</tr>
<tr>
<td>Fe-O</td>
<td>1.94</td>
<td>1</td>
<td>3.9x10$^3$</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>B Fe-N</td>
<td>2.03</td>
<td>4</td>
<td>3.6x10$^3$</td>
<td>-0.6</td>
<td>6.6</td>
</tr>
<tr>
<td>Fe-O</td>
<td>1.92</td>
<td>1</td>
<td>5.0x10$^3$</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>C Fe-N</td>
<td>1.98</td>
<td>4</td>
<td>6.1x10$^3$</td>
<td>1.8</td>
<td>6.4</td>
</tr>
<tr>
<td>Fe-O</td>
<td>2.11</td>
<td>1</td>
<td>6.3x10$^3$</td>
<td>-3.3</td>
<td></td>
</tr>
<tr>
<td>D Fe-N</td>
<td>1.99</td>
<td>5</td>
<td>3.8x10$^3$</td>
<td>0.9</td>
<td>3.4</td>
</tr>
<tr>
<td>Fe-O</td>
<td>2.13</td>
<td>1</td>
<td>3.4x10$^3$</td>
<td>-3.1</td>
<td></td>
</tr>
<tr>
<td>E Fe-N</td>
<td>2.03±0.015</td>
<td>4</td>
<td>3.0x10$^3$</td>
<td>-0.3</td>
<td>3.3</td>
</tr>
<tr>
<td>Fe-O</td>
<td>1.92±0.02</td>
<td>1</td>
<td>3.7x10$^3$</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>Fe-O</td>
<td>2.11±0.03</td>
<td>1</td>
<td>-1.5x10$^{-2}$</td>
<td>-4.3</td>
<td></td>
</tr>
</tbody>
</table>

*a Each solution for a set of models is designated by a bold upper-case letter.  
b $r$, Fe-ligand distance in angstroms.  
c N, number of ligands, held constant.  
d $\Delta \sigma^2$, difference in Debye-Waller factor (model-protein) in square angstroms ± 45%.  
e $\Delta E_0$, difference in threshold energy (model-protein) in electron volts ± 2.5 eV.
were (i) the proximal ligand distance of WT SWMb and MbH93Y is \(-0.2\,\text{Å}\) longer than that of HEC; (ii) the sixth ligand distance of WT SWMb is \(-0.14\,\text{Å}\) shorter than that of HEC; and (iii) MbH93Y has no sixth ligand.

The results for the first coordination shell analysis for WT SWMb-CN are summarized in Table 6. The $\Sigma R^2$ for the three-atom consistency test (Solution C) is more than a factor of 2 smaller than those of the two-atom-type fits (Solutions A and B). Error analysis of each bond length value indicated that $2.00 \pm 0.015\,\text{Å}$ is the average Fe-N$_p$ bond length and that $2.10 \pm 0.02\,\text{Å}$ and $1.87 \pm 0.03\,\text{Å}$ are probable solutions for the axial ligands within the first coordination shell.

Similarly, the results for the first coordination shell for MbH93Y-CN are provided in Table 7. Again the three-atom-type consistency test (Solution C) is more than a factor of 2 smaller than those of the two-atom-type fits (Solutions A and B). Therefore, after error analysis of each bond length value, the final solution for the first coordination shell is $2.07 \pm 0.02\,\text{Å}$ (Fe-O), $1.84 \pm 0.03\,\text{Å}$ (Fe-CN), and $1.96 \pm 0.015\,\text{Å}$ (average Fe-N$_p$).

Finally, the results for the first coordination shell for HEC-CN are summarized in Table 8. The three-atom-type consistency test (Solution C) is about 30% smaller than that with an N of 5/1 (Solution A). The resulting final solution after error analysis of each bond length for the first coordination shell was $2.12 \pm 0.02\,\text{Å}$ (Fe-O), $1.87 \pm 0.03\,\text{Å}$ (Fe-CN), and $2.01 \pm 0.015\,\text{Å}$ (average Fe-N$_p$).

Among these three cyanide-bound proteins, the only significant differences in bond lengths were (i) the proximal bond length of MbH93Y-CN is \(-0.05\,\text{Å}\) shorter
Table 6: Results of the Two-Atom-Type Fitting Procedure and Three-Atom Consistency Test for the First Coordination Shell of Cyanide-Ligated Wild-Type Sperm Whale Myoglobin at pH 7.0.

<table>
<thead>
<tr>
<th>Model</th>
<th>( r^b )</th>
<th>( N^c )</th>
<th>( \Delta \sigma^2^d )</th>
<th>( \Delta E_0^e )</th>
<th>( \Sigma R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.98</td>
<td>5</td>
<td>5.7 \times 10^{-3}</td>
<td>-3.9</td>
<td>7.2</td>
</tr>
<tr>
<td>Fe-N</td>
<td>2.10</td>
<td>1</td>
<td>1.3 \times 10^{-2}</td>
<td>-1.2</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2.03</td>
<td>5</td>
<td>6.3 \times 10^{-3}</td>
<td>-6.2</td>
<td>8.0</td>
</tr>
<tr>
<td>Fe-N</td>
<td>1.89</td>
<td>1</td>
<td>1.2 \times 10^{-2}</td>
<td>-3.6</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2.00 \pm 0.015</td>
<td>4</td>
<td>6.8 \times 10^{-3}</td>
<td>-1.9</td>
<td>3.0</td>
</tr>
<tr>
<td>Fe-N</td>
<td>2.10 \pm 0.02</td>
<td>1</td>
<td>8.5 \times 10^{-3}</td>
<td>-6.6</td>
<td></td>
</tr>
<tr>
<td>Fe-C</td>
<td>1.87 \pm 0.03</td>
<td>1</td>
<td>8.5 \times 10^{-3}</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

* Each solution for a set of models is designated by a bold upper-case letter.  
  
* \( r \), Fe-ligand distance in angstroms.  
  
* \( N \), number of ligands, held constant.  
  
* \( \Delta \sigma^2 \), difference in Debye-Waller factor (model-protein) in square angstroms \( \pm 45\% \).  
  
* \( \Delta E_0 \), difference in threshold energy (model-protein) in electron volts \( \pm 2.5 \text{ eV} \).

Table 7: Results of the Two-Atom-Type Fitting Procedure and Three-Atom-Consistency Test for the First-Coordination Shell of Cyanide-Ligated MbH93Y at pH 7.0.

<table>
<thead>
<tr>
<th>Model</th>
<th>( r^b )</th>
<th>( N^c )</th>
<th>( \Delta \sigma^2^d )</th>
<th>( \Delta E_0^e )</th>
<th>( \Sigma R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.95</td>
<td>5</td>
<td>4.2 \times 10^{-3}</td>
<td>-2.2</td>
<td>9.8</td>
</tr>
<tr>
<td>Fe-N</td>
<td>2.06</td>
<td>1</td>
<td>5.1 \times 10^{-3}</td>
<td>-5.4</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1.98</td>
<td>5</td>
<td>3.7 \times 10^{-3}</td>
<td>-3.1</td>
<td>9.9</td>
</tr>
<tr>
<td>Fe-N</td>
<td>1.89</td>
<td>1</td>
<td>6.1 \times 10^{-3}</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1.96 \pm 0.015</td>
<td>4</td>
<td>5.5 \times 10^{-3}</td>
<td>-2.7</td>
<td>4.2</td>
</tr>
<tr>
<td>Fe-O</td>
<td>2.07 \pm 0.02</td>
<td>1</td>
<td>2.4 \times 10^{-3}</td>
<td>-5.2</td>
<td></td>
</tr>
<tr>
<td>Fe-C</td>
<td>1.84 \pm 0.03</td>
<td>1</td>
<td>1.0 \times 10^{-3}</td>
<td>9.6</td>
<td></td>
</tr>
</tbody>
</table>

* Each solution for a set of models is designated by a bold upper-case letter.  
  
* \( r \), Fe-ligand distance in angstroms.  
  
* \( N \), number of ligands, held constant.  
  
* \( \Delta \sigma^2 \), difference in Debye-Waller factor (model-protein) in square angstroms \( \pm 45\% \).  
  
* \( \Delta E_0 \), difference in threshold energy (model-protein) in electron volts \( \pm 2.5 \text{ eV} \).
Table 8: Results of the Two-Atom-Type Fitting Procedure and Three-Atom Consistency Test for the First-Coordination Shell of Cyanide-Ligated Horse Erythrocyte Catalase at pH 7.0.

<table>
<thead>
<tr>
<th>Model</th>
<th>$r^b$</th>
<th>$N^c$</th>
<th>$\Delta G^2$ $^d$</th>
<th>$\Delta E_o$ $^e$</th>
<th>$\Sigma R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.04</td>
<td>5</td>
<td>-1.1x$10^{-3}$</td>
<td>-4.2</td>
<td>7.5</td>
</tr>
<tr>
<td>Fe-N</td>
<td>1.89</td>
<td>1</td>
<td>7.7x$10^{-3}$</td>
<td>-1.4</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1.97</td>
<td>5</td>
<td>-1.2x$10^{-3}$</td>
<td>-3.0</td>
<td>8.7</td>
</tr>
<tr>
<td>Fe-N</td>
<td>2.12</td>
<td>1</td>
<td>4.4x$10^{-3}$</td>
<td>-7.7</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2.01±0.015</td>
<td>4</td>
<td>5.1x$10^{-3}$</td>
<td>-2.8</td>
<td>5.3</td>
</tr>
<tr>
<td>Fe-O</td>
<td>2.12±0.02</td>
<td>1</td>
<td>5.4x$10^{-3}$</td>
<td>-7.8</td>
<td></td>
</tr>
<tr>
<td>Fe-C</td>
<td>1.87±0.03</td>
<td>1</td>
<td>4.6x$10^{-3}$</td>
<td>3.4</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Each solution for a set of models is designated by a bold upper-case letter. $^b$ $r$, Fe-ligand distance in angstroms. $^c$ $N$, number of ligands, held constant. $^d$ $\Delta G^2$, difference in Debye-Waller factor (model-protein) in square angstroms ± 45%. $^e$ $\Delta E_o$, difference in threshold energy (model-protein) in electron volts ± 2.5 eV.

than that of HEC-CN and (ii) the average the Fe-$N_p$ bond length of MbH93Y-CN is -0.04 Å shorter than in WT SWMb-CN and HEC-CN.

When each of these three proteins (WT SWMb, MbH93Y, and HEC) are compared with their respective cyanide-ligated forms, the following differences in bond lengths were revealed: WT SWMb and WT SWMb-CN differed in their Fe-distal ligand and average Fe-$N_p$ bond lengths; MbH93Y and MbH93Y-CN differed in their proximal Fe-O and average Fe-$N_p$ bond lengths; and HEC and HEC-CN differed in their Fe-distal ligand and proximal Fe-O bond lengths.
Reactions of Myoglobins with $H_2O_2$. The huge difference in the structure of the active sites of HEC and MbH93Y should lead us to dismiss the possibility that the corresponding different functions between HEC and SWMb are due solely to the different proximal ligand at the distal site. It was tempting to speculate that as a result of this mutation, Compound I (Fita and Rossmann, 1985) could not be formed and that this protein is incapable of any catalytic activity. Unlike HEC, MbH93Y was neither able to form a Compound I-like species nor was it able to decompose $H_2O_2$. And unlike the native SWMb (Powers et al., 1987) which formed a Compound II-like spectrum (Figure 10a), upon addition of 2.00 or 0.25 molar equivalents of $H_2O_2$, MbH93Y did not form myoglobin peroxide. After 8 minutes of exposure to $H_2O_2$, haemochromagen assay revealed the heme in native SWMb to be completely degraded (Figure 10b; Keilin and Hartree, 1950). Regeneration of the Soret peak upon treatment of SWMb by L- (+)-ascorbic acid following exposure to $H_2O_2$ indicated that the heme could be restored before the peroxide had time to completely oxidize the heme (Figure 10c). On the other hand, following exposure to $H_2O_2$, the heme in MbH93Y [Fe$^{2+}$-Fe$^{3+}$ couple, $E^\circ = -190$ mV (Adachi et al., 1991)] was not reducible by a small amount of ascorbate (Figure 10d), but was reducible by a small amount of sodium dithionite (Figure 10e; recall absorption spectra, Figure 8). Haemochromagen assay revealed the presence of degraded hemes when ascorbate was the reductant (Figure 10d), but intact hemes were indicated when dithionite was the reductant (Figure 10e). Perhaps because Fe$^{2+}$-Fe$^{3+}$ couple of $E^\circ_{MbH93Y} = -190$ mV and because dithionite is a stronger reductant
Figure 10: Optical absorption spectra of the effects of exposure of native sperm whale myoglobin or MbH93Y to 2 molar equivalents of H$_2$O$_2$. Spectra are identical whether 2 or 0.25 molar equivalents were used. All reactions were executed in 20 mM phosphate buffer, pH 7.0, at 25°C. (a) Minute-by-minute decay of the Soret band of native SWMb upon exposure to 2 molar equivalents H$_2$O$_2$ where [native SWMb] = 7.25 µM and [H$_2$O$_2$] = 14.5 µM. (b) Haemochromagen assay reveals the presence of broken hemes after prolonged exposure to 0.25 molar equivalents H$_2$O$_2$ where [native SWMb] = 13.37 µM and [H$_2$O$_2$] = 3.34 µM. (c) Regeneration of the Soret band of the native protein is evident with the addition of a small amount of L-(+)-ascorbic acid, following exposure to 0.25 molar equivalents H$_2$O$_2$ (4.62 µM), where [native SWMb] = 18.5 µM. (d) Absorption spectra of the effects of exposure of MbH93Y to 0.25 molar equivalents H$_2$O$_2$ with respect to the protein. All reactions were executed in 20 mM phosphate buffer, pH 7.0, at 25°C. Exposure to 0.25 molar equivalents H$_2$O$_2$, followed by a small amount of L-(+)-ascorbic acid, which was unable to restore the Soret peak. Haemochromagen assay revealed the presence of broken hemes. (e) Same conditions as in (d), except that sodium dithionite was the reductant. Haemochromagen assay revealed the presence of intact hemes.
than ascorbate, dithionite is able to reduce the MbH93Y heme, electronically protecting the heme system from the damaging oxidative effects of $H_2O_2$. Since ascorbate is not sufficient to reduce the MbH93Y heme, the prosthetic group is not protected from the damaging oxidative effects of $H_2O_2$.

In addition, an attempt was made to determine the ferric-ferryl ($Fe^{3+}$-$Fe^{4+}$) redox couple of MbH93Y in the same manner that Adachi et al. (1991) used for the ferrous-ferric ($Fe^{2+}$-$Fe^{3+}$) redox couple of the human MbH93Y. In metmyoglobin, when $H_2O_2$ oxidizes the porphyrin, a red-shift of the Soret band is observed, yielding a Compound II-like (myoglobin peroxide) spectrum. However, when MbH93Y was exposed to a 16-fold molar excess of potassium iridate or a 33-fold molar excess of ethyl hydrogen peroxide, no such red-shift resulted (Figure 11). These results indicate that in
Figure 11: Results of the studies to determine the Fe$^{3+}$-Fe$^{4+}$ redox couple of MbH93Y. In all experiments below, the [MbH93Y] = 6 μM.

(a) Experiment in which a 3.33-fold molar excess, followed by a 15.15-fold molar excess of the oxidant, potassium hexachloroirridate, was added to MbH93Y. Note the absence of any shift of the Soret band.

(b) Experiment in which a 15.15-fold molar excess of potassium hexachloroirridate (relative to MbH93Y) was added to MbH93Y, followed by a 32.36-fold molar excess of ethyl H$_2$O$_2$. Again, note the absence of any spectral change. (c) For the sake of clarity, the spectrum of ethyl H$_2$O$_2$ is shown alone with MbH93Y.
MbH93Y, either the ferryl intermediate is unstable or the redox potential for the Fe$^{3+}$-Fe$^{4+}$ is too high (> 1 V) to be determined in this manner.

*Cyanide Binding to Myoglobins.* The binding affinity and kinetics of CN$^-$ to native 2(and/or WT) SWMb and MbH93Y were calculated from the titration of each protein with increasing concentrations of KCN, whose resultant reactions were followed spectroscopically (Figure 12) over time. A comparison of the CN$^-$ binding affinity and kinetics of the native (and/or WT) SWMb and MbH93Y reveals striking differences between the two proteins. Table 9 shows an average difference of approximately three orders of magnitude in binding affinity ($K_D$) between MbH93Y and the WT protein; that is, the latter binds CN$^-$ in the micromolar range (Ver Ploeg et al., 1971; King et al., 1992) while MbH93Y requires millimolar concentrations of CN$^-$. Specifically, at the lower pH values of 5.5 and 6.0, there was a $-10^2$-fold difference between the native protein and the mutant; at pH 6.5, 7.0, and 7.5, this difference increased to three orders of magnitude; and at pH 8.0, there was $-10^4$-fold difference in $K_D$ between the native protein and MbH93Y. Specific pH-dependent differences in $K_D$ between the MbH93Y and WT SWMb were not identical, but comparable (Table 9). In addition, unlike the native or WT proteins, which always reached 100% saturation in formation of the MbCN complex, MbH93Y reached only 44-86% saturation, depending on the pH.

Provided in Table 10 and Figure 13 are the graphically calculated apparent pseudo-first order rate constants ($k_{-1(\text{app})}$) for CN$^-$ binding (Vega-Catalan et al., 1986) to the native and mutant myoglobins. The native SWMb displays approximately the
Figure 12: Typical optical absorption scans of the titration of (a) wild-type sperm whale myoglobin and (b) MbH93Y with increasing concentrations of KCN at pH 7.0. With the addition of each aliquot of known amount of KCN, the reaction was followed spectroscopically at the Soret wavelength until the reaction was complete. The spectrum at the resultant Soret wavelength and its corresponding absorbance were then recorded. The difference in absorbance (ΔA) was then used in the calculations (Recall Chapter II, Experimental Procedures). The titration experiments were performed at pH values of 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0; but since the calculations and resultant binding affinities and kinetics depend only on the ΔA and on the relative concentrations of total myoglobin and total cyanide and are not visually revealed in the titration spectra of each experiment at each pH, the spectra at pH 7.0 were chosen to illustrate a sample of a typical absorption scan of the titration of each of the myoglobins with KCN.
Absorbance

Wavelength (nm)

(a)

(b)
Table 9: Dissociation Constants ($K_D$, M) and Corresponding Changes in Free Energy ($-\Delta G$, kcal mol$^{-1}$) for Native and Wild-Type Sperm Whale Myoglobin and MbH93Y at pH Values of 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The SEM = Standard Error of the Mean.

<table>
<thead>
<tr>
<th>pH</th>
<th>Native SWMb</th>
<th>WT SWMb</th>
<th>MbH93Y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_D \times 10^{-5}$</td>
<td>$K_D \times 10^{-3}$</td>
<td>$K_D \times 10^{-3}$</td>
</tr>
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<td></td>
<td>$\pm$ SEM</td>
<td>$\pm$ SEM</td>
<td>$\pm$ SEM</td>
</tr>
<tr>
<td></td>
<td>$-\Delta G$</td>
<td>$-\Delta G$</td>
<td>$-\Delta G$</td>
</tr>
<tr>
<td>5.5</td>
<td>2.44</td>
<td>3.00</td>
<td>6.19</td>
</tr>
<tr>
<td>6.0</td>
<td>2.08</td>
<td>1.56</td>
<td>3.96</td>
</tr>
<tr>
<td>6.5</td>
<td>0.570</td>
<td>1.43</td>
<td>5.43</td>
</tr>
<tr>
<td>7.0</td>
<td>0.451</td>
<td>0.439</td>
<td>8.55</td>
</tr>
<tr>
<td>7.5</td>
<td>0.240</td>
<td>0.346</td>
<td>8.18</td>
</tr>
<tr>
<td>8.0</td>
<td>0.522</td>
<td>0.281</td>
<td>11.7</td>
</tr>
</tbody>
</table>

The same pH-dependent trend of $k_{-1\, \text{app}}$ for CN$^-$ binding reported earlier (Ver Ploeg and Alberty, 1968, 1971; Vega-Catalan et al., 1986; Ikeda-Saito et al., 1992). The MbH93Y, however, also displays a kinetic dependence on pH, but only at the extreme values examined. The pH-optima for the rate of CN$^-$ association appears to be at pH 5.5, or, at least, below pH 6.0 (Table 10, Figure 13). We were unable to accurately evaluate the rate constant for CN$^-$ dissociation ($k_{-1\, \text{app}}$) from either native (or WT) or MbH93Y due to the large degree of uncertainty associated with the intercept (Vega-Catalan et al., 1986). This indicates that the reaction is not easily reversible and goes nearly to completion (Ver Ploeg and Alberty, 1968). Furthermore, unlike the native protein, which displays classic pseudo first-order kinetics, the rate of CN$^-$ binding to MbH93Y was biphasic at all pH values examined. Only the rapid phase is reported...
Table 10: Apparent Pseudo-First Order Rate Constants ($k_{1(app)}$, M$^{-1}$sec$^{-1}$) for Wild-Type Sperm Whale Metmyoglobin and MbH93Y at pH Values of 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0.

<table>
<thead>
<tr>
<th>pH</th>
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<th>6.0</th>
<th>6.5</th>
<th>7.0</th>
<th>7.5</th>
<th>8.0</th>
</tr>
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<tbody>
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<td>WT</td>
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<td>72.16</td>
<td>94.16</td>
<td>73.34</td>
<td>145.59</td>
</tr>
<tr>
<td>MbH93Y</td>
<td>22.04</td>
<td>6.64</td>
<td>5.02</td>
<td>4.19</td>
<td>5.26</td>
<td>1.76</td>
</tr>
</tbody>
</table>

in Figure 13 and is still many orders of magnitude lower than that of the WT form.

*Cyanogen Bromide-Modified Myoglobins.* Figure 14 displays the UV/visible absorption spectra of the reaction of CNBr with native SWMb (Figure 14a) and MbH93Y (Figure 14b). Modification of the distal His in the native protein is clearly evident by the dramatic blue shift of the Soret peak, indicating conversion from hexacoordination to pentacoordination (see below; Figure 14a; Jajczay, 1970) and by the fact that subsequent addition of H$_2$O$_2$ to the reaction leads to a dramatic decrease in the rate at which the Soret peak decreased, indicative of heme degradation (compare to Figure 10a). Modification of His64 in MbH93Y is also evidenced by a decrease in the rate of heme degradation by H$_2$O$_2$, but results in a Soret peak that is only very slightly red-shifted, indicating that the mutant protein was originally pentacoordinate before the introduction of CNBr.
Figure 13: Time course decay curves for the reaction of native sperm whale myoglobin or MbH93Y with cyanide at the pH values of 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The decay curves were followed at the Soret peak $\lambda$ (Table 1) for each respective protein for 10 minutes to make sure the reaction was complete. Calculations of the pseudo-first order rate constants and apparent rate constants [$k_{+1(\text{app})}$, M$^{-1}$sec$^{-1}$, mean ± standard error of the mean (SEM)] of the bimolecular reaction of the WT SWMb or MbH93Y plus CN$^-$ were performed as described by Vega-Catalan et al. (1986), where the plots of $k_{\text{obs}}$ vs. [CN$^-$] displayed linear correlation coefficients between 0.90-1.00. For the sake of clarity, the plot of the native SWMb is not shown. Experimental conditions were identical to those for determining the $K_D$'s (Table 9), since both $K_D$ and $k_{+1(\text{app})}$ can be calculated from the same experiment. From the current titration curve, due to only one sharply defined pH-optima (pH 5.5), only an approximation of the $pK_a$ could be made, which appears to be between 5.5 and 6.0.
Figure 14: Optical absorption spectra of the reaction of cyanogen bromide modification of the distal histidine in native sperm whale myoglobin or MbH93Y. In Panel a, [SWMb] = 1.34 - 4.07 µM and in Panel b, [MbH93Y] = 1.44 - 3.22 µM. For either protein, [CNBr] = 615 - 645 µM and [H₂O₂] = 1 - 2 µM. Panel a: (a) Unmodified native SWMb; (b) 7 minutes, 35 seconds (7:35) after the addition of 615 µM CNBr; (c) Addition of 1.08 µM H₂O₂; (d) 3:30 after the addition of H₂O₂; (e) 7:00 after the addition of H₂O₂; (f) 15:00 after the addition of H₂O₂; (g) Addition of solid grains of L-(+)-ascorbic acid. The rate of distal His modification by CNBr was calculated by following the decrease in the absorbance at 409 nm. Calculation of the observed pseudo-first order rate constant, \( k_{obs} = 1.326 \times 10^{-2} \pm 4.01 \times 10^{-3} \text{ sec}^{-1} \), was linear (Vega-Catalan et al., 1986) in a plot of absorbance changes over time; correlation coefficient, R = 1.00. Panel b: (a) Unmodified MbH93Y; (b) 4:20 after the addition of 645 µM CNBr; (c) Addition of 1.08 µM H₂O₂ at 10:37 after the addition of CNBr; (d) 3:23 after the addition of H₂O₂; (e) Addition of solid grains of L-(+)-ascorbic acid 6:33 after the addition of H₂O₂. Obviously, no kinetics were calculated due to the lack of any appreciable Soret peak shift. Also note the absence of any discernable isosbestic point.
Moreover, the addition of H₂O₂ to the CNBr-modification reaction generates a sharp isosbestic point in the Soret peak in SWMb (Figure 14a), which strongly indicates that the metaquo SWMb is being converted from a hexacoordinate to a pentacoordinate species. On the other hand, the lack of Soret peak shift and the absence of an isosbestic point in the CNBr-modified MbH93Y reaction, following the addition of H₂O₂, clearly indicate MbH93Y was already pentacoordinate, in agreement with the XAS results.

**Acid↔Alkaline Transitions of Myoglobins.** Shown in Figure 15 are the acid↔alkaline transition curves of the native and MbH93Y proteins. Consistent with previously reported values for native SWMb, the pKₐ of the acid→alkaline transition and that of the alkaline→acid transition was found to be 8.9 (Figure 15a; Hartzell et al., 1968; Shire et al., 1974a, b; Morishima et al., 1978; Yamazaki et al., 1978) and 6.5 (Yamazaki et al., 1978), respectively. However, while the alkaline→acid transition for the mutant protein yielded a pKₐ of 6.5 (Figure 15e), the pKₐ of the acid→alkaline profile yielded a value of ca. 12.0 (Figure 15c). The stability constants for the formation of the hydroxyl complexes for native SWMb and MbH93Y were calculated to be 5.1 and 2.0, respectively. However, while native SWMb yielded the typical Hill Plot of n ~1.00 (Figure 15a), MbH93Y exhibited a Hill Plot that was biphasic in nature (Figure 15c,e), suggesting that some sort of general heme-linked protonation coupled with some degree of protein denaturation occurs.
Figure 15: Forward and reverse titration curves of native sperm whale myoglobin and MbH93Y. The forward and reverse titrations were executed with increasing concentrations of NaOH and HCl, respectively, of (a) 3-4 µM native SWMb starting at pH 6.54 and (b) at pH 8.03. In (a), the inset reveals a Hill Plot with a slope of n ~ 1.00. (c) 2-4 µM MbH93Y starting at pH 8.15, titrating through to pH 12.72, and then back to pH 7.1, all within the same reaction cuvette. (d) Back-titration of MbH93Y starting at pH 12.8. (e) Back-titration of MbH93Y starting at pH 12.73 and continuing through to pH 5.67; only the acidic side of the curve is shown since the basic side is identical to that shown in (d). Back-titration of MbH93Y starting at pH 9.38 and continuing through to pH 5.41 is also identical to the curve displayed in (e) (Data not shown). In (e), the inset reveals a biphasic Hill Plot. See text for explanation.
Forward Titration of Native SWMb

Back-Titration of Native SWMb

Forward and Reverse Titration of MbH93Y

Reverse Titration of MbH93Y
Figure 15 (Continued)

(e) Reverse Titration of MbH93Y

% Change Absorbance

pH

0 2 4 6 8 10 12 14 16 18

Polaroid for MbH93Y

log (C_0/C)

pH 9.5 10 10.5 11 11.5 12 12.5 13

Hill Plot for MbH93Y
Spectroscopy. We used optical absorption and XAS spectroscopies to probe the active sites of MbH93Y, WT SWMb, and HEC and compared the mutant protein to the two controls. In the optical absorption spectra, a blue-shift of the Soret peak from 409 nm (WT) to 403 nm (Figure 8, Table 1) for MbH93Y strongly suggests pentacoordination and reflects the fact that the phenolic oxygen fifth ligand possesses more negative charge than the imidazole WT fifth ligand. This fifth ligand phenolic oxygen exhibits a trans effect through the heme Fe, enabling it to repel a ligand (e.g., an oxygen atom of a water molecule) from binding to the sixth position on the distal side of the heme. Our XAS results, which are shown in Figure 16, also show a pentacoordinate heme in MbH93Y. Furthermore, the proximal bond length in WT SWMb did not change as a result of the mutation, which is significantly different from that of HEC. That is, simply replacing the fifth ligand with a Tyr does not alter the bond distances in the active site. However, the negative charge density of the phenolic oxygen atom results in an electrostatic repulsion of water through the Fe bond and pentacoordination.

Normally, at the low temperatures (~ -130 - -100°C) at which XAS data are recorded (Powers, 1982), met SWMb (Chance et al., 1986b) and catalase (Chance et al., 1984) are indeed liganded at the distal site. In the crystal structure of bovine liver catalase (23°C), however, the sixth-coordination site is vacant (Murthy et al., 1981), which is in agreement with resonance Raman spectroscopy (4°C) studies (Chuang et
Figure 16: EXAFS-derived bond distances of the active sites of wild-type sperm whale myoglobin, MbH93Y, horse erythrocyte catalase, and of their respective cyanide-ligated complexes. Distances are in angstroms and the errors are provided in the text.
al., 1988; Sharma et al., 1989). However, other peroxidases are known to become hexacoordinate at low temperatures such as lactoperoxidase (Manthey et al., 1986), cytochrome c peroxidase (Smulevich et al., 1991) and lignin peroxidase (Sinclair et al., 1992).

The hemoglobin mutant, Iwate (mutation of the proximal His → Tyr on the α chains of hemoglobin), is also pentacoordinate (Nagai et al., 1983) and binds O₂ poorly (Greer, 1971), as does MbH93Y. Similarly, in my hands, the oxy-form of MbH93Y was impossible to achieve (Table 1), indicating that simple ligand binding at the distal site is influenced by the identity of the proximal axial ligand (Goldsby et al., 1986).

The average Fe-to-pyrrole nitrogen (Fe-Nₚ) bond lengths of MbH93Y-CN were significantly different (outside the error) from that of either met SWMb-CN or HEC-CN. However, unlike the met forms, where the MbH93Y proximal bond is longer than that in HEC, the proximal bond length of the MbH93Y-CN is shorter than that of HEC-CN.

Comparison between each protein and its respective cyanide-ligated form revealed that WT SWMb and its cyanide-ligated form were significantly different in their Fe-Nₚ and distal bond lengths (although this difference is close to the total error), MbH93Y and its cyanide-ligated form were significantly different in their Fe-Nₚ and proximal bond lengths, and HEC and HEC-CN were significantly different in both axial bond lengths. When CN⁻ binds the Fe³⁺ in MbH93Y, there is considerable π-electron withdrawal through the Fe to the CN⁻ ligand. This results in electron withdrawal from the π-orbitals of the heme system, pulling the phenolate ligand inward, shortening the
proximal bond, as well as the Fe-N$_p$ bonds, pulling the Fe into the plane of the heme
and culminating in a low-spin complex. Alternatively, the distal His, as a result of the
proximal mutation, may not be as free to swing as in the WT protein. If the distal His
is able to form an H-bond with the nitrogen of the bound CN$^-$, some of the electron
density of the antibonding $\pi^*$ orbitals of the CN$^-$ will be drawn away from the Fe
bond. This is consistent with the determination of the greatly increased pK$_a$ of $\sim$12.0
(Figure 15c).

Oxidation of Myoglobins by $H_2O_2$. In order to test the functional consequences of
this single His-to-Tyr mutation, it was necessary to determine if the MbH93Y can form
a Compound I-type intermediate, functionally different from myoglobin peroxide
(George and Irvine, 1952, 1953, 1956), but similar to that formed by catalases and
peroxidases. As established earlier by resonance Raman spectroscopy (Morikis et al.,
1990) and our studies at low and room temperatures (Figure 16), the most striking
difference between the MbH93Y and WT SWMb or HEC is the absence of a distal
ligand in the mutant protein (see Frauenhoff and Scott, 1992). The proximal Tyr
lowers the Fe$^{2+}$-Fe$^{3+}$ redox potential by $\sim$135 mV in human myoglobin (Adachi et al.,
1991) compared to WT human myoglobin. In terms of active site structure, probably
the most important property responsible for the high degree of activity found in HEC
is the complex H-bonding network on the proximal side (Fita and Rossmann, 1985).
This highly polar environment, in addition to the short Fe-O bond length of 1.90 Å
(Figure 16; Murthy et al., 1981; Chance et al., 1984) and the localized negative charge
on the deprotonated phenolic oxygen (Fita and Rossmann, 1985), probably contributes
to the extremely low redox potential of this enzyme \( \text{E}^{0'} < -500 \text{ mV} \) (Adachi et al., 1991)]. In MbH93Y, whose proximal bond length is unchanged as a result of the mutation and still is significantly longer than that in HEC (Figure 16), the through-Fe bond trans effect towards the distal side is much weaker compared to that in HEC, but stronger than that in WT SWMb. In addition, the active site pocket of myoglobin is much less polar (Poulos, 1988) than that of HEC, so MbH93Y is less capable of forming an appreciable H-bonding network on the proximal side like that in catalase (Fita and Rossmann, 1985). Obviously, since WT SWMb does not have this through-Fe trans electrostatic repulsion, this protein is much better able to accommodate a ligand bound at the distal site. Because one edge of the heme in WT SWMb and in MbH93Y is exposed to the solvent (Takano, 1977), the prosthetic group in these proteins is more susceptible to oxidation by \( \text{H}_2\text{O}_2 \). However, in WT SWMb, whose distal site is occupied by \( \text{H}_2\text{O} \), the heme is much more sterically protected (e.g., Guillard et al., 1991) from \( \text{H}_2\text{O}_2 \) (Tetreau et al., 1990; McArthur and Davies, 1993) than is the mutant protein. Prolonged exposure of WT SWMb to \( \text{H}_2\text{O}_2 \) (over an 8-minute period) resulted in a Compound II-like spectrum (Figure 10a). At shorter times, ascorbate was able to reduce the Fe and restore the Soret peak (Figure 10c). However, the vacancy of the distal site in the MbH93Y left the heme open to oxidative attack by \( \text{H}_2\text{O}_2 \). The maximum absorbance of the Soret peak plummeted within less than 1 minute and was not retrievable by reduction with ascorbate (Figure 10d). Dithionite red-shifted the Soret peak 20 nm (Figure 10e), 7 nm shorter than normally
produced with this reductant (cf. Figure 8, Table 1) and was unable to restore the Soret peak.

Furthermore, because the substitution of a Tyr for a His in the heme pocket is not isosteric (Creighton, 1984), it is possible that in addition to the trans electronic repulsive effects of the phenolate oxygen, there is also a heme pocket rearrangement, both of which may contribute to favoring fifth-coordination in MbH93Y. Indeed, this substitution may disrupt the H-bonding network among the hydroxyl of Ser92, NϵH of His93, one of the two propionate groups, His97 and Leu89 on the proximal side (Biram et al., 1993; Smerdon et al., 1993). Although it is possible that the phenolic oxygen atom can form a H-bonding network on the proximal side as it does in catalase (Fita and Rossmann, 1985), it is unlikely that Tyr93 would have the same H-bonding constraints of the WT His93 residue. The proximal ligand, Tyr93, may now be more free to move about, compared to WT His93, possibly culminating in a more exposed heme (Smerdon et al., 1993) and greatly enhancing the susceptibility of the heme to oxidation by H₂O₂.

When a heme protein (e.g., catalase, cytochrome c peroxidase) is exposed to H₂O₂, O₂ is evolved. Indeed, many investigators routinely use O₂ evolution as a measure of dismutation activity (e.g., Adachi et al., 1993). However, the globins differ from the peroxidases and catalases inasmuch as the number of reaction cycles with H₂O₂ completed by the globins is severely limited (ca. 8, King and Winfield, 1963; Tajima and Shikama, 1993). Also, these proteins are altered in the reaction as well (King and Winfield, 1963). When WT SWMb was exposed to H₂O₂, there was not only a
gradual drop in the maximum absorbance of the Soret peak, but also a slight red-shift yielding a Compound II-like spectrum (Figure 10a; King and Winfield, 1963), which is indicative of a ferryl intermediate, similar to that of catalase (Nicholls and Schonbaum, 1963). After 10-15 minutes, haemochromagen assay revealed broken hemes, indicating complete oxidation by $\text{H}_2\text{O}_2$ (Figure 10b; Keilin and Hartree, 1950). However, if reductant was added early in the reaction, the Soret peak was restored (Figure 10c).

On the other hand, when MbH93Y was exposed to $\text{H}_2\text{O}_2$, there was a rapid, drastic, and irreversible loss of the Soret peak (Figure 10d; also Adachi et al., 1993), which may be due to a sterically accessible heme via the vacancy at the distal site (Figure 16) and/or disruption of the H-bonding network on the proximal side. In WT SWMb, it has been suggested that the ferryl state is governed by the accessibility of $\text{H}_2\text{O}$ in the pocket, bound at the distal site (Whitburn, 1987). In addition, unlike in WT SWMb, it is possible that the strong electron-donating phenolate ligand in MbH93Y does not allow production of a porphyrin $\pi$ cation radical/Fe$^{IV}=\text{O}$ complex (Dounce and Sichak, 1988). The absence of this cation radical could result in a destabilization of the the Fe$^{IV}=\text{O}$ intermediate (Yamazaki and Nakajima, 1988), possibly resulting in a net negative charge in the immediate vicinity of the Fe, increasing the overall negative charge density in the heme, and ultimately disrupting the $\pi$-conjugated system.

**Binding by Cyanide.** Like BLC (Fita and Rossmann, 1985) and the hemoglobin mutants, Hyde Park (mutation of the proximal His $\rightarrow$ Tyr in the $\beta$ chains of
hemoglobin; Greer, 1971) and Iwate (mutation of the proximal His → Tyr in the α chains of hemoglobin; Nagai et al., 1983, 1989), MbH93Y is also high-spin. As in the reactions with H₂O₂ discussed above, the strong electron-donating effects of the phenolate ligand (a weak field ligand) can prevent CN⁻ from binding at the distal site in MbH93Y and are manifested by an affinity constant, which average three orders of magnitude higher than those for WT SWMb (Table 9). This result is further reflected by the inability of the mutant to achieve 100% saturation or formation of the metcyano complex. When CN⁻ approaches, the Fe in MbH93Y undergoes σ-electron donation from the proximal side and π-electron withdrawal from the distal side, leading to a repulsion of the incoming CN⁻ ligand and a resulting lower affinity, compared to the native or WT SWMb. Native (and WT) SWMb also has the ability to repel CN⁻ by the same mechanism; however, imidazole(ate) is a stronger field or electron-withdrawing ligand than phenolate, but is weaker than CN⁻. The π-acid ligand, therefore, is able to complement the field strength of the imidazole, both ultimately contributing to the low-spin state of the heme. Our XAS shows that upon CN⁻ binding to the mutant, both the proximal ligand bond and the Fe-N_p bond distances shorten (Figure 16), which are results consistent with the π-withdrawal from the distal site.

Furthermore, in native SWMb, Ver Ploeg et al. (1971) proposed that CN⁻ attacks the distal site via a dissociation-association mechanism, with H₂O leaving before CN⁻ binds. Both native and WT SWMb, perhaps due to the occupied distal site, take up to 10 minutes to reach equilibrium upon binding CN⁻. In MbH93Y, however, there is no leaving group to dissociate before CN⁻ binds.
Biphasic kinetics of CN· binding to MbH93Y is exhibited at all pH values examined. There is a rapid phase, which is completed within 10-15 seconds. A slow phase follows, probably due to some conformational change within the heme pocket, which reaches equilibrium within 5 minutes and is reminiscent of the slow phase of a conversion of reduced, low-spin cyano-lactoperoxidase to a high-spin cyano complex (Manthey et al., 1986). The rapid phase is still many orders of magnitude slower than the pseudo first-order kinetics displayed by the WT protein (Table 10; Ikeda-Saito et al., 1992) and is consistent with the observation of approximately three orders of magnitude more CN· required to reach equilibrium with the mutant protein than with WT.

**pH Effects.** The pH dependence of the kinetics of CN· binding and dissociation has been the focus of a number of studies utilizing site-directed mutations in the distal pocket (Bellelli et al., 1990a; Ikeda-Saito et al., 1992). However, in our study of the proximal mutation, we observed a simple bimolecular reaction where Mb+ + CN· \( \rightleftharpoons \) MbCN and \( K_D = \frac{k_{-1}}{k_{+1}} \) (Vega-Catalan et al., 1986), where the forward rate constant, \( k_{+1(app)} \), and \( K_D \) both differ significantly between the native (or WT) SWMb and MbH93Y. As discussed in Chapter III, we were unable to accurately calculate the rate of the dissociation, the \( k_{-1(app)} \). However, in the presence of dithionite, Bellelli et al. (1990a) detected an unstable, transient intermediate, whose rate of decomposition to the unligated SWMb was inversely proportional to pH. These investigators tested two different point mutations of the distal His, substituted by a Gly or a Val residue, which exhibited large decreases in the rate of the decomposition reaction at acidic pH. Thus,
our study of the rate of the forward binding reaction and that of Bellelli et al. (1990a) of the rate of the dissociation reaction both show a pH dependence at acidic pH.

In my study, the failure to observe any significant dependence on pH, of $K_D$ or the $k_{+1(app)}$, with the mutant protein except between the extreme pH values examined may have been due to the buffer effects of 0.1 M ionic strength MES/MOPS. It has long been known that the type and strength of the buffer influences the behavior of native SWMb (Ver Ploeg and Alberty, 1968; Ver Ploeg et al., 1971). Although both MES and MOPS are Good's buffers, an appropriate study to undertake in the future would be to characterize MbH93Y under a wide variety of buffers, ionic strengths and pH values, perhaps in smaller intervals than at every half-unit as was performed in this study (Ver Ploeg and Alberty, 1968; Ver Ploeg et al., 1971). Nevertheless, the observed differences in $k_{+1(app)}$ at pH 5.5 and 8.0 lend support to our earlier results of enhanced negative charge density trans to the phenolate oxygen. At pH 8.0, CN$^-$ anion has over 300 times more OH$^-$ ions with which to compete than at pH 5.5. And since OH$^-$ is a much weaker field ligand than CN$^-$, the observation of a higher rate of association and lower dissociation constant at the lower pH is not at all surprising. At pH 5.5, the [H$^+$] is over 300 times higher than at pH 8.0, thereby protonating the distal His, which swings out of the way (Ramsden and Spiro, 1989) and facilitates CN$^-$ binding.

**Cyanogen Bromide Modification of Myoglobins.** Steric modification of the distal His by CNBr has been demonstrated to be an effective means to probe the dynamics of ligand binding in the distal pocket of heme proteins (Jajczay, 1970;
Morishima et al., 1989; Bracete et al., 1991). Specifically, the imidazolyl N-H of His64 in SWMb is substituted by a N-CN moiety (Morishima et al., 1989 and references cited therein), thereby disrupting any potential for the distal His to form a H-bond with a ligand (Phillips and Schoenborn, 1981). Although the heme has been converted from six-coordinate to five-coordinate, indicated by the prominent blue-shift of the Soret and the isosbestic point upon exposure to H₂O₂ (Figure 14a), the heme is now more protected from the damaging, oxidative effects of H₂O₂ than in unmodified hexacoordinate SWMb. This oxidative reaction by H₂O₂, manifested by a rapid, irreversible loss of the Soret peak seen in Figure 10a, is much slower in the CNBr-modified SWMb. This could occur by one or both of the following mechanisms: (i) the binding of CNBr to the distal His prevents this residue from swinging down in its usual gate-like fashion, which provides large ligand entry into the pocket (Johnson et al., 1989); or (ii) modification of the histidyl imidazolyl NH to N-CN could sterically constrain this residue, thus preventing H-bonding to incoming ligands, including H₂O (and H₂O₂), at the distal side (Phillips and Schoenborn, 1981; Morishima et al., 1989). In other words, CNBr binding to His64 could prevent this residue from swinging out of the way as quickly as it would in unmodified SWMb, thereby delaying, but not completely preventing, H₂O₂ from damaging the heme.

Cyanogen bromide modification of His64 in MbH93Y resulted in the same decrease in the rate of heme degradation by H₂O₂ (Figure 14b), relative to unmodified MbH93Y (Figure 10e). Equally important, this finding corroborates the optical absorption and XAS results of a vacancy at the distal site in MbH93Y (Figure 16) and
lends credence to the phenomenon of the strong, through-Fe, electron-donating effects of the phenolate oxygen. That is, the strong, trans-labilizing effects of the phenolate oxygen will serve to help neutralize the positive charge on the Fe, thereby decreasing the residence time (Schonbaum, 1982) and affinity of a ligand, e.g., H₂O, CN⁻, at the distal site. Cyanogen bromide substitution of a N-CN on the distal imidazolyl can only help maintain or reinforce the five-coordinate structure of this protein via one or both of the steric mechanisms discussed above for the native protein and is manifested by the absence of any significant shift of the Soret band (Figure 14a).

Acid↔Alkaline Transitions of Myoglobins. Like the peroxidation and the CN⁻-binding profiles, the acid↔alkaline transition for MbH93Y is also drastically different from that of the native SWMb. The ionization constant of 8.9 for native SWMb has been cited as the midpoint potential of the protolytic exchange of a H⁺ between an Fe-bound OH⁻ ligand and the distal imidazole in metMb (Morishima et al., 1978; Yamazaki et al., 1978).

However, several surprises were revealed in the characterization of the acid↔alkaline transition of the MbH93Y. In light of the three orders of magnitude difference in the basic pKₐ between the native protein and MbH93Y, it is surprising that there was no difference in the acidic pKₐ for H⁺ dissociation between the native SWMb and mutant protein. The shape of the forward titration (acid→alkaline) curve for MbH93Y indicated a pH-dependence much more complex than that for the native protein. Unlike the reverse titration for native SWMb, the alkaline→acid titration for
MbH93Y was not reversible and the Hill Equation yielded a curve that was biphasic over the pH range 9-12.

Importantly, the drastically increased pKₐ of MbH93Y (~12.0) relative to native SWMb (8.9) in the relatively nonpolar active site is expected, due to the through-Fe negative charge density imposed by the phenolate oxygen atom. Specifically, the charge neutralization of the Fe³⁺ inhibits deprotonation and gives rise to the observed increased pKₐ of the distal His. The active sites of catalase and SWMb are drastically different in polarity. However, they share the common feature of the fifth ligand in MbH93Y. Due to the polarity of the respective active sites, the pKₐ of the distal His in catalase, which is oriented roughly parallel (Fita and Rossmann, 1985) instead of perpendicular to the heme as in SWMb, is expected to decrease (Fita and Rossmann, 1985) relative to free His in solution.

In addition, the increased pKₐ of 12.0 for MbH93Y relative to the native SWMb means that more than three orders of magnitude in the concentration of OH⁻ ions are required to titrate MbH93Y and would be consistent with a population of protonated distal imidazoles 3-fold higher than those which are not protonated. Such protonation of the distal His represents removal of a major steric barrier to ligand binding (Ramsden and Spiro, 1989).

The fact that the pKₐ's of both proteins are virtually identical on the acid side (Figure 15b, e), but so different on the alkaline side (Figure 15a, c), strongly indicates that in MbH93Y, unlike the native protein, simple association/dissociation reactions are not taking place between H⁺ and OH⁻ ions. More likely, MbH93Y is undergoing
some sort of electrostatically imposed local or global conformational change—a change perhaps similar to the binding of larger ligands by native SWMb (Johnson et al., 1989). Such a change is supported by the biphasic nature of the Hill plot between pH 9-12. Overall, the Hill constant was $n = 1.50$, but inspection of the inset of Figure 15e clearly indicates that such a calculation is erroneous as are any conclusions about cooperative effects. Rather, the biphasic nature of the Hill Plot of MbH93Y (Figure 15e, inset), as well as the irreversibility of the forward and reverse titrations (Figure 15c), probably represents some combination of general heme-linked protonation and/or proton dissociation and protein denaturation. Again, just as in the case of CN$^-$ binding, an appropriate study to undertake next would be to examine the behavior of MbH93Y in the presence of various buffers and differing ionic strengths similar to those performed on native SWMb (Ver Ploeg and Alberty, 1968; Ver Ploeg et al., 1971; Shire et al., 1974a, b; Janssen et al., 1972).

Inspection of Figure 15e of the forward and reverse titrations of MbH93Y executed in the same sample reveals that the mutant is probably not as stable as the native form. That is, the failure of the reverse titration curve towards the acidic side to follow the same route as that of the forward titration towards the basic side indicates that some denaturation of the protein may have occurred. Such denaturation would be accurately represented by the difference between the two curves. Therefore, it appears that unlike native SWMb, which (i) titrates reversibly between pH 5-10 (Antonini and Brunori, 1971), (ii) is stable even at pH 12.9 (Uyeda and Peisach, 1981), (iii) has its Fe-His bond able to withstand an environment of pH 4.0, and (iv) maintains its
integrity even at pH 2.6 in the CO-ligated form (Sage et al., 1991a, b; 1992), the conformational changes MbH93Y undergoes could ultimately culminate in partial or complete unfolding, eventually leading to denaturation. This indicates that the mutant is not as stable as its WT counterpart. Such pH-associated conformational changes have been extensively studied (Shimada and Caughey, 1982; Yang and Honig, 1993) and in the case of myoglobins, do not necessarily all involve deprotonation of a distal H$_2$O molecule (Morishima et al., 1978; Yamazaki et al., 1978), but perhaps may involve some residue near the Fe (Giacometti et al., 1975).

Recently, Biram et al. (1993) mutated the Ser92 to a Leu residue on the proximal side in pig metMb and found an increased affinity for both N$_3^-$ (Biram et al., 1993; Smerdon et al., 1993) and OH$^-$ relative to the WT protein. Importantly, this mutation of the Ser to a Leu disrupts the H-bonding network on the proximal side, allowing His93 greater freedom to rotate laterally as well as to move towards the Fe (Biram et al., 1993). Thus, by removing some of the steric constraints on the proximal side, Biram et al. (1993) were able to decrease the pK$_a$ of the distal His in the S92L mutant relative to WT. In striking contrast, in our MbH93Y mutation, the huge increase in pK$_a$, reflected by a 2.5-fold lower formation constant relative to native SWMb represents an electronic influence that (i) largely overshadows most steric considerations since the side chain of Tyr is only a little larger than that of His (Creighton, 1984) and (ii) is at the limiting pK$_a$ for a weak acid ligand as predicted by Schonbaum (1982).
Summary. All of the results in this study can be explained in terms of the EXAFS-derived bond lengths, the steric and electronic implications for the mutation of a His to a Tyr residue, and the polarity or ionic environment of the surrounding active site.

Compared with the respective bond distances in WT SWMb, both Fe-N_p and the Fe-proximal ligand bond distances remained virtually unchanged. Compared to HEC, the Fe-N_p distance is also about the same in MbH93Y, but the Fe-proximal ligand bond length is significantly shorter in HEC. Strikingly, however, unlike WT SWMb and HEC, the distal site in MbH93Y is vacant. Compared to WT SWMb, the stronger through-Fe electrostatic repulsion of the phenolate oxygen atom in MbH93Y is most likely responsible for the vacancy at the distal site. However, MbH93Y, while possessing the same axial ligand as that in catalase, does not have the same complex H-bonding network present in HEC, which is considered to be responsible for the high catalytic/catalatic activity of this enzyme. In addition, compared to HEC, whose proximal bond length is significantly shorter than in either the WT or the mutant myoglobins, the longer Fe-N_e bond in the globins does not confer any true catalytic activity onto these proteins. The shorter proximal bond distance in HEC, compared to that of the globins, may also play a prominent role in the activity of this enzyme.

Among the cyanide-ligated proteins, the proximal and the Fe-N_p bonds of MbH93Y-CN are shorter than those of WT SWMb-CN. This is probably due to weaker σ-donor interaction and π back-bonding of the phenolate in MbH93Y-CN and consequently less electronic overlap along the ππ orbital directions and shortening the
Fe-N_p bonds relative to the proximal imidazole in the WT SWMb-CN. In HEC-CN and in WT SWMb-CN, despite the different proximal ligands and active site environments, the distal, Fe-N_p, and proximal ligand distances were no different between the two cyanide-ligated proteins. The effect CN^- has on each protein is to generate the low-spin state of the heme via electronic repulsion between the two axial ligands through the Fe, resulting in shorter Fe-N_p bond lengths compared to their respective met forms.

Unlike WT SWMb, MbH93Y was unable to form myoglobin peroxide. In fact, the heme in MbH93Y undergoes irreversible and much more rapid oxidation by 2-molar equivalents of H_2O_2. These results may be explained in terms of an active site, which is much more highly exposed by the vacancy at the distal site and disruption of the H-bonding network on the proximal side.

When HCN approaches the Fe, the metal in MbH93Y undergoes \sigma-donation from both the proximal and distal sides, as well as \pi back-bonding from the Fe in both directions, but more strongly towards the phenolate ligand. This ultimately leads to an electrostatic repulsion of the incoming CN^- ligand and results in lower rate constants and binding constants within the pH range between 5.5 and 8.0, compared to that of either native or WT SWMb. Again, the highly polar active site of HEC and its short Fe-proximal ligand bond distance enables this protein to delocalize the negative charge of the phenolate ligand, which allows CN^- to bind tightly.

Steric modification of the distal His enabled us to probe the dynamics of the binding pocket, using the UV/visible absorption spectra of the reaction to
biochemically confirm the optical absorption and EXAFS results of a pentacoordinate heme in MbH93Y.

Both the native and MbH93Y displayed the same acidic pKₐ's of 6.5, while their basic pKₐ's differed by more than three orders of magnitude. The pKₐ of 12.0 for the mutant is again primarily attributed to the strong, through-Fe, negative charge density of the phenolate oxygen atom. In addition, unlike native SWMb, the irreversibility of the forward and reverse titration curves in MbH93Y, as well as the peculiar shape of the curves, may indicate a local or more global conformational change in the globin. This apparent greater susceptibility to changes in the immediate ionic environment would be consistent with a more exposed heme, revealed by the spectroscopic studies, and the possible disruption of the H-bond network on the proximal side.

Conclusions. The mutation of the proximal His to a Tyr residue in SWMb is the logical first step for rational redesign of a heme protein. Following molecular dynamics simulations and energy minimization calculations, the next logical step(s) to undertake would be to mutate the heme pocket of SWMb in order to introduce a H-bonding network in conjunction with the existing proximal mutation; such a H-bonding network would be designed by mutating certain key residues in SWMb that correspond to the homologous site or position that exists in catalase. The structural and biochemical consequences of these mutations could then be evaluated. The ability to form Compound I would be the prerequisite to the long-range goal of conferring onto SWMb the type, and if possible, the degree of enzymatic activity present in catalase
and CcP. To this end, the type of H-bonding seen in the active site of catalase could ideally be duplicated in that of SWMb.

The current use of heme proteins in biosensors and clinical and biochemical assays and their potential use in biomolecular electronics and the degradation of toxic wastes are only a few examples of their broad utility. Heme proteins are ideal candidates for use as biomolecular electronic materials, because of their unique electron transfer properties. Several investigators have been exploiting this potential in this respect using site-directed mutagenesis for general investigations of electron transfer and to improve monolayer formation in the production of a "protein chip" (Stayton et al., 1989; Walker, 1991). For example, in biosensor applications, horseradish peroxidase has long been used as part of the assay for cholesterol; heme proteins have potential as integral parts of many biosensors (Ball et al., 1986; Hill, 1987). Two of the key problems in biosensor technology have been specificity of the interaction of the probe with the molecule of interest and the stability of the probe. My studies presented in this work and the next logical steps of heme protein rational redesign, such as the introduction of a H-bonding network, will ultimately increase our understanding of heme protein catalysis and facilitate these needed improvements.
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