
**Synthesis, Physical
Characterisation and Solution
Chemistry of
N-Acetyl-Cobalt(III)-
Microperoxidase 8**

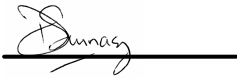
-Desigan Sannasy-

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

March 2006

Declaration

I declare that this is my own unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

A handwritten signature in black ink, appearing to read 'D. Sannasy', is written over a solid black horizontal line.

D. Sannasy

March 2006

Abstract

This dissertation describes the synthesis, physical characterisation and solution chemistry of $\text{NAC-Co}^{\text{III}}\text{MP8}$, a biomimetic model compound of vitamin $\text{B}_{12\text{a}}$, synthesised from the haemotapeptide derived from horse heart cytochrome *c*. Peptic and tryptic digestion of horse heart cytochrome *c* removes much of the globular protein encapsulating the iron porphyrin prosthetic group. The resulting haemotapeptide fragment retains residues 14 to 21 of the parent cytochrome (MP8) via thioether linkages to Cys-14 and Cys-17. Reductive demetalation of MP8 yielded the metal free MP8. This was treated with cobaltous acetate in an aerated aqueous solution to produce $\text{Co}^{\text{III}}\text{MP8}$. $\text{Co}^{\text{III}}\text{MP8}$ was acetylated by treatment with acetic anhydride and yielded *N*-acetyl-Co(III)-microperoxidase 8 ($\text{NAC-Co}^{\text{III}}\text{MP8}$). It is well established that acetylation reduces aggregation of these haempeptides. The starting materials and products of each step during synthesis were characterised by UV-visible absorption spectroscopy, high performance liquid chromatography (HPLC) and fast atom bombardment-mass spectroscopy (FAB-MS). MP8 free base and Co(III)-MP8 were also analysed using luminescence spectroscopy.

The molar extinction coefficients of $\text{NACCo}^{\text{III}}\text{-MP8}$ in aqueous and ionic medium were determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) and UV-visible absorption spectroscopy. The extinction coefficient, ϵ , of $\text{NACCo}^{\text{III}}\text{MP8}$ ($\lambda = 420 \text{ nm}$, pH 7.00, 25 °C) in distilled water and 1.0 M NaClO_4 was $1.80 \pm 0.01 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and $1.66 \pm 0.01 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. Beer's law studies show that $\text{NAC-Co}^{\text{III}}\text{MP8}$ remains monomeric in aqueous solution up to concentrations of at least 35 μM .

The spectroscopic changes observed for $\text{NAC-Co}^{\text{III}}\text{MP8}$ during the course of a spectrophotometric titration are very similar to those observed for $\text{NAC-Fe}^{\text{III}}\text{MP8}$, with both being consistent with six successive ionisations. By analogy with $\text{NAC-Fe}^{\text{III}}\text{MP8}$, we attributed the first ($\text{p}K_1 = 2.0 \pm 0.3$) to the coordination of the *c*-terminal carboxylate group (Glu-21) of the appended polypeptide. The second acid range transition ($\text{p}K_2 = 2.8$

± 0.1) for $\text{NACo}^{\text{III}}\text{MP8}$ involved the deprotonation of the cationic His-18 and concomitant replacement of the *c*-terminal carboxylate by the neutral heterocyclic base. The third and fourth pK_a 's are attributed to the ionisation of the haem propanoic acid groups ($\text{pK}_3 = 3.9 \pm 0.03$) and ($\text{pK}_4 = 7.5 \pm 0.03$). Ionisation of the cobalt-bound water molecule above neutral pH was assigned to $\text{pK}_5 = 9.2 \pm 0.04$. Finally, we attributed pK_6 (12.1 ± 0.03) to the ionisation of the coordinated histidine *trans* to the OH⁻ to form the histidinate complex ($\text{His}^- \text{-Co}^{\text{III}} \text{-OH}^-$).

A principal aim of this work was to demonstrate that the kinetics and the thermodynamics of the ligand substitution reactions of $\text{NAC-Co}^{\text{III}}\text{MP8}$ can be studied spectrophotometrically; a comprehensive investigation of these reactions will be undertaken by others. Towards this end the formation constants between $\text{NAC-Co}^{\text{III}}\text{MP8}$ and *N*-methylimidazole and pyridine were determined. We observed the formation of a bis-substituted complex in the reaction of $\text{NAC-Co}^{\text{III}}\text{MP8}$ with the ligands, but only mono-substitution with $\text{NAC-Fe}^{\text{III}}\text{MP8}$ and B_{12a} . We attribute this first ligand binding to the replacement of the axial water molecule, and the second replacement of the axial histidine residue. The absence of the second reaction with $\text{NAC-Fe}^{\text{III}}\text{MP8}$ and B_{12a} suggest that the $\text{Co}^{\text{III}}\text{-N(His)}$ bond in $\text{NAC-Co}^{\text{III}}\text{MP8}$ is significantly weaker than the $\text{Fe}^{\text{III}}\text{-N(His)}$ and $\text{Co}^{\text{III}}\text{-N(dimethylbenzimidazole)}$ bond, respectively. When comparing the formation constants of $\text{NAC-Fe}^{\text{III}}\text{MP8}$, $\text{NAC-Co}^{\text{III}}\text{MP8}$ and B_{12a} , we found that the value of $\log K_1$ for $\text{NAC-Co}^{\text{III}}\text{MP8}$ for these ligands is significantly higher than that reported for $\text{NAC-Fe}^{\text{III}}\text{MP8}$ and B_{12a} .

Kinetics studies of $\text{NAC-Co}^{\text{III}}\text{MP8}$ with *N*-methylimidazole and methylamine were investigated. The data obtained did not follow conventional pseudo-first order kinetics; instead there was some evidence for biphasic kinetics. In the reaction of *N*-methylimidazole with $\text{NAC-Co}^{\text{III}}\text{MP8}$, we observed that the rate of reaction is virtually independent of the concentration of the incoming ligand. The results can be explained if the mechanism proceeds through a purely dissociative mechanism, i.e., if the rate of the reaction is controlled by the rate at which, firstly, the water molecule dissociates from the Co^{III} centre and, secondly, the histidine dissociates from the metal. The second order rate

constant, k_2 , could not be determined since the rate of reaction is independent of *N*-methylimidazole concentration. In the reaction of methylamine with *N*Ac-Co^{III}MP8, we observed that the rate of reaction is dependent on the concentration and participation of the incoming ligand. We propose that the displacement of water and histidine by methylamine involves an interchange mechanism (I_d), where the bond forming and bond breaking occur simultaneously, and thus the rate of reaction becomes dependent on the concentration of the incoming ligand.

The results showed that the rate of reaction for methylamine with *N*Ac-Co^{III}MP8 was faster than with *N*-methylimidazole. We attributed these differences in rate constants to the size of the incoming ligands. *N*-methylimidazole is a secondary amine and is relatively more bulky than methylamine which is a primary amine; therefore it is easier for methylamine to attach to the metal centre compared to *N*-methylimidazole.

For comparison, the rate of reaction of B_{12a} with *N*-methylimidazole and methylamine was determined. The results show that the rate of the reaction between *N*Ac-Co^{III}MP8 and B_{12a} with *N*-methylimidazole and methylamine are significantly different. Furthermore, we observe only mono-substitution in B_{2a} and bisubstitution in *N*Ac-Co^{III}MP8. Overall, the results presented in this work do give a general indication on how thermodynamically stable a Co^{III} ion is in a porphyrin ring and also to a very limited extent show that a porphyrin does not confer the same kinetic lability on the Co^{III} ion as the corrin ring.

This work is dedicated to God and my loving Family:

My Parents: Jason and Rani Sannasy

My Brother: Gregory

My Grandparents: Ma and Nine Daddy, Umma and Shinah

My Uncle: Vis

My Fiancé: Ying

Thanks to all of you for the love and support throughout the years.

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Abbreviations

A	Associative mechanism
AR	Analytical reagent
BDE	Bond dissociation energy
BP	Biologically pure
CAPS	3-[Cylohexylamino]-1-propanesulfonic acid
CD	Circular dichroism
CHES	3-[<i>N</i> -Cylohexylamino]-ethansulfonic acid
CMD	Classical molecular dynamics
CN (Im)Cbl	Cyanoimidazolyl-cobamide
Co	Cobalt
Cor	Corrin
CP	Chemically pure
CR	Commercial reagent
Cyt <i>c</i>	Cytochrome <i>c</i>
D	Dissociative mechanism
DAC	Diaquacobinamide
ESR	Electron spin resonance
ETC	Electron transport chain
FAB-MS	Fast atom bombardment-mass spectroscopy
GR	General reagent
Hb	Haemoglobin
HOMO	Highest occupied molecular orbital
HPLC	High performance liquid chromatography
HS	High-spin
I	Interchange mechanism
I _a	Associative interchange mechanism
ICP-OES	Inductively coupled plasma–optical emission spectroscopy
I _d	Dissociative interchange mechanism
Im	Imidazole

LMCT	Ligand to metal charge transfer
LS	Low-spin
LUMO	Lowest unoccupied molecular orbital
Mb	Myoglobin
MD	Molecular dynamic
Me	Methyl
MRI	Magnetic resonance imaging
MM	Molecular mechanical
MES	4-morpholineethanesulfonic acid monohydrate
MOPS	3-[N-Mopholino]-propanessulfonic acid
MP	Microperoxidase
MP11	Fe(III)-microperoxidase-11
MP8	Fe(III)-microperoxidase-8
<i>N</i> -Ac-Co ^{III} MP8	<i>N</i> -Ac-Co(III)-microperoxidase-8
<i>N</i> -AcMP8	Acetylated MP8
NAD(P)H	Nicotine adenine dinucleic coenzyme
NBA	Nitro benzoic acid
NMR	Nuclear magnetic resonance
PDB	Protein data base
Por	Porphyrin
QM	Quantum mechanical
RDS	Rate determining step
Tris	Tris-(hydroxymethyl)-aminomethane
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
$?H^\ddagger$	Enthalpy of activation
$?S^\ddagger$	Entropy of activation

Chapter 1 Introduction

1.1 Bioinorganic Chemistry

1.1.1 General Overview

Although bioinorganic chemistry is a relatively new field, reports on metals bound to proteins and enzymes date way back into the nineteenth century and may be found in earlier centuries if we replace the terms “proteins” and “enzymes” with “animal or plant tissues”.¹ Scientists by nature self-sort into groups and disciplines. The artificial division of the study of chemical compounds into organic and inorganic chemistry has long been established. Bioinorganic chemistry seems to contradict this artificial division. In the early nineteenth century chemistry was still divided into “organic” chemistry, which included only substances isolated from living organisms, and “inorganic” chemistry of “dead matter”. This distinction became meaningless after Wöhlers’ synthesis of “organic” urea from inorganic ammonium cyanate in 1828. Nowadays, organic chemistry is defined as the chemistry of carbon compounds, especially of hydrocarbons and their derivatives, with possible inclusion of certain heteroelements such as N, O or S regardless of the origin of the material.²

Bioinorganic or biological inorganic chemistry is the discipline dealing with the interaction between inorganic substances and molecules of biological interest. It spans many facets of other fields, since it involves the role, uptake, and fate of elements essential for life, response of living organisms to toxic inorganic substances, the function of metal-based drugs, the synthetic production of functional models, the production of pharmaceutical contrast agents in medical applications, the development of theoretical models, and so on.³ Bioinorganic chemistry is a relatively new branch of chemistry and only became an independent field after 1960.⁴ It is, however, a rapidly expanding interdisciplinary field that has developed through contributions from chemical physics (characterisation techniques), molecular biology (supply materials), pharmacology (drug-inorganic substance interactions), medicine (diagnostic aids and tumour therapy), environmental and human toxicology (toxicity of inorganic compounds), crystallography and spectroscopy (characterisation techniques) and chemistry (specifically bio-

coordination chemistry).^{1,4} Figure 1.1 is a diagrammatic representation of the far reaching, interdisciplinary nature of bioinorganic chemistry. The growing interest in bioinorganic chemistry research has become apparent with the emergence of journals that are solely devoted to this field of chemistry.

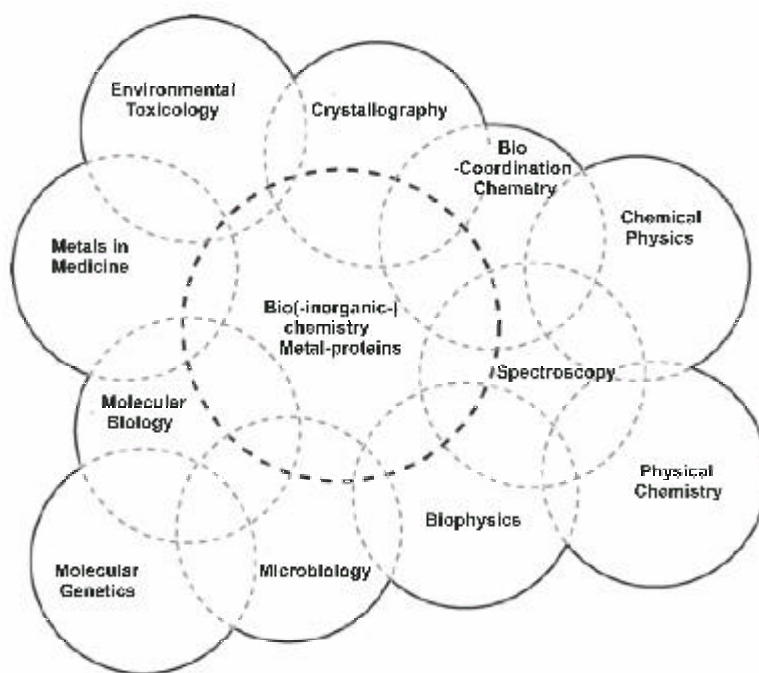


Figure 1.1: Tentative view of overlapping fields in bioinorganic chemistry.¹

1.1.2 Metal Ions in the Chemistry of Life

There are approximately thirty elements essential for life.⁵ These may occur in macroscopic, microscopic or trace amounts.⁶ Of these thirty elements, many are metal ions involved in life-sustaining biological systems and biochemical processes. These metal ions play a pivotal role in about one third of enzymes.⁷ Ions can modify electron flow in the substrate or enzyme, thus effectively controlling an enzyme-catalysed reaction. They can serve to bind and orient the substrate with respect to the functional groups in the active site, and they can provide a site for redox activity if the metal has several valence states. Many molecules that are involved in the above mentioned processes rely on metal ions for their activity.⁸ Evolution has made efficient use of the available metals and their properties to allow a range of inorganic reactions to be accessible to biological systems. The periodic table illustrated in Figure 1.2 highlights the elements that are necessary for life.

IA	IIA	IIIA	IVA	VA	VIA	VIIA	VIII	VIII	VIII	IB	IIB	IIIB	IVB	VB	VIB	VIIA	0
(H)																	He
Li	Be											B	C	N	O	F	Ne
(Na)	(Mg)											Al	Si	P	S	Cl	Ar
(K)	(Ca)	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	Ge	As	Se	Br	Kr
Rb	Sr	Y	Zr	Nb	Mo	Tc	Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te	I	Xe
Cs	Ba	Ln	Hf	Ta	W	Re	Os	Ir	Pt	Au	Hg	Tl	Pb	Bi	Po	At	Rn
Fr	Ra	Ac	Th	Pa	U												

Figure 1.2: Periodic Table of biological elements in life.⁷

Key: Circled Elements = Bulk Biological Elements

Boxed Elements = Essential Trace Elements

Dashed Box Elements = Possible Trace Elements Showing Weaker
Evidence for Involvement in Biology

The term “essential” is given to an element that would cause severe, irreversible damage if it were completely eliminated from the organism.⁴ Some elements such as As, Br, and Sn have not been unequivocally determined to be essential since they are present in such low concentrations that experiments are difficult to carry out. The normal concentration range of essential metals is narrow and deficiencies or excesses of such metals cause pathological changes in an organism.⁷

The alkali metals sodium and potassium are involved in the relay of electric pulses in the nervous system of a number of animals. The alkaline earth metals, magnesium and calcium, are responsible for teeth and bone structure in animals. Magnesium is also important element in the capture of solar energy by plants. The metal ion is bound in a highly conjugated ring system, the chlorin ring, which is bound to a macromolecule and together they form chlorophyll as shown in Figure 1.3. Chlorophyll and other pigments such as the carotenoids, work together to absorb a wide range of energies in the visible spectrum.⁸

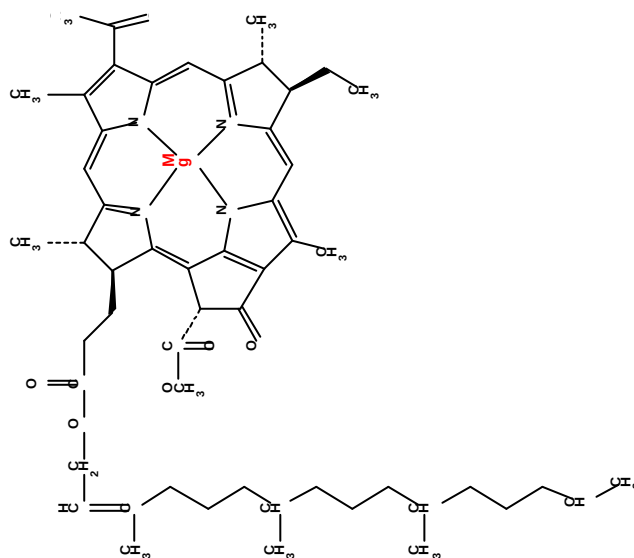


Figure 1.3: The structure bacteriochlorophyll *a*.

As this project revolves around a particular question in the biological chemistry of two specific transition metals namely, iron and cobalt, the focus in this section is primarily on them.

1.1.2.1 Aspects of the Chemistry of Iron: Properties and Special Characteristics

Iron is the most abundant metal both in and on the Earth.⁹ As far as biology is concerned, it is one of the most important metals in the first transition series as it is the transition metal found in greatest abundance in biological systems. It constitutes one third of the Earth's mass and is present from crustal rocks, to ocean and river waters, and to the organisms that exist in those environments.^{10, 11} The atmosphere as we know, abundant in oxygen, has developed over billions of years from a reducing atmosphere that was too hostile to support most living organisms. The only organisms that could exist were those that could survive in an anaerobic environment. In this reducing atmosphere iron was present in the ferrous state and was gradually oxidised to the ferric state as oxygen became more prevalent. This increase in oxygen concentration was brought about the blue-green algae and the photosynthetic bacteria.¹²

➤ Redox, Geometric and Magnetic Properties

The oxidation states of iron have been noted to range from -2 to +4, the most common being Fe^{II} and Fe^{III} in an aqueous environment.¹³ The change in atmospheric composition as described above, has had a dramatic effect on the biology and biochemistry of iron due to the different properties of the most common oxidation states of iron.

An interesting property of iron is its ability to be oxidised/reduced over a wide range of potentials depending on its coordination environment. Redox potentials are an indication of the relative stability of the oxidation state of iron in the presence of particular ligands. Iron is found in the first row of transition metals in the periodic table. Since the valence electrons are in the outer lying *d* orbitals, Fe^{II} is a d^6 and Fe^{III} is a d^5 metal ion.¹⁴

There are a wide range of geometries adopted by both Fe^{II} and Fe^{III} . The sizes of the ferrous and ferric ions also affect the geometry of their complexes. The increase of electrons in *d* orbitals as iron is reduced causes greater repulsion between those electrons,

resulting in a slight increase in the ionic radius. A structural consequence of this is seen in haemoglobin where the difference in the ferric and ferrous ionic radius dictates whether the ion will fit into the porphyrin ring, this plays an important role in haemoglobin function.¹⁵ A key feature of it is the behaviour of an individual iron porphyrin complex as it goes from the deoxy to the oxygenated state. In the deoxy state the haem complex is distinctly domed, with the iron atom $\sim 0.5 \text{ \AA}$ out of the porphyrin mean plane and with the Fe-N bond to histidine some 8° off perpendicular as shown in Figure 1.4.⁶ However, when an oxygen molecule becomes bound the iron comes into, or nearly into, the porphyrin plane and the Fe-N becomes more near to perpendicular.

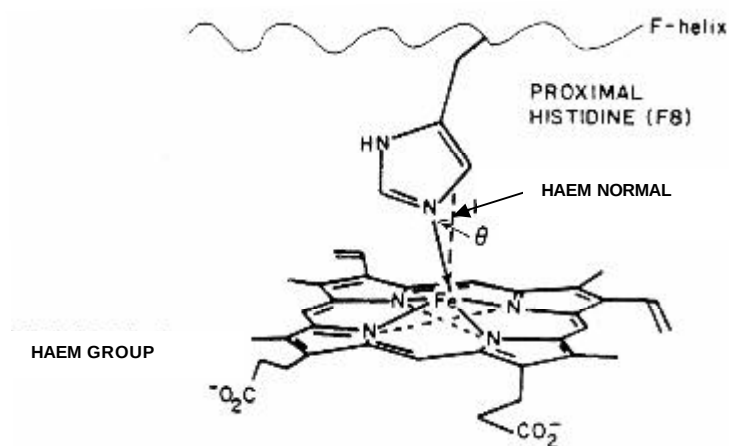


Figure 1.4: The structure of the deoxy haem group.⁶

Distortions from octahedral geometries arise when there is uneven distribution of electrons in the d orbitals. This occurs when the symmetry about the metal centre is decreased. All of the above mentioned redox, geometric and magnetic properties of the Fe^{II} and the Fe^{III} ions play a pivotal role in the structure, function and operation of the active sites in haemproteins.⁶

➤ Special Characteristics

Commonly, Fe^{II} (d^6) and Fe^{III} (d^5) complexes are high spin as quite strong ligand fields are required to induce low spin electron configurations. High spin Fe^{II} has a 5D ground term, which splits into the ground states 5T_2 and 5E . Ligand field ($d-d$) transitions ($^5T_2 \rightarrow ^5E$) in octahedral complexes of Fe^{II} occur in the visible region of the electronic spectra.

High spin octahedral Fe^{III} has an orbitally non-degenerate 6A_1 ground term arising from 6S ground term. Due to the single orbital occupancy of high spin Fe^{III} , there are no excited states of the same multiplicity and therefore all $d-d$ transitions are spin-forbidden to the T states, 4T_1 and 4T_2 , and to a nearly degenerate pair, 4A_1 and 4E . The intensities of the ligand field transitions $^6A_1 \rightarrow ^4T_1$ (4T_2) are so low that the $d-d$ band transitions are rarely observed in the electronic absorption spectra of high spin Fe^{III} complexes. Simple monomeric compounds of high spin Fe^{III} have little or no colour at all. Fe^{III} is electron deficient and can participate in the ligand-to-metal charge transfer (LMCT) processes that have much greater intensity than $d-d$ transitions.⁵ Strong colours arise from LMCT or intraligand transitions.

In the presence of strong octahedral fields there is a stabilisation of the low spin ($S = \frac{1}{2}$) state. The intermediate spin state of ($S = \frac{3}{2}$) may be produced in fields of low symmetry. High spin Fe^{II} and all spin states of Fe^{III} are paramagnetic and therefore ESR active. The iron atom has a nuclear spin of $I = \frac{1}{2}$.⁶ Although high spin Fe^{II} is paramagnetic, it has an even number of electrons. There is no guarantee of seeing an ESR signal because the ground term is not a Kramer's doublet.

1.1.2.2 Aspects of the Chemistry of Cobalt: Redox and Magnetic Properties

Cobalt is a first row transition metal and is found in oxidation states from +1 to +3. Co^{III} is unstable in simple compounds but is much more stable in larger low spin complexes.⁵ Typical geometries for cobalt range from tetrahedral to octahedral throughout the range of oxidation states.¹⁴ The electronic structure of Co^{III} is low spin d^6 except for very weak ligands such as the fluoride ion.⁵ Thus, Co^{III} complexes are diamagnetic in the ground state. Co^{III} complexes have characteristic electronic spectra with bands in the visible region due to transitions from the ground state ($^1A_{1g}$) to the excited singlet states ($^1T_{1g}$ and $^1T_{2g}$) representing the d-d transitions.⁵ Due to its diamagnetic electron configuration, Co^{III} is ESR inactive.

1.2 Metalloproteins and their Significance

Many transition elements have been found to serve as prosthetic groups (which are active sites of proteins) or as co-factors in metal activated proteins.¹⁵ Numerous essential biological functions require metal ions and most of these occur in metalloproteins. Metal ions are critical to the function of many proteins, including stabilising their structure. In fact, one third of all proteins are metalloproteins, using metal ions such as iron, copper, zinc, molybdenum and vanadium.¹⁶ Metalloproteins are an important class of biomolecules that play a central role in many biochemical processes, including respiration, metabolism, nitrogen fixation, photosynthesis, nerve transduction, muscle contraction, signal transduction, and protection against mutagenic agents. They also serve as catalysts in a wide variety of reactions ranging from electron transfer to the insertion of oxygen into carbon-hydrogen bonds. Thus, the role of the metal ions in metalloproteins is two fold, as it serves both functional and structural roles.^{17, 18} In performing these actions the metal can function alone, in a cluster or associated with complex ring systems.¹⁸

Without the appropriate metal ion, a reaction catalysed by a particular metalloenzyme would proceed very slowly if at all.¹⁹ The enzymes provide an arrangement of amino acid side-chain functional groups which orient themselves to form an appropriate sized hole (depending on the metal) to bind the metal. In addition, preferred groups on the enzyme side chains further bind the required metal ion to increase stability. The optimal number of such binding groups is chosen for the particular metal ion, together with an environment of appropriate polarity in the binding site. Metal ions may be bound by main-chain amino and carbonyl groups, but specific binding is achieved by the amino acid side chains, particularly the carboxylate groups of aspartic and glutamic acid, and the ring nitrogen atom of histidine. Other side chains that bind metal ions include tryptophan (ring nitrogen), cysteine (thiol), methionine (thioether), serine, threonine, tyrosine (hydroxyl groups), and asparagine and glutamine (carbonyl groups, less often amino groups). There are no general set of rules that describe how a metal will behave in an enzyme.²⁰ In essence the function of the protein is to anchor, control and modify the fundamental chemistry of the metal.

In recent years, the analysis and classification of metalloenzymes and metalloproteins at the interface between chemistry and biology has accelerated. Although a variety of spectroscopic techniques have been used for many years to probe the metal centres in metal containing proteins, progress is particularly due to the recent developments in x-ray diffraction methods, mass spectroscopy and NMR spectroscopy. Table 1.1 shows some of the metalloproteins that have already been elucidated.

Table 1.1: Some metalloproteins and their functions.¹⁸

Metal	Protein	Function
Cu	Azurin	Electron transport protein in <i>Alcaligenes xylosoxidans</i>
	Rusticyanin	Electron transport protein in <i>Thiobacillus ferrooxidans</i>
	Nitrite Reductase	Reduces nitrite in the denitrification pathway of <i>Alcaligenes xylosoxidans</i>
	Superoxide Dismutase	Scavenges superoxide free radicals
	Ceruloplasmin	A ferroxidase found in the blood
	Haemocyanin	Oxygen transport in the blood of molluscs and arthropods
Fe	Haemoglobin	Oxygen transport in the blood
	Myoglobin	Oxygen storage in muscle tissue
	Transferrins	Iron transport protein found in the blood
	FixL	Haem oxygen sensor protein symbiotic bacterium <i>Rhizobium meliloti</i>
	Cytochromes	Participate in important electron transfer reactions in ETC

Fe	Peroxidase	Protect the body against uncontrolled oxidation by hydrogen peroxide
	Cytochrome P450	Activation of molecular oxygen with insertion of one of its atoms into the substrate and reduction of the other to form water
Mo	Nitrogenase	Catalyses the ATP dependent reduction of dinitrogen to ammonia
	ModA	Periplasmic molybdate binding proteins <i>Azotobacter vinelandii</i>
	ModE	Molybdate dependent transcriptional regulator from <i>Escherichia coli</i>
Mn	Catalase	Reduces hydrogen peroxide to molecular oxygen and water
Ni	Urease	Hydrolysis of urea to ammonia and carbon dioxide
V	Nitrogenase	Catalyses the ATP dependent reduction of dinitrogen to ammonia
Zn	Superoxide Dismutase	Scavenges superoxide free radicals

1.3 Porphyrins

1.3.1 Basic Structure

The basic structures of porphyrin consist of four pyrrole units joined by methine bridges. In a haemprotein, the iron binds to the porphyrin ring and is also covalently linked by axial binding to at least one endogenous ligand. These macromolecules are relatively rigid, but flexible, and the porphyrin cavity is able to bind nearly all metal ions. They are ideal building blocks for self-assembled structures due to their pre-organised geometry, ease of substitution and selective axial ligand coordination chemistry. Porphyrins are susceptible to several chemical reactions typical of aromatic compounds. The porphyrin ring is a planar moiety belonging to the D_{4h} space group with an extensive delocalised p-electron system.²¹ The porphyrin macrocycle is an aromatic system containing 22 p-electrons, but only 18 of them are involved in any one delocalization pathway and thus conform to the Huckel's $(4n + 2)$ rule for aromaticity. Many haemproteins contain protoporphyrin IX. Table 1.2 shows the different porphyrins that arise from the substitution that occurs at the peripheral positions in the stable porphine shown in figure 1.5.²¹

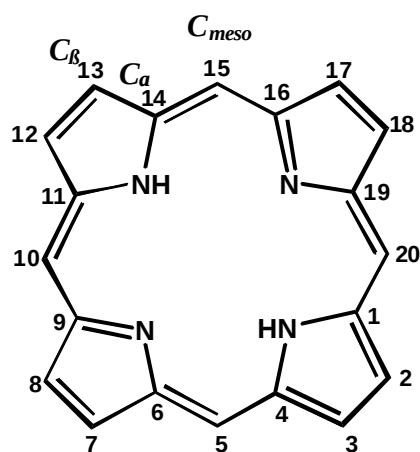


Figure 1.5: The porphine moiety.

Table 1.2: Different Types of Naturally Occurring Porphyrins.²¹

Porphyrin Name	Substituents at C _β Position No.							
	2	3	7	8	12	13	17	18
Porphyrin <i>a</i>	M	P	P	F	M	Hfar	M	V
Porphyrin <i>b</i> (porphyrin IX)	M	P	P	M	M	V	M	V
Porphyrin <i>c</i>	M	P	P	F	M	C	M	C
Deuteroporphyrin IX	M	P	P	F	M	H	M	H
Mesoporphyrin	M	P	P	F	M	E	M	E
Coproporphyrin IX	M	P	P	F	M	P	M	P
Hematoporphyrin	M	P	P	M	M	SA	M	SA
Uroporphyrin II	A	P	P	A	A	P	A	P
Aetioporphyrin III	M	E	M	E	M	E	M	E
Chlorocruporphyrin IX	M	P	P	M	M	F	M	V

Key: A = -CH₂COOH

E = -C₂H₅

H = -H

M = -CH₃

SA = -CH(OH)CH₃

C = CysteinyI S

F = -CHO

Hfar = C₁₇H₂₉O

P = -CH₂CH₂COOH

V = -CHCH₂

In addition to the several electrophilic substitutions that can be performed on porphyrins, porphyrins can be metalated and demetalated. Different metals (for example, Zn, Mn, Cu, and Ni) can be inserted into the porphyrin cavity using various metal salts. These metals can then be removed with various acids.²¹

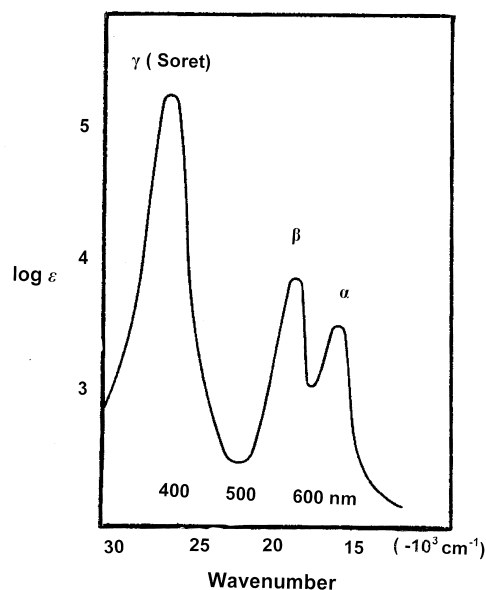


Figure 1.6: A typical absorption spectrum observed for porphyrins.²²

One of the most distinctive properties of haemproteins is their absorption of visible and ultraviolet (UV) light.²³ The three spectral bands that appear in the visible region are due to transitions between p^* (vacant orbitals) and p (occupied orbitals). The electronic spectra are dominated by the absorption of the haem moiety, which allows two porphyrin $p \rightarrow p^*$ transitions that are mixed together by interelectronic repulsion to give an intense signal at 410 nm (called the γ or Soret band) and two less intense signals between 500 nm and 600 nm (called α - and β -bands). These bands can be explained using Simpson's "perimeter free-electron" model where the porphyrin is idealised as an 18-membered circular ring. Figure 1.6 shows a typical absorption spectrum observed for porphyrins. However, more recently, there have been a number of reviews that have described the success of the four-orbital model which is based on the earlier free-electron model and the simple Huckel's molecular-orbital model.²³ The variations of the peripheral substituents on the porphyrin ring often cause minor changes to the intensity and wavelength of these absorptions.^{22, 23}

1.3.2 Iron Porphyrins

Iron porphyrins form the prosthetic group (active site) of the biologically important haemproteins. These haemproteins are macromolecules that play very important roles in various biological processes, which include oxygen transportation and storage in living tissues (as in haemoglobin and myoglobin), the reduction of hydrogen peroxide (as in the catalase and peroxidase enzymes), the reduction of oxygen (as in oxidases), the transport of electrons (as in the cytochromes *b* and *c* in the electron transport chain), and the oxygenation of organic substrates (as in cytochrome P-450). More specifically, haemoglobin and myoglobin serve as reversible oxygen transfer proteins, oxidases and peroxidases are involved in irreversible, covalent transformation of substrates, and the cytochromes *b* and *c* function as reversible one-electron transfer agents. Even though these haemproteins have diverse functions, all haemproteins possess a common prosthetic group composed of an iron porphyrin complex. Although the remainder of the protein plays a key role in modulation of their biological activity, there is evidence that the biological function of the haemproteins is determined by the conformational and electronic properties of the haem group, modulated by the protein.²³

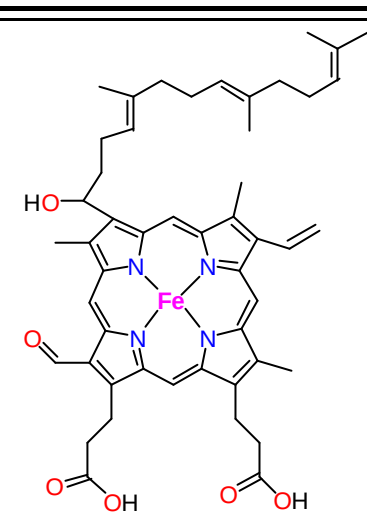
1.4 Haemproteins

Haemproteins (which contain iron porphyrins) are ubiquitous in nature and serve many roles as mentioned above. They can be classified according to their biological function, by haem iron coordination, by haem type, and further by sequence similarity. Studies have showed that the hydrophobic interior, hydrogen bonding interactions, and polar and non-polar interactions play an integral role in the correct folding around the haem. Consequently, there has been much experimental and theoretical effort directed towards the elucidation of the detailed nature of the haem group. Haemproteins are the only proteins to have an intimate relationship between structure, spectra and function. Thus, there exists a well-defined relationship between properties of the active site and the function of the protein.²⁴

1.4.1 The Cytochromes

Cytochromes are haemproteins whose specific biological function is electron and/or proton transfer by virtue of a reversible valency change of their haem iron.²⁵ They are known to play a major role as electron carriers in the electron transport chain (ETC). Although this definition takes into account both biological and chemical features and is wide enough to encompass both non-oxidizable and oxidizable cytochromes, it is however too wide to include the catalase and peroxidase group of haemproteins. There are however seven main groups of cytochromes that can be distinguished by their haem type (see Table 1.3).²⁶

Table 1.3: Different cytochromes classified by their haem type.²⁶

Haem type	Example	Haem Structure
Haem <i>a</i>	Cytochrome <i>c</i> oxidase	

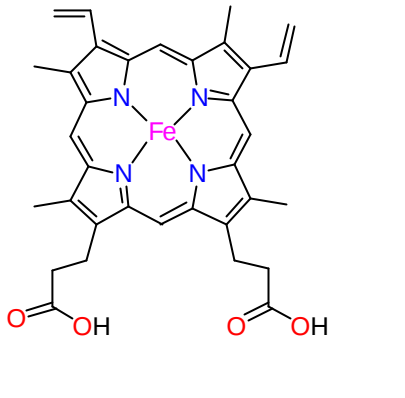
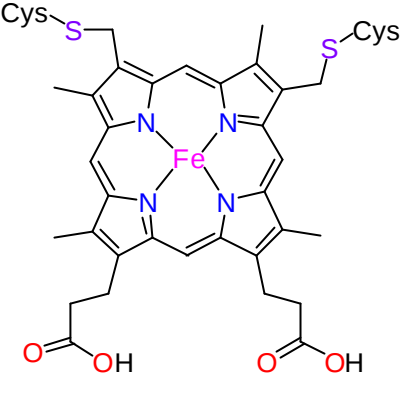
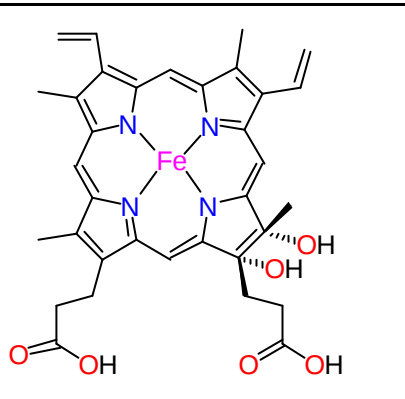
Haem <i>b</i>	Cytochromes <i>b</i>	 The structure shows a central iron atom (Fe) coordinated by four nitrogen atoms (N) in a porphyrin-like ring. The ring has two vinyl groups (CH=CH₂) at the top and two propionate side chains (CH₂CH₂COOH) at the bottom. The iron atom is colored pink, and the nitrogen atoms are blue.
Haem <i>c</i>	Cytochrome <i>c</i>	 The structure is similar to Haem b, but the two vinyl groups at the top are replaced by two propionylcysteine side chains (CH₂CH₂CO-S-Cys). The iron atom is pink, and the nitrogen atoms are blue.
Haem <i>d</i>	Cytochromes <i>d</i>	 The structure is similar to Haem b, but the propionate side chain on the right is replaced by a hydroxymethyl group (CH₂OH). The iron atom is pink, and the nitrogen atoms are blue.



Figure 1.7: 3D Structure of oxidised horse heart cytochrome *c*.²⁷

PDB code: 1AKK

In this project cytochrome *c* (cyt *c*) was used as the starting material for the preparation of microperoxidase 8, so it is important to highlight its significance in nature. Figure 1.7 illustrates the 3D structure of cyt *c*. Cyt *c* mediates single electron transfer between integral membrane complexes in the respiratory chain of eukaryotes.¹⁸ It is a protein ubiquitous to all eukaryotic organisms and is found associated with the inner mitochondrial membrane. There have been numerous structural studies elucidating the structure of cyt *c* using both X-ray crystallography and NMR spectroscopy.^{18,28} Although there is a vast amount of work that has been published on cytochromes, this project is not focussed on the biochemistry nor the bioenergetics of the cytochromes, but rather on the haem component of the cytochromes.

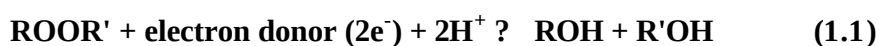
1.4.2 The Peroxidases and Catalases

Peroxidases and catalases are distributed widely in animals and plants. However, peroxidases are found more commonly in plants.

➤ The Role and Biochemistry of Peroxidases

Peroxidases are enzymes which play an important role in large and diverse numbers of physiological processes in organisms including humans. They are haem-containing enzymes that use hydrogen peroxide (H₂O₂) or other substrates as the electron acceptor to

catalyze a number of oxidative reactions. As such, they are classified as oxidoreductases. Toxic molecules such as superoxide and hydroxyl radicals can be found in cells due to the presence of oxygen. These are by-products of aerobic respiration. They are eliminated by a number of enzymes present inside the cell. Superoxide, for example, is destroyed by superoxide dismutase. The degradation, however, produces more hydrogen peroxide, which is, in turn, destroyed by peroxidase. Peroxidases reduce H_2O_2 to water while oxidizing a variety of substrates. Thus, peroxidases are oxidoreductases that use H_2O_2 as electron acceptor for catalyzing different oxidative reactions. The overall reaction is as follows:



For many of these enzymes the optimal substrate is hydrogen peroxide, but others are more active with organic hydroperoxides such as lipid peroxides. The nature of the electron donor is very dependent on the structure of the enzyme. For example, horseradish peroxidase can use a variety of organic compounds as electron donors and acceptors. Horseradish peroxidase has a broad and accessible active site and many compounds can reach the site of the reaction. For an enzyme such as cytochrome *c* peroxidase, the compounds that donate electrons are very specific, because there is a very closed active site. Cytochrome *c* peroxidase is used as a soluble, easily purified model for cytochrome *c* oxidase.^{4, 29}

On the basis of sequence and structure similarity, haem-dependent peroxidases can be separated into two families: the family of animal peroxidases and the family of plant, fungal and bacterial peroxidases.^{30, 31}

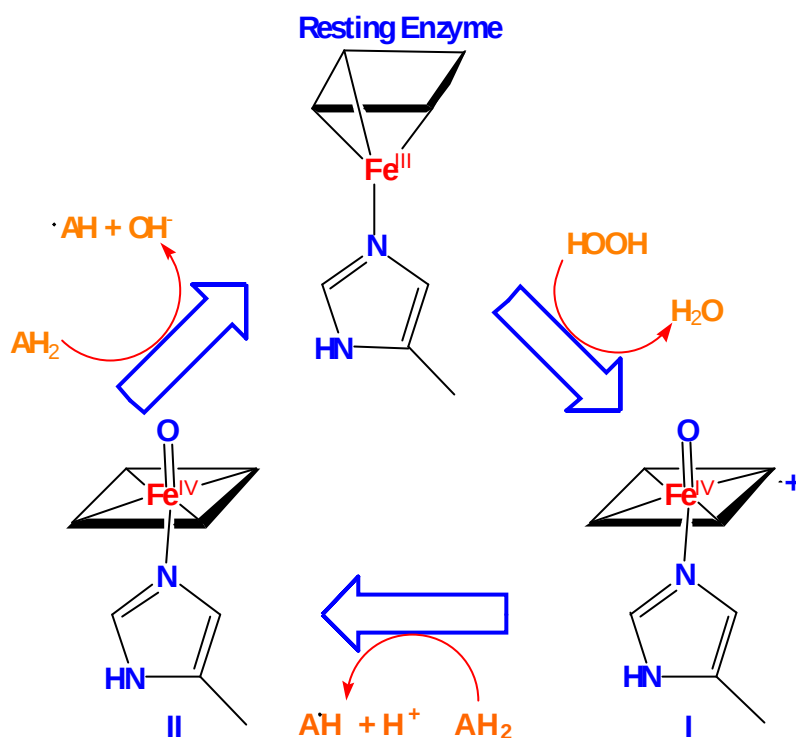
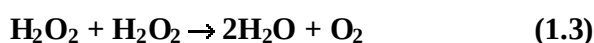


Figure 1.8: Reaction mechanism of most haem peroxidases.^{4, 29}

In this mechanism, the enzyme reacts with one equivalent of H_2O_2 to give compound **I**, a porphyrin π -cation radical containing Fe^{IV} . This is a two-electron oxidation/reduction reaction where H_2O_2 is reduced to water and the enzyme is oxidised. One oxidising equivalent resides on iron, giving the oxyferryl ($\text{Fe}^{\text{IV}}=\text{O}$) intermediate and the second resides on the porphyrin which becomes a p-radical cation. Compound **I** then oxidises an organic substrate to give a substrate radical ($\cdot\text{AH}$). Compound **I** undergoes a second one-electron oxidation reaction yielding compound **II**, which contains an oxyferryl centre coordinated to a normal (dianionic) porphyrin ligand. Finally, compound **II**, is reduced back to the native ferric state with concomitant one electron substrate reduction. The overall charge on the resting state and compound **I** is +1, while compound **II** is neutral.^{4, 29}

➤ The Role and Biochemistry of Catalases

Catalase is a haem-containing enzyme that catalyses reaction (1.2). The R represents hydrogen, an alkyl or acyl group and the HQOH is a two electron donor. Catalases can utilise hydrogen peroxide (H_2O_2) both as an electron acceptor and an electron donor yielding molecular oxygen (O_2) and water in the disproportionation reaction (1.3).



The reaction cycle of the catalases begins with the high spin ferric (Fe^{III}) state which reacts with the molecule of peroxide to form compound I intermediate, a porphyrin cation radical containing Fe^{IV} . This is followed by oxidation of an electron donor which returns compound I to the native resting state. Both the resting state and compound I of catalase are neutral.

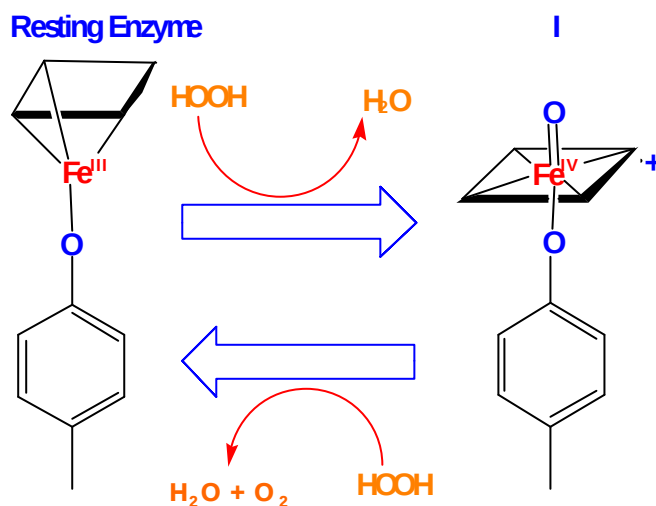


Figure 1.9: Schematic of catalytic cycle of Catalase.

Most catalases have haem *b* as the prosthetic group; however, a number of fungal and bacterial catalases contain chlorin type haem (haem *d*).³²⁻³⁴ In all catalases the haem iron

fifth (proximal) ligand is a Tyr residue; a His residue essential for catalysis is located on the distal side of the haem. It is thought that its function is to deprotonate H_2O_2 , which is bound to H^+ (on His) and HO_2^- (on Fe^{III}). In all catalase structures, a water molecule located close to the sixth coordination of the haem has been observed.³⁵⁻³⁸

1.4.3 The Cytochromes P450

Cytochromes P450 (P450s) are a superfamily of haem-thiolate enzymes that mainly catalyse the monooxygenation of a wide array of apolar substrates.¹⁸ This is a two electron oxidation of substrate with one of the dioxygen derived O atoms inserted into a C-H bond of the substrate.³⁹ Cytochromes P450 are isolated from all living organisms, including bacteria, fungi, plants, insects and vertebrates.⁴⁰ P450s are involved in metabolising a wide variety of hydrophobic compounds. In mammals, P450 substrates range from endogenous substances such as cholesterol, steroids, and lipids, to exogenous ones like environmental pollutants and drugs.

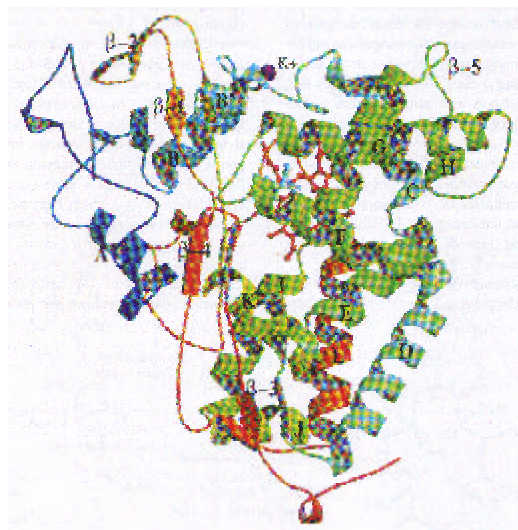


Figure 1.10: Ribbon of P450 camphor-substrate complex structure.⁴¹

PDB code: P450BM-3

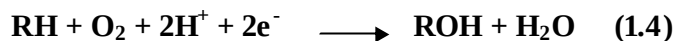
➤ Physical Properties

The P450s are haemproteins that are made up of between 400 and 500 amino acids and contain a single haem prosthetic group.^{42, 43} The distal axial ligand of this haem moiety is formed by a cysteine residue. In the haem system, six-fold coordinated Fe^{III} is generally found to be LS and five-fold coordinated Fe^{III} is found in the HS state.⁴⁴ This is due to the fact that the ionic radius is larger for HS Fe^{III} than for LS⁴⁵ and in the HS state the Fe^{III} moves out of the plane of the porphyrin ring as the central cavity is too small. Furthermore, it was also thought that the five coordinate Fe^{III} moves out of the porphyrin cavity to maximise orbital overlap of the donor atom of the proximal ligand. The nature of the axial haem ligands also has an important effect on the LS-HS balance. A strong axial field will bring about a relatively large d-orbital splitting, favouring the LS state. This can be seen in practice if one compares the UV absorption spectra of CO complexes with haemoglobin (histidine axial ligand) and P450 (cysteine ligand). The axial ligand field strength due to histidine is much stronger than that due to cysteine⁴⁶ and correspondingly the UV absorption band due to the $d \rightarrow d$ transition in CO-hemoglobin occurs at 420 nm, while that due to CO-P450 occurs at 450 nm.⁴⁷

Ligand-free P450s exhibit a Soret absorption maximum in a UV spectrum at approximately 420 nm. This is associated with the LS state of the Fe^{III} . Spectral⁴⁸, NMR⁴⁹ and crystallographic⁵⁰ data indicate that a water molecule forms a sixth axial ligand of the Fe^{III} in the substrate-free form, thus stabilising the LS state of the ion. The HS and LS states are not independent, but exist in equilibrium. The differences in the absorption and extinction coefficients between the two bands at 390 and 420 nm allow the equilibrium constant between the spin states and hence the fraction of HS character to be determined. The equilibrium between HS and LS is only one of a number of microequilibria which must be considered in a full description of the substrate binding reaction.⁵⁰⁻⁵³ The equilibrium between these states may be affected by many factors such as the pH of the solution or changes in the conformation of the enzyme which alter the nature of the binding of a ligand.

➤ Catalytic Cycle

The principal physiological role of the P450 superfamily of enzymes is that of a monooxygenase. The catalytic reaction can be summarised as follows



where RH can be one of a large number of possible substrates.

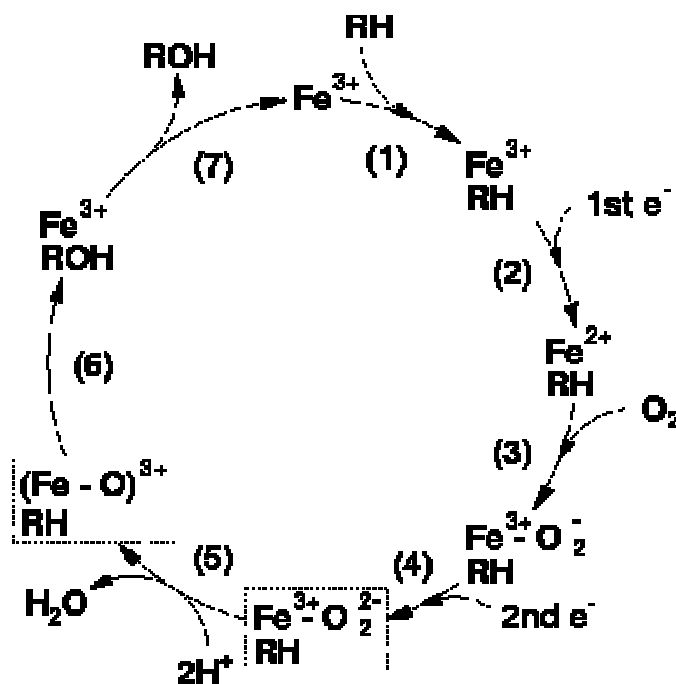


Figure 1.11: The catalytic cycle of cytochrome P450.

* The intermediate states enclosed in a dashed box have not been directly observed and are hypothetical.

(1) Substrate binding

The binding of a substrate to a P450 causes a lowering of the redox potential by approximately 100 mV,^{54, 55} which makes the transfer of an electron favourable from its redox partner, NADH or NADPH. This is accompanied by a change in the spin state of the haem iron at the active site. It has also been suggested that the binding of the substrate brings about a conformational change in the enzyme which triggers an interaction with the redox component.⁵⁵

(2) The first reduction

The next stage in the cycle is the reduction of the Fe^{III} ion by an electron transferred from NAD(P)H via an electron transfer chain.

(3) Oxygen binding

An O_2 molecule binds rapidly to the Fe^{II} ion forming $\text{Fe}^{\text{II}} + \text{O}_2$. There is evidence to suggest that this complex then undergoes a slow conversion to a more stable complex $\text{Fe}^{\text{III}} - \text{O}_2^-$.⁵⁶ A comparison between the bond energies of O_2 , O_2^- , and O_2^{2-} suggest that the $\text{Fe}^{\text{III}} - \text{O}_2^{2-}$ complex is the most favourable starting point for the next stage of the reaction to occur.⁵⁷

(4) Second reduction

A second reduction is required by the stoichiometry of the reaction. This has been determined to be the rate-determining step of the reaction.⁵⁸ However, evidence from resonance Raman spectroscopy indicates the presence of a superoxide (O_2^-) complex.⁵⁹ There is evidence to suggest that this complex then undergoes a slow conversion to a more stable complex $\text{Fe}^{\text{III}} - \text{O}_2^{2-}$.⁵⁶

(5) O_2 Cleavage

The O_2^{2-} reacts with two protons from the surrounding solvent, breaking the O-O bond, forming water and leaving an $(\text{Fe}-\text{O})^{3+}$ complex.

(6) Product formation

The Fe-ligated O atom is transferred to the substrate forming a hydroxylated form of the substrate.

(7) Product release

The product is released from the active site of the enzyme which returns to its initial state.

The structures of the transitional states following processes (4) and (5) have never been directly observed and are hypotheses based by analogy to other haemproteins and often conflicting experimental evidence.

1.4.4 Haemoglobin (Hb) and Myoglobin (Mb)

➤ Biological Roles

It is well known that invertebrates and vertebrates have different dioxygen coordination strategies. Before dioxygen can be metabolised, it has to be taken up (reversibly) from the atmosphere and transported to oxygen-depleted tissues where it must then be stored until needed.⁴

The physiological roles of Hbs in vertebrates are the transport of oxygen from the lungs (or gills in fish) to the body tissues, enhancement of carbon dioxide transport in the opposite direction and the regulation of blood pH. Since each haem can bind one oxygen molecule, four molecules of oxygen bind to the Hb tetramer. The affinity for the first oxygen is low but the affinity rises with the number of bound oxygen molecules. This is known as the haem-haem interaction or homotropic allosteric effect.¹⁸

The physiological roles of Mbs are to buffer the oxygen concentration in the respiring tissues. It is a haemprotein that contributes to intracellular oxygen storage and transcellular facilitated diffusion of oxygen. It is normally found in muscle tissue where continuous oxygen is supplied. The reversibility of oxygen binding is preserved only when the haem is kept in the ferrous state which is stabilised by the hydrophobic environment of the protein.⁶⁰ The affinity of myoglobin for oxygen lies between that for Hb, which releases oxygen during its passage through respiring tissues, and for the cytochromes that make use of molecular oxygen in oxidative respiration.¹⁸

➤ **Molecular Structures**

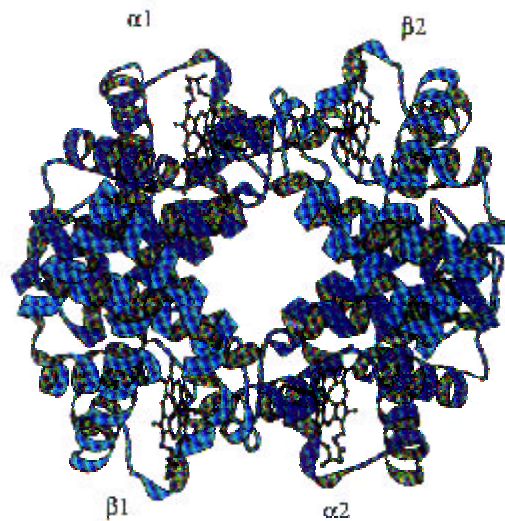


Figure 1.12: Schematic representation of deoxy T state Hb structure.⁶¹

PDB code: 1HGA

In 1959, Perutz obtained the first electron density map of horse oxyHb, which resulted in the determination of its crystal structure.^{62, 63} Hb consists of four protein units, each of which has an iron protoporphyrin IX group. Two subunits have the same amino acid sequence and are designated as α -subunits. The other two are designated as β -subunits; they are similar but not exactly the same as the α -subunits. Despite their differences between amino acid sequences, both subunits have the same fold, which is completely made up of α -helices. Thus, the four-unit composition of Hb serves a pivotal biological role as mentioned earlier. Hb has an overall compact globular structure with a diameter of 50-55 Å, while its subunit is approximately 47 x 35 x 22 Å³ in size. The helices have been traditionally named A through to H from the N- to the C-terminus.¹⁸

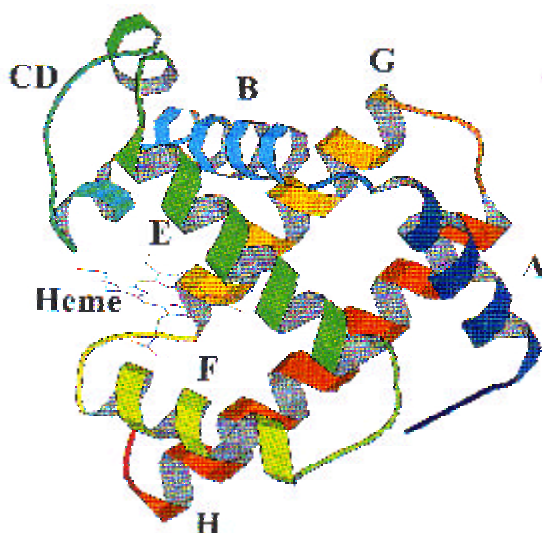


Figure 1.13: The overall structure of myoglobin.⁶⁴
PDB code: 2MBW

In 1960, Sir John Kendrew and his co-workers published the first solved crystal structure of Mb and consisted of a number of right-handed α -helices.⁶⁵ It was the first protein structure to be revealed at the atomic level. Mb consists of a single peptide chain combined with an iron porphyrin. It comprises eight helices packed in the pattern that leaves a pocket for haem. These helices are typically named from A to H. This helical haem-binding domain is known as the globin fold. The tertiary structure of the protein is kept intact by sulphide bridges, hydrogen bonds, van der Waals forces and ionic interactions.⁶⁶ The overall shape of the molecule is best described as an oblate ellipsoid with dimensions $45 \times 45 \times 25 \text{ \AA}$.¹⁸

1.5 The Microperoxidases (MPs)

1.5.1 Background

One of the greatest challenges facing bioinorganic chemists and biochemists is explaining how the protein controls and modifies the basic properties of the active site to give rise to such wide-ranging functions.⁶⁷ One method of doing this is to compare the properties of protein-free porphyrins and the haemproteins. However, with this approach come two main complications. The first is solubility limitations, where porphyrins like haemin have limited solubility in aqueous and neutral solutions⁶⁸ and tend to aggregate in

aqueous alkaline solutions.⁶⁹ Thus work had to be performed in non-aqueous solvents⁷⁰⁻⁷³ and detergents^{74, 75} to ensure a monomeric system. These non-aqueous solvent conditions for studying the physical and chemical properties of haemproteins are inadequate and are not physiologically relevant. The second complication is that many haemproteins possess either one or two histidine residues as axial ligands of the metal ion; these include Hb, Mb, peroxidases and the different cytochromes *c*, *b*, *f* and cytochrome *c* oxidase.⁶⁷ The addition of ligands like imidazole and its derivatives to solutions of iron porphyrins almost always results in the formation of the bis-imidazole complex with only transient existence of the mono-imidazole species because of the thermodynamic drive from HS to LS.^{76, 77}

Thus, the need arose to synthesise model haemproteins whose iron porphyrin active site incorporated the ligand combination found in the protein itself. Various model haempeptides with a variety of axial ligands were synthesised. These include (i) haempeptides prepared by adding small mono-, di-, and tri-peptide groups to the propionic acid side chains or other suitably functionalised groups of the porphyrin; (ii) synthetic haempeptide fragments in which the invariant Cys₁₄-X₁₅-X₁₆-Cys₁₇-His₁₈ sequence of the cytochrome *c* is attached to the haem periphery and (iii) the range of haempeptide fragments that may be obtained by treating cytochrome *c* with suitable proteolytic enzymes.⁶⁷ The haempeptides in (ii) and (iii) incorporate a His residue as an axial ligand, and therefore afford the possibility of mixed ligation of the haem iron (i.e. the formation of the mono- and bis-complexes together).⁶⁷ However, the flexible ligand-bearing side-chains attached to the porphyrin periphery has the tendency to dimerise and form higher aggregates in solution.⁷⁸

1.5.2 Preparation of Microperoxidases

Microperoxidases (MPs) are haem-based minenzymes that form a new generation of biomimics. They consist of a haem *c* cofactor covalently bound to an oligopeptide, which contains histidine, the axial ligand to the haem iron. It is obtained by controlled

proteolytic digestion of horse heart cytochrome *c*. It is an appealing model for mimicking the structure and the function of the active site of haem-containing enzymes since it retains an axial histidine ligand.⁷⁹ The haempeptides provide an opportunity for exploring aspects of the fundamental chemistry of an iron(III) porphyrin in which one of the axial coordination sites of the metal ion is occupied by a His residue which is from the parent protein and the other position contains a readily replaceable water molecule.⁶⁷ This provides an opportunity for quantitatively studying in aqueous solution the coordination chemistry of an iron porphyrin with a single accessible coordination site.⁸⁰ Like many iron porphyrins, microperoxidase-8 is monomeric only at very low concentrations in aqueous solution.^{81,82}

Although MP8 is commercially available, it is expensive. The hydrolysis of cytochrome *c* was historically performed to help determine the structure of the parent protein.⁸³⁻⁸⁵ The first haempeptides that were isolated MP11 (residues 11-21), MP9 (residues 14-22) and MP10 (residues 13-22).⁸⁵⁻⁸⁷ Further proteolysis resulted in the formation of MP8 (residues 14-21) and MP6 (residues 14-19).^{88, 89} The peptides are usually prepared by digestion of ferric cytochrome *c* with appropriate enzymes (in this project for the preparation of MP8, the enzymes trypsin and pepsin were used). This is then followed by size-exclusion or ion exchange chromatographic techniques to separate the different MPs (in this project we separated the different MPs using a Sephadex G-50 size exclusion column). Analytical high performance liquid chromatography (HPLC) can be used to purify MP preparations effectively. The products can be characterised or identified using a multitude of characterization techniques including amino acid analysis⁹⁰, FAB-MS, liquid secondary ion mass spectrometry⁹¹, UV-vis spectrophotometry⁶⁷ and NMR.⁹²

Recently, there has been a considerable amount of work and development in the area of biomimetic models for haemproteins. Most of the chemical and biochemical models show catalytic activities closely related to the activities of the proteins they mimic. Thus, metalloporphyrin models can act as oxygen binding and/or oxygen transport molecules; they can transfer electrons, and also catalyse peroxidase and/or monooxygenase

reactions.⁸⁰ The biomimetic modelling approach is the key to unlocking many facts and theories that scientists dealing with proteins have encountered through the decades.

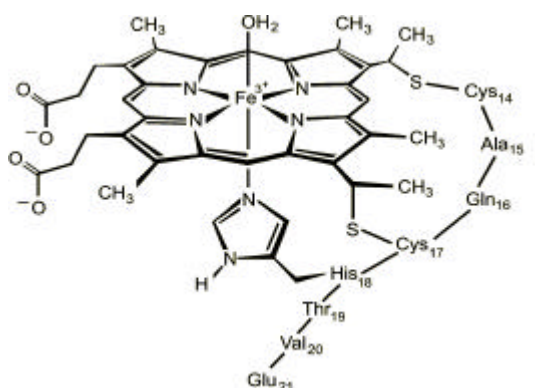


Figure 1.14: The structure of the Fe^{III} -MP8, the ferric haemoctapeptide from cytochrome *c*.

1.5.3 Solution Behaviour of the Microperoxidases

1.5.3.1 Solution Structure

Although there have been many attempts, none of the MPs have been crystallised; thus no solid state data are available. However, there has been a study of the ^1H NMR spectroscopic study of the monomeric cyanide complex MP11 which has suggested that most of the amino acid side chain from Lys-13 to Glu-21 lies in an α -helix conformation beneath the coordinated Fe^{III} atom and the proximal His 18 ligand in the shift cone produced by the diamagnetic ring current of the porphyrin ring.⁹³ This suggestion was later confirmed by Mondeli *et al.*⁹⁴ who studied monomeric MP11 in the paramagnetic, Fe^{III} low spin state by NMR-restrained molecular dynamics calculations in water-trifluoroethanol and water-methanol mixtures with various iron distal ligands at different temperatures. Ten structures obtained showed the polypeptide side chain preferred the right-handed helical secondary structure.

In another study involving MP11 it was found that at low pH, His-18 becomes protonated and does not coordinate Fe^{III} . As the solution pH is raised His-18 is deprotonated and coordinates Fe^{III} with a pK_a of 3.4 at 25 °C.⁹⁵ In addition to this a second pK_a of 5.8 was found in the acid region which was attributed to the deprotonation and intermolecular coordination of either the $\alpha\text{-NH}_2$ group of Val-11 or the $\epsilon\text{-NH}_2$ group of Lys-13.⁹⁵ However, more recently there were three pK_a s observed in the acidic region and the neutral region for MP11 at 3.4, 5.8 and 7.6 at 20 °C, and attributed to the deprotonation and intermolecular coordination of His-18, to the deprotonation and intermolecular coordination of $\alpha\text{-NH}_2$ group of Val-11 and to the deprotonation and intermolecular coordination of the $\epsilon\text{-NH}_2$ group of Lys-13, respectively.⁹⁶

Most studies reported on MP11 reveal a severe problem due to intermolecular coordination and aggregation of the haempeptide, and compromised the findings.⁶⁸ Similarly, physical studies on aqueous solutions of MP8 show its behaviour is also complicated by the aggregation phenomena and the solutions of MP8 have been found to deviate from Beer's Law as a result of dimerisation at concentrations as low as 2 μM at pH 7.00 at 25 °C⁹¹; since the intensity of the Q_y band at 494 nm in the visible region of the electronic spectra increased at the expense of the charge-transfer band at 622 nm, which is indicative of the conversion of the high-spin aqua complex to the low spin species, thus it was concluded that dimerisation by intermolecular coordination of $\alpha\text{-NH}_2$ group of the Cys-14 was likely. Supporting this evidence were the results obtained by Urry and Pettegrew⁹⁷ who used optical rotary dispersion (ORD), circular dichroism (CD), and difference absorption spectroscopy with a range of solutions of MP8. It was found that dimerisation of MP8 also involved p-p interactions between the haem groups.

1.5.3.2 Aggregation in Aqueous Solution

In 1955, Ehrenberg *et al.* demonstrated that at pH 8.7 the haempeptides became extensively aggregated with an apparent molecular weight of 10 000.⁸⁸ These workers also showed that at pH 2.3, no such aggregation occurred and proposed that this was due

to the intermolecular coordination of α -NH₂ group of the terminal Val-11 residue. Furthermore, the addition of exogenous ligands such as His, ammonia and imidazole, which compete for the distal coordination site was shown to disperse the aggregation.^{88,89} Further evidence for pH dependant aggregation phenomena of MP11 was confirmed by Harbury and Loach⁸⁹, who demonstrated that the aggregation was concentration dependent. The authors also discovered that the acetylation of Val-11 and the ϵ -NH₂ group of Lys-13 eliminated aggregation, which suggested that the observed aggregation was due to intermolecular coordination of the amino groups of the peptide chains.^{88, 89}

In addition, further evidence that aggregation of MP11 in aqueous solution occurs due to intermolecular coordination of the free amino groups of the polypeptide chains was provided by magnetic studies^{90, 98-101}, which all show that Fe(III) is low spin above pH 6. Aron *et al.*¹⁰² studied the aggregation of MP8 (due to the intermolecular coordination of the *N*-terminal Cys-14 residue) qualitatively in aqueous and aqueous methanolic solutions at pH 7.00 and 12.00 by observing deviations from Beer's Law that occurs in the concentration of MP8 increased. The workers proposed that the deviations could be accounted for by a simple equilibrium between two monomers and a dimer.¹⁰² Similar results were obtained by Baldwin *et al.*¹⁰³ who demonstrated that MP9 is monomeric in 50% aqueous methanol solution up to a concentration of 35 μ M. It was thought that aggregation of MP9 occurred due to intermolecular coordination of Lys-22 to the Fe^{III} centre of an adjacent molecule.¹⁰⁴

It has well been established that the aggregation of MP8 and MP11 aqueous solutions occur due to non-covalent π -interactions between the distal faces of two or more haempeptides.^{97, 105} Urry and coworkers^{97, 105} studied this second form of aggregation by means of optical rotation, CD and absorbance spectroscopy, and demonstrated that aggregation could be minimised by both dilution of the haempeptide and by elevated temperatures. It was suggested that dimerisation of MP11 proceeds through a head-to-tail alignment of the haem planes, whereas dimerisation in MP8 was thought to be through the stacking of haems.

1.5.3.3 Overcoming Aggregation

Monomerisation in aqueous solution may be achieved by using alcohols or detergents to disperse aggregates, or simply by using microperoxidase solution concentrations $< 2 \mu\text{M}$. Although these techniques are commonly used to ensure that MPs remain monomeric in solution, these conditions are far from ideal.⁶⁸ It has long since been established that rate constants, equilibrium constants, and other physical parameters measured in mixed solvent systems usually depend on the permittivity of the medium; thus it becomes more difficult to compare the results of the model studies in mixed solvent systems with data obtained on studies of haemproteins in aqueous solution. Furthermore, the low concentrations required to ensure monomeric conditions limit physical studies to UV-vis spectroscopy using long pathlength cells.⁶⁸

In 1959, Harbury and Loach¹⁰⁶ reported that the acetylation of the $\alpha\text{-NH}_2$ group of the terminal Val-11 residue and $\epsilon\text{-NH}_2$ group of Lys-13 minimised aggregation of MP11 in aqueous solution. Further evidence that acetylation of MP8 prevents aggregation was provided by the ESR data collected by Yang and Sauer.¹⁰⁷ A further suggestion by Wang and Wart¹⁰⁸ suggested that acetylation prevents intermolecular coordination in their studies of MP8 using optical and resonance Raman spectroscopy. However good the above results, they failed to provide conclusive evidence that acetylation prevents aggregation.

The most comprehensive study that showed definitive evidence that acetylation of the Cys-12 of the α -amino group of MP8 prevents aggregation was provided in a detailed solution chemistry study by Marques and Munro.¹⁰⁹ Marques and co-workers demonstrated that acetylated MP8 (*N*-AcMP8) remains monomeric in aqueous solution at concentrations below $3 \times 10^{-5} \text{ M}$, with a self-association constant an order of magnitude lower than that for MP8,¹⁰² showing evidence that the *N*-acetyl protecting group diminishes intermolecular coordination. These results are in agreement with those of Wang *et al.*¹¹⁰ who reported that *N*-AcMP8 forms aggregates above concentrations of $1 \times 10^{-5} \text{ M}$.

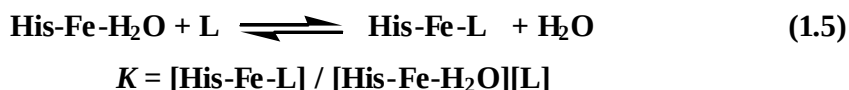
1.5.4 Haempeptide Acid Dissociation Constants

It has been well established that for all haempeptides for which the solution chemistry has been investigated there are corresponding pH dependant electronic spectra.⁹¹ However, in most cases the pK_a s associated with these spectral changes are complicated by formation of aggregates. In the study carried out by Marques and Munro¹⁰⁹ who investigated the solution chemistry of *N*-AcMP8 as a monomeric aqueous solution, *N*-AcMP8 was found to undergo six spectroscopically observed pH dependant transitions. The authors suggested that the first transition ($pK_a = 2.1$) is due to the deprotonation of the *c*-terminal carboxylic group, which subsequently displaces the proximal H_2O ligand of the bis-aqua complex. Furthermore, it is reported that the electronic spectra of these two species show that both are predominantly high spin. The second transition occurs when the pH is raised above 3.21, and is attributed to the deprotonation of the His-18 with subsequent binding to the lower axial coordination site of the ferric centre. The electronic spectra of the third species suggest that it is predominantly high spin, but also has some low spin character. The third and fourth pK_a s at 4.95 and 6.1, respectively, are thought to be due to the deprotonation of the two propionic acid groups, with the first also corresponding to the ionisation of the *c*-terminal carboxylic acid group of the peptide chain. It is however probable that these two processes are closely overlapped and not completely resolved. The pK_a at 9.59 is attributed to ionisation of the water molecule occupying the distal position, and the final pK_a at 12.71 corresponds to the deprotonation of the bound His-18 forming the histidinate complex (His-Fe(III)-OH⁻). The electronic spectra show that Fe(III) in *N*-AcMP8 is predominantly in the $S = 5/2$ state at pH 7, while the hydroxo complex at pH 10.5 is an equilibrium mixture of the $S = 5/2$ and $S = 1/2$ states. Above pH 12.71, the ferric ion is predominantly in the low spin state.

1.5.5 Ligand Binding Studies of Microperoxidases

There have been many studies aimed at understanding the chemistry that occurs at the active site of haemproteins, and to elucidate the effect of the protein on the chemistry of the prosthetic group, a wide range of ligand binding studies have been performed on both

the native haemproteins and their corresponding model compounds. By comparing the formation constants of a given ligand by a haemprotein and by a MP, it provides vital information about the effect of the protein on the ligand binding properties of the iron porphyrin.⁹¹ The model compound, in this case MP, serves as useful tool for the prosthetic group of haemproteins such as haemoglobin, myoglobin and the peroxidases. However, these model compounds have to be monomeric in solution. In these systems, the lower distal His-18 ligand is retained as part of the amino acid side chain and the upper axial ligand is a readily replaceable water molecule. This makes it possible for a ligand substitution to be investigated for a wide variety of exogenous ligands. This process is defined by Equation 1.5:



The nature of the distal ligand in haemproteins and MPs determines the spin states of the complexes. Ligands that produce predominantly low spin complexes includes cyanide^{100, 107, 111, 112}, imidazole and its derivatives^{100, 107, 110, 111, 113-115}, and pyridine and its derivatives^{82, 116}. Weaker field ligands such as azide^{107, 111, 117, 118}, and sulphite¹¹⁴ produce complexes that are in spin equilibrium. Predominantly high spin complexes are formed with weak field ligands such as cyanate¹¹⁹ and fluoride^{100, 107, 118} anions. Table 1.4 shows a comparison of equilibrium constants for various ligands coordinated by MP8 and various haemproteins. K_{rel} , which is defined as $K_{\text{haemprotein}}/K_{\text{MP8}}$, is generally much larger for the anionic ligands than for neutral ones. The distal environments of most haemoglobins and myoglobins contain the side chains of Val, Phe, and the distal His.

Table 1.4: A comparison of the ligand formation constants for various haemproteins, and for ferric MP8.¹²⁰

Ligand (L)	Haempeptide/ Haemprotein	Log <i>K</i>	<i>K</i> _{rel} ^b	Reference
F ⁻	MP8	-0.022		121
	Sperm whale Mb ⁺ ^c	1.85	74	122
	Human Hb ⁺	1.77	62	122
	<i>Aplysia</i> Mb ⁺ ^d	1.7	53	123
SCN ⁻	MP8	-0.52		120
	Human Hb ⁺	2.6	1300	122
	Horse Mb ⁺	2.42	870	124
	<i>Aplysia</i> Mb ⁺	1.05	37	123
NCO ⁻	MP8	0.083		120
	Sperm whale Mb ⁺	3.3	1600	122
	Human Hb ⁺	3.19	1300	125
	Horse Mb ⁺	2.58	310	124
N ₃ ⁻	MP8	1.45		120
	Sperm whale Mb ⁺	4.98	3400	126
	Human Hb ⁺	5.41	9100	122
	Horse Mb ⁺	4.46	1000	124
	<i>Aplysia</i> Mb ⁺	3.4	89	123
	<i>D. dendriticum</i> Hb ⁺ ^e	3.1	45	122
	Hb ⁺ M _{Hyde Park} ^f	5.17	5200	122
	Hb ⁺ M _{Iwate} ^f	5.27	6600	122
Imidazole	MP8	4.45		102
	Sperm whale Mb ⁺	2.1	0.0045	127
	Human Hb ⁺	2.6	0.014	128
	Horse Mb ⁺	1.6	0.0015	129
	<i>Aplysia</i> Mb ⁺	2.3	0.0071	127
2-methyl imidazole	MP8	2.61		120
	Horse Mb ⁺	1.3	0.049	129
Benzimidazole	MP8	2.71	0.12	120
	Sperm whale Mb ⁺	1.8		130
CN ⁻	MP8	7.58		112
	Sperm whale Mb ⁺	8.4	6.6	130
	Human Hb ⁺	8.74	14	131

^a The equilibrium constant for the process defined in Equation 4.

^b $K_{\text{rel}} = K_{\text{haemprotein}} / K_{\text{MP8}}$

^c Mb⁺ and Hb⁺ refer to the ferric (met) myoglobin and haemoglobin, respectively.

^d In myoglobin for the mollusc *Aplysia limacine* the distal His is replaced by Val.

^e *Dicrocoelium dendriticum* is a small liver fluke; the distal His is replaced by Gly.

^f Mutant Hb; in Hb M_{Hyde Park} the distal His of the β chains is replaced by Tyr; in the Hb M_{Iwate}, the same mutation conditions but in the α chains.

1.5.6 Demetalation of Haempeptides

In order to gain new insights into the structure and function of metalloproteins, the iron in the iron porphyrins has been substituted with other metals. Iron has been substituted with manganese in MP8.^{79, 80} In other studies iron has been substituted with copper, zinc, manganese, nickel and cobalt in cytochrome *c*.¹³² There are three methods of iron abstraction that have been reported. The first method involves removing the entire haem moiety from the polypeptide chain and substituting it by another porphyrin containing the desired metal.¹³³ This route is both complicated and quite involved, with many steps. The second method involves the removal of iron by treatment with hydrogen fluoride at temperatures below 0 °C.^{80,132} This path is hazardous and not user friendly as it involves the use of hydrogen fluoride. The final and much safer method which was adopted involved the removal of iron by reductive demetalation reported by Primus *et al.*⁷⁹ In the project the insertion of the cobalt ion is accomplished by refluxing the free base (metal-free MP8) with cobaltous acetate which is followed by oxidation in air.

1.6 Cobalt Corrinoids: Basic Structure

Much of the chemistry discussed in this project revolves around the unique nature of the corrin ring and why life has preserved this apparently evolutionary antique. The corrinoids are a group of compounds containing four reduced pyrrole rings joined into a macrocyclic ring by links between their α positions; three of these links are formed by a one-carbon unit (methylidyne radicals) and the other by a direct (Ca-Ca bond). They include various B₂ vitamins, factors and derivatives based upon the corrin skeleton, C₁₉H₂₂N₄.¹³⁴

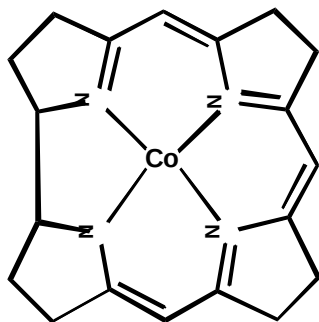


Figure 1.15: The basic structure of the cobalt corrinoids.

1.7 Vitamin B₁₂

1.7.1 Background

Vitamin B₁₂ is a vital biological compound as it is involved in several biochemical processes, particularly in bacteria. The nature of the biologically active forms of vitamin B₁₂, which involve a cobalt-to-carbon σ -bond, is most remarkable. This organometallic bond is a source of some very interesting coordination chemistry found in the derivatives of vitamin B₁₂.⁴ It is not fully understood what vitamin B₁₂ does in cells, but it is known to be essential in preventing pernicious anaemia, a fatal disease of the red blood cells.¹³⁵ The history of the discovery of vitamin B₁₂ and its biological function can be traced back to the early 1920s when Minot and Murphy, two American physicians, started to cure pernicious anaemia patients with a raw liver diet, and Castle observed that the stomach juice contains a protein factor he called *intrinsic factor*, which strongly enhances the curing effect of the liver or its extracts administered orally.^{136,137} Following this discovery, and for the next twenty years, liver was the main source of this unknown curing factor which was prepared, as time passed, in more and more concentrated form. Finally, in 1948 the isolation of pure crystalline factor was announced by two independent teams from the United States and England.^{138, 139}

It was Folkers and his co-workers at Merck Laboratories and Smith and Parker at Glaxo Laboratories who purified and isolated pure crystals of vitamin B₁₂. Small red crystals of

vitamin B₁₂ were then grown by Lester Smith and given to Dorothy Hodgkin for crystal structure analysis. All that was known at that stage was the approximate empirical formula. A crystal structure on a molecule of this size and complexity had never been attempted before, it was a huge and complex task, since crystal structure determinations were not the routine tasks that they are today, and the techniques were still being developed, both the X-ray and the computer equipment were tedious and difficult to use. Thus the X-ray crystal structure which emerged from this study between 1950 and the early 1960s was the first determination of a chemical formula by x-ray diffraction, and the first determination of the structure of a metalloenzyme.^{138,139} However, it is possible to trace several lines of study, independent in origin, which were well underway to the discovery of vitamin B₁₂.

1.7.2 Structure of Vitamin B_{12a}

The cobalt corrinoids contain a cobalt ion in the +3 oxidation state coordinated in the equatorial plane by four nitrogen atoms of the corrin macrocycle.²³ Vitamin B₁₂ is the only known biomolecule with a stable carbon-metal bond as shown in Figure 1.16. The core of the molecule is a corrin ring with various attached side chains. The ring consists of 4 pyrrole subunits, joined on opposite sides by a C-CH₃ methylene link, on one side by a C-H methylene link, and with the two of the pyrroles joined directly. It is thus like a porphyrin, but with one of the bridging methylene groups removed. The nitrogen of each pyrrole is coordinated to the central cobalt atom. The sixth ligand below the ring is a nitrogen of a 5,6-dimethylbenzimidazole. The other nitrogen is linked to a five-carbon sugar, which in turn connects to a phosphate group, and thence back onto the corrin ring via one of the seven amide groups attached to the periphery of the corrin ring. The base ligand thus forms a 'strap' back onto the corrin ring.

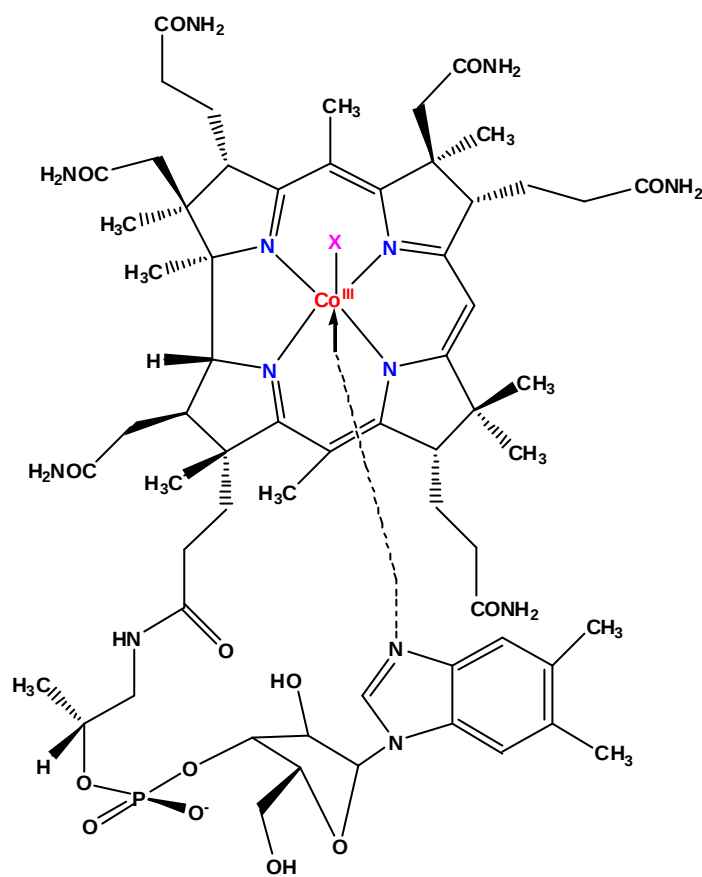


Figure 1.16: The structure of the vitamin B_{12a}.

Generally, vitamin B₁₂ and other cobalt corrinoids crystallise in the orthorhombic space group $P2_12_12_1$ with four molecules per unit cell. The coordination geometry is tetragonally distorted pseudo-octahedral, with four equatorial nitrogen donors from the corrin ring. The corrin ring is quite flexible and folds upward about the C10-Co axis. This fold is quantified as the fold angle, defined as the angle between the normal to the least squares planes through N21-C4-C5-C6-N22-C9-C10 and through C10-C11-N23-C14-C15-C16-N24.¹⁴⁰ One important finding from the structural studies of substituted cobalamins (R-Cbl's) is the so called "inverse trans effect"¹⁴¹ for alkyl-Cbl's in which increasing s-donation from R leads to an increase in the length of both the upper (β) Co-L bond and the lower (α) Co-NB3 bond despite expectations to contrary. There have been several studies investigating and describing many different aspects of the chemistry of vitamin B₁₂ that are beyond the scope of this project. These special properties and features are described in detail in a review by Brown¹⁴²

1.7.3 Chemistry of Vitamin B₁₂ and its derivatives:

Ligand Substitution and Kinetic Studies

The inorganic chemistry of the cobalt corrinoids (derivatives of vitamin B₁₂) is vital for mankind's existence and therefore attracts much attention. The kinetics of the reactions of aquacobalamin (vitamin B_{12a}) with imidazole, 4(5)-methyl imidazole, 2-methyl imidazole, imidazole 4(5)-lactic acid, histamine and histidine were studied as a function of pH at 25 °C, 1.0 M ionic strength (KCl) by UV-Vis spectrophotometry.¹⁴³ It was found experimentally that the 2-methyl imidazole and the analogous 4-substituted tautomers of asymmetrically substituted imidazoles, compared to imidazoles where tautomerism was impossible (1-methyl imidazole, 1,5-dimethyl imidazole), inconsequential (imidazole), or where the 5-substituted tautomer was the greatly predominant species in solution (neutral histamine, anionic histidine), replaced H₂O insignificantly slowly due to the steric repulsion between the annular substituents and the corrin ring. Thus it was concluded that the rate of substitution of H₂O varies linearly with the p*K_a* of the incoming ligand. The positive deviation of imidazole itself from this series was attributed to the possibility of its reaction through two annular coordination sites. On the other hand, the negative deviation of 4(5)-lactic acid, cationic histamine and neutral histidine from the linear relationship was attributed to the presence of two tautomeric forms in solution and this provided the means of finding the equilibrium constant, *K_T*, for interconversion between these species.¹⁴³

The thermodynamics of the coordination of imidazole and its derivatives by aquacobalamin were also investigated.¹⁴⁴ From the fits of the relevant equations to the experimental data and from direct spectrophotometric titrations, it was shown that coordination decreases the p*K_a* of the imino group of the imidazoles from >14 to 10 due to the polarization by the metal ion. Also, the p*K_a*s of the pendant amino acids of the histamine and histidine decreased from 9.96 and 9.18 to 8.89 and 7.99, respectively, on coordination. This was attributed to the destabilization of the conjugate acids by coulombic repulsion with the residual charge at the metal centre.¹⁴⁴

In another study, the activation parameters for the reaction of aquacobalamin with small anionic and neutral ligands were determined.¹⁴⁵ From the temperature dependence of the rate constants, activation enthalpies and entropies were determined for the reaction of aquacobalamin with SCN^- , $\text{S}_2\text{O}_3^{2-}$, NO_2^- and HSO_3^- . These parameters, as well as those determined previously for CN^- , HCN , N_3^- and HN_3 , demonstrated that there are very small differences that exist between most small anionic and neutral ligands in their reaction with B_{12a} . There were also exceptions, though; CN^- and SO_3^{2-} had small and large values for $\Delta H^\ddagger$ and $\Delta S^\ddagger$, respectively. These effects were attributed to a possible nucleophilic participation of these ligands in the transition state of the reaction.¹⁴⁵

Ligand substitution reactions of aquacobalamin with primary amines have also been investigated.¹⁴⁶ Enthalpies and entropies of activation were determined from the temperature variation of the spectrophotometrically determined pH-independent rate constants for the reaction of nine neutral primary amines with aquacobalamin in aqueous solution. There were compensating changes in $\Delta H^\ddagger$ and $\Delta S^\ddagger$ for this series of ligands, with $\Delta H^\ddagger$ decreasing from approximately 81 to 58 kJ mol⁻¹ as $\Delta S^\ddagger$ decreased in parallel from approximately 46 to -46 J K⁻¹mol⁻¹. It was also reported that there was no isokinetic relationship for the series. Plots of $\Delta H^\ddagger$ and $\Delta S^\ddagger$ against $\text{p}K_a$ revealed that the ligands fall into two distinct classes. Ligands in the first class have significantly larger $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values than those in the second class. This was ascribed to ligands in the second class interacting by hydrogen bonding to the acetamide side chains of the corrin ring thus allowing their amino groups to be favorably orientated for interaction with the metal ion.¹⁴⁶

The rate determining step (RDS) for a purely dissociative process is the first step. Thus the overall rate of the reaction should be sensitive to the nature of the leaving group (X), but insensitive to the entering group (Y). Neither purely associative nor dissociative mechanisms are generally found. The difference between I_a and I_d is the degree of bond formation to the entering ligand, Y. If bond making is more important in transition state 2 then the mechanism is I_a . Conversely, if bond breaking is more important then the mechanism is I_d .¹⁴⁷

The kinetics of the reaction of aquacobalamin with azide anion and hydrazoic acid, producing azidocobalamin, was investigated as a function of ligand concentration, pH, temperature and pressure, by stopped-flow spectrophotometry.¹⁹ The pH dependant rate constants and activation parameters were determined. However, after a concerted effort to try and explain the data obtained, it was not possible to distinguish between a limited dissociative and a dissociative interchange mechanism.¹⁴⁸

The kinetics of the reaction of aquacobalamin with *N*-donor ligands, glycine and methyl glycinate were investigated as a function of ligand concentration, pH and temperature at constant ionic strength by conventional and stopped-flow spectrophotometry.¹⁴⁹ The pH profile for the reaction with glycine was bell-shaped; this demonstrated that B_{12a} reacts only with deprotonated ligand and that hydroxocobalamin is kinetically inert. At 25 °C, it was reported that the ligands react at about the same rate, but the enthalpies and entropies of activation determined for glycine and methyl glycinate showed that this was coincidental and due to a compensating change in the two parameters.¹⁴⁹

The kinetics of substitution of bound H₂O in aquacobalamin by six different ligands, namely hydroxylamine, methyl glycinate, pyridine, 4-methylpyridine, imidazole and histamine were investigated as a function of ligand concentration and temperature by stopped-flow spectrophotometry at constant ionic strength and pH.¹⁵⁰ In all six cases the observed pseudo-first-order rate constants for the ionization of bound H₂O in B_{12a} showed saturation at high ligand concentrations after correcting for the protonation of the *N*-donor ligand. It was shown that there was a compensating change in the $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values of the saturating rate constant, k_{sat} , but there was no general isokinetic relationship for the ligands. It was concluded that the dependence of the value of the saturating rate constant k_{sat} , and specifically, the dependence of $\Delta H^\ddagger$ and $\Delta S^\ddagger$ for this rate constant, on the entering ligand indicated that the rate-limiting step in the reaction was not unimolecular release of H₂O from B_{12a}. The results were interpreted in terms of a dissociative interchange mechanism with the nucleophilic participation of the ligand in the transition state. The compensating effect was then explained on the basis of the extent of bond formation between Co and the ligand in the transition state. Molecular mechanics

techniques and molecular volume calculations were used to show that the steric bulk of ligands played an important role in controlling its rate of reaction at the metal centre.¹⁵⁰

The position of hydroxide in the thermodynamic *trans* influence order of the cobalt corrinoids were investigated.¹⁵¹ Diaquacobinamide (DAC) was prepared by reduction of aquacyanocobinamide with zinc and oxidation using aqueous HCl. The macroscopic acid dissociation constants pK_{Co1} and pK_{Co2} for coordinated H_2O in DAC were found spectrophotometrically to be 5.91 ± 0.04 and 10.30 ± 0.24 at $25^\circ C$; from their temperature dependence it was found that $\Delta H = 45 \pm 4$ and $27 \pm 2 \text{ kJ mol}^{-1}$, and $\Delta S = 40 \pm 14$ and $-109 \pm 8 \text{ J K}^{-1} \text{ mol}^{-1}$, for pK_{Co1} and pK_{Co2} , respectively. The equilibrium constants for the reaction of cobinamide with cyanide were determined in the pH range 8-12 at $25^\circ C$, $\mu = 1.0 \text{ M (NaClO}_4\text{)}$. It was reported that two CN^- ligands bind to aquahydroxocobinamide and the pH dependence of the observed equilibrium constants could be accounted for on the assumption that both dihydroxocobinamide and hydroxocyanoamide were inert, while the equilibrium constants for the reaction of the labile species aquahydroxocobamide and aquacyanocobamide with HCN were small compared to those for the reaction with CN^- anion. A fit of the data showed that the macroscopic equilibrium constant, $\log \beta_2$, for the reaction of 2 CN^- with the two diastereomers of aquahydroxocobinamide to produce dicyanocobinamide is 19.0 ± 0.1 . Thus the macroscopic $\log K$ for the binding of CN^- to the two diastereomers of aquahydroxocobinamide was 11 since $\log K$ for the binding of CN^- to aquacyanocobamide is 8. The equilibrium constants for the binding of azide, pyridine, *N*-methylimidazole and 3-amino-1-propanol to aquahydroxocobinamide were determined at $25^\circ C$, $\mu = 1.0 \text{ M (NaClO}_4\text{)}$, pH 12. In these cases only a single ligand was bound; the macroscopic $\log K$ values were *N*-methylimidazole, 6.06 ± 0.04 ; 3-amino-1-propanol, 4.66 ± 0.04 ; pyridine, 4.19 ± 0.01 ; and azide, 3.45 ± 0.04 . A comparison of these results with others available showed that hydroxide was above water and 5,6-dimethylbenzimidazole, but below cyanide, sulfite and alkyl ligands in the *trans* influence order of the cobalt corrinoids.¹⁵¹

The kinetics of substitution of bound H_2O in aquahydroxocobinamide by cyanide, azide, pyridine and *N*-methylimidazole were investigated as a function of ligand concentration

and temperature by stopped-flow spectrophotometry at pH 12.0.¹⁵² The observed pseudo-first-order rate constants showed the onset of saturation with ligand concentration for all ligands. The relative insensitivity of rate on the identity of the incoming ligand suggests a dissociative intimate mechanism. Furthermore, the observation that the saturation rate constant, k_{sat} (and the activation parameters $\Delta H^\ddagger$ and $\Delta S^\ddagger$), depended on the identity of the ligands indicated that the incoming ligand participates in the transition state. This allowed the mechanism of the reaction to be identified as a dissociative interchange.¹⁵²

Further evidence for a dissociation interchange mechanism and factors controlling reaction rates in ligand substitution reactions of aquacobalamin were determined.¹⁵³ The substitution of H_2O in aquacobalamin by the anionic ligands I^- , $\text{S}_2\text{O}_3^{2-}$, NO_2^- , SCN^- and N_3^- were determined as a function of ligand concentration and temperature by stopped-flow spectrophotometry at constant ionic strength and pH. The observed pseudo-first-order rate constants saturated to a limiting value, k_{sat} , at high ligand concentration which is consistent with reactions proceeding through a dissociative activation pathway. The value of k_{sat} depended on the entering ligand and the activation parameters for k_{sat} , $\Delta H^\ddagger$ and $\Delta S^\ddagger$, were directly correlated [$\Delta H^\ddagger$ varied from 26 ± 1 (I) to 83 ± 4 (N_3^-) kJ mol^{-1} while $\Delta S^\ddagger$ varied from -92 ± 4 (I) to 94 ± 13 (N_3^-) $\text{J K}^{-1} \text{mol}^{-1}$]. This is inconsistent with a limiting dissociative (D) mechanism which requires k_{sat} , and hence $\Delta H^\ddagger$ and $\Delta S^\ddagger$, to be independent of the identity of the entering ligand. Thus the mechanism was found to be a dissociative interchange (I_d) mechanism. Furthermore, there was a direct correlation between $\Delta H^\ddagger$ (and hence $\Delta S^\ddagger$) and the total Mulliken population of the donor atom of the entering ligand, but there was no correlation between these activation parameters and the cone angle subtended by the ligand at the metal atom. These results suggested that for anionic ligands, in contrast with neutral N-donor ligands, electronic rather than steric effects were primarily responsible for controlling reaction rates. It was also concluded that for anionic ligands, which were capable of bonding through two different donor atom types the reaction, occurred primarily through the donor atom with the higher electron density.¹⁵³

The ligand substitution reactions of coordinated H_2O in aquacobalamin by OCN^- , SCN^- , SeCN^- , NO_2^- , $\text{S}_2\text{O}_3^{2-}$ and N_3^- in NaNO_3 medium were investigated.²⁵ In the preceding report,¹⁵² it was reported that the activation parameters for N-donor ligands investigated did not correlate to the Mulliken population; the activation parameters for these species correlated instead with the steric demands of the ligand at the metal ion. Thus it suggests that the steric demands can override the electronic effects. According to recent studies, it has now emerged that there is a potential problem with these suggestions. To date the ionic adjustor used in all these studies was KCl, because it is UV-transparent it allows standard combination glass electrodes to be used for the measurement of pH, and little spectroscopic evidence was found for the binding of Cl to Co^{III} . Following this another report has shown that at high chloride concentrations there is a significant retardation of reaction rates which is attributed to the formation of the more substitution-inert chloro complex. Although these findings do not invalidate the preceding conclusions reached concerning the ligand substitution reactions of aquacobalamin, i.e. there is now a general agreement that they proceed through an interchange mechanism under dissociative activation, however it brings into question any conclusions based on the absolute values of the rate constants and their activation parameters. In this study the previous suggestions have been reconsidered concerning the identification of the reacting atom of the entering ambidentate ligand. Attempts have been made to rationalize the nature of the outer-sphere or precursor complex formed between aquacobalamin and the incoming ligand. The second-order rate constants were corrected for pH effects, for the reactions have been determined as a function of temperature, which lead to the calculation of the activation parameters. The identity of the donor atom of the ambidentate ligands in the rate determining processes, k_{II} , was considered and the correlations found between $\Delta H^\ddagger_{k_{\text{II}}}$, the enthalpy of activation, and $\Delta S^\ddagger_{k_{\text{II}}}$, the entropy of activation, respectively. It was found that the Mulliken population on the donor atom, and the energy of the highest occupied molecular orbital (HOMO) had a σ symmetry. The parameters $\Delta H^\ddagger_{k_{\text{II}}}$ were strongly dependant on the HOMO energy, and more weakly dependant on the electron density on the donor atom, while the converse was true for $\Delta S^\ddagger_{k_{\text{II}}}$.¹⁵⁴

It is surprising to notice that the Co^{III} in vitamin $\text{B}_{12\text{a}}$ shows unusual kinetic lability. Co^{III} is a classical example of a kinetically inert metal ion, which is largely due to the high ligand field stabilization energy associated with its low spin d^6 electron configuration. It is now generally accepted that the ligand substitution reactions of vitamin $\text{B}_{12\text{a}}$ proceed through a dissociative interchange mechanism (I_d), despite earlier suggestions that the mechanism is strictly dissociative (D).¹⁵⁵ According to this study, the distinction between a limiting D mechanism and an interchange I_d mechanism for an octahedral complex lies in one being to demonstrate directly or indirectly the existence of the five coordinate intermediate. This distinction becomes possible when the pseudo first-order rate constant shows saturation with the concentration of the incoming ligand. The kinetic results obtained for vitamin $\text{B}_{12\text{a}}$ were surprising for two reasons. Firstly, not only is the Co^{III} encapsulated by the small corrin ring (corrins, unlike porphyrins, have a direct linkage between the A and D ring which results in a smaller macrocyclic cavity) but also the substituents on the periphery of the corrin ring provide a formidable steric barrier to the incoming ligand. However, an I_d mechanism requires nucleophilic participation by the incoming ligand in the transition state. Secondly, the reactions are surprisingly fast for Co^{III} , which is the classical example of an inert transition metal ion. A comparison of representative second-order rate constants for the substitution of H_2O by ligands in Co^{III} complexes with four N-donor equatorial ligands showed that the approximate lability ratio of the metal ion towards substitution in corrin, porphyrin, cobaloxime and ammine systems was $10^9 : 10^6 : 10^4 : 1^8$. According to the findings this showed clear evidence for a *cis* labilising effect of the corrin ring, which was suggested to be a consequence of the electronic properties of the *cis* ligand that have a direct and marked effect on the kinetics of the ligand binding properties in the axial position. This paper aimed to address these two questions. Firstly, investigate the ability of the corrin ring to perturb the properties of the metal ion, thus usual inertness of the $d^6 \text{Co}^{\text{III}}$ had been modified by replacing the H atom at C10 with an electron withdrawing group like a nitroso group. This compound called 10-nitrosoaquacobalamin was then characterized by NMR and UV-vis spectroscopy and by FAB-MS. Secondly, the steric bulk of the departing ligand was increased by making it iodide. Following this, the kinetics of its substitution by three representative ligands, N_3^- , $\text{S}_2\text{O}_3^{2-}$ and imidazole were investigated, to determine whether

an increase in the bulk of the departing ligand was sufficient to switch from the I_d to the D mechanism. The pK_a and activation parameters were determined and compared to B_{12a} . It was found that the electron withdrawing NO group deactivated the metal ion towards ligand substitution, and that neither pyridine nor N_3^- caused a displacement of the coordinated H_2O , and it was concluded that either the reactions were very slow or the $\log K$ had been decreased by at least 1 and 4 orders of magnitude for the coordination of these two ligands, respectively. Hence, it was reported that the electronic structure of the corrin ring could directly influence the axial ligand binding properties of the metal.¹⁵⁵

Ligand substitution reactions of the vitamin B_{12} analog cyanoimidazolyl-cobamide, CN(Im)Cbl, with cyanide were studied.¹⁵⁶ Cyanide substitutes imidazole (Im) in the α -position more slowly than it substitutes dimethylbenzimidazole in cyanocobalamin (vitamin B_{12}). The kinetics of displacement of Im by CN^- showed saturation behaviour at high cyanide concentration; the limiting rate constant was found to be 0.0264 s^{-1} at $25\text{ }^\circ\text{C}$ and is characterised by the activation parameters: $\Delta H^\ddagger = 111 + 6\text{ JK}^{-1}\text{ mol}^{-1}$, $\Delta V^\ddagger = +9.3 + 0.3\text{ cm}^3\text{ mol}^{-1}$. These parameters are interpreted in terms of an I_d mechanism. The equilibrium constant for the reaction of CN(Im)Cbl with CN^- was found to be $861 + 75\text{ M}^{-1}$, which is significantly less than that obtained for the reaction of cyanocobalamin with CN^- which was reported to be $0.99 + 0.05\text{ M}^{-1}$, which is about 0.9 pH units higher than that obtained previously in the case of cyanocobalamin.¹⁵⁶ According to the above studies it seems that Co(III)'s lability is conferred on it by the corrin ring.

1.8 Comparison of Porphyrin and Corrin

1.8.1 Structural and Functional

Despite their different functions, the structures of porphyrin and corrin are quite similar. The porphine ring has D_{4h} symmetry, with four pyrrole rings connected by methine bridges. As can be seen in Figure 1.17, the only two things that distinguish the two ring systems are the absence of one of the four methine bridges in the corrin, a feature that lowers symmetry to C_{2v} , and ten saturated carbon atoms at the periphery of the corrin ring, which eliminates the conjugation of the outer part of the ring and lowers the symmetry to C_1 .¹⁵⁷ An important aspect of the corrin ring, when compared to the porphyrin, is the relative flexibility of the corrin system. The corrin ring is also less flat when viewed from the side than is a porphyrin ring. This adds up to some considerable differences between the chemistry of a cobalt porphyrin and a cobalt corrin. In addition, the corrin only has a conjugated chain around part of the ring system, whereas a porphyrin is delocalised around the whole four pyrrole rings.

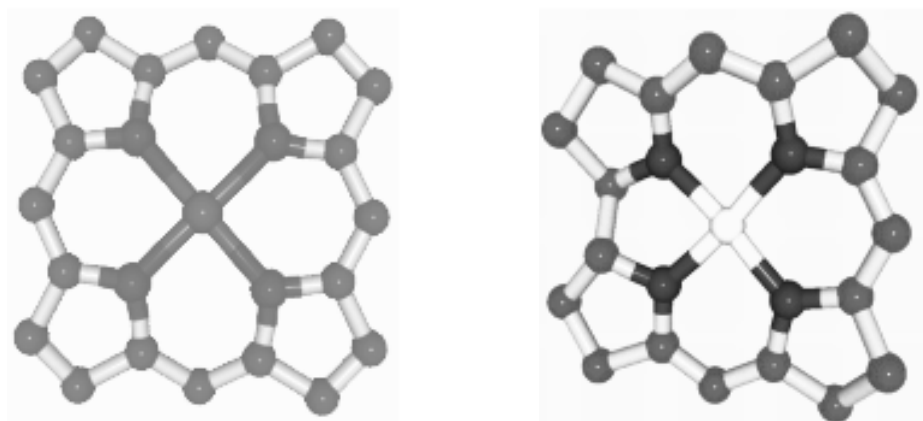


Figure 1.17: The porphyrin (left) and corrin (right) ring systems.¹⁵⁷

It is quite clear that nature seems to have a preference for iron as the central metal ion in porphyrin cofactors, whereas cobalt is normally found in corrins. Among the first row transition metals, cobalt has the lowest abundance in sea water together with scandium,⁷ but it is still present in the ubiquitous coenzyme B₁₂. Cobalt resides between iron and

nickel in the first row of the d-block. It has common oxidation states of +2 and +3, as does iron, but cobalt may even be reduced to a formal oxidation number of +1 *in vivo*, a property that iron does not possess.¹⁵⁸ On the other hand, iron porphyrin complexes are well known for their accessible high-valency states (formally Fe^{+4} and Fe^{+5}); the first plays an important role in the function of the haem oxidases such as P450 (see section 1.4.3).¹⁵⁹

The corrins exist in nature in the form of cobalamins. The B_{12} coenzymes contain a corrin ring with a d^6 low-spin Co^{+3} ion in their octahedral resting states. In most cobalamin-dependant enzymes, the imidazole side chain of the histidine residue coordinates to the cobalt ion. In another group of enzymes, the cobalt binds to the pendant dimethylbenzimidazole group of the coenzyme, the properties of which are quite similar to those of an imidazole ligand.¹⁶⁰ The second axial site is occupied by a methyl or 5'-deoxyadenosyl group; these form an organometallic Co-C bond. This bond is broken during the catalytic cycle to form either a five-coordinate Co^{+2} intermediate and an adenosyl radical, or a four-coordinate Co^{+1} ion, where the imidazole ligand has dissociated and the methyl group has been transferred to a nucleophilic substrate.^{161, 162}

Haem enzymes show a larger variation in their axial ligands (His, Cys, Met, Tyr, Glu, Asp, amino terminal, or exogenous ligands), depending on their function.¹⁶² The haem group can be either five-coordinate with an open coordination site where a substrate binds, or six-coordinate with one or more ligands from the protein. However, the most common ligand is a histidine/imidazole group, as is present, for example, in myoglobin, haemoglobin, peroxidases, haem oxygenase and most types of cytochromes.⁴

1.8.2 Chemical Properties

Recently, density functional calculations have been used to compare various geometric, electronic and functional properties of iron and cobalt porphyrin and corrin species.¹⁵⁷ The aim of the study was to investigate how the chemical properties of cobalt and iron, as well as porphyrin and corrin, differ. Specifically, the authors wanted to understand why

cobalt is associated with corrins and iron with porphyrins in nature. This project aims to further investigate this observation. The investigation was concentrated on one typical reaction for each of the two coenzymes, namely, the breaking of the Co-C bond as it is a typical reaction example of coenzyme B₁₂ metabolism, and electron transfer as it is a typical reaction of the haem-containing cytochromes.¹⁵⁷

Others have addressed similar questions.^{7, 23, 163-165} These investigations suggested that corrin was selected to fit the smaller Co^{III} ion.^{7, 23, 163} Others have emphasised the flexibility of the corrin ring as an important factor in the labilisation of the Co-C bond.^{165, 166} Williams *et al.* have proposed that the low spin Co^{II} is unique among the available first-row transition metal ions to provide a stable direct one electron radical.¹⁶⁸ On the other hand Pratt *et al.* have attributed the choice of cobalt to the low 3d to 4s/4p promotion energy of Co^{II}, which gives strong Co-C bonds, because the 3d orbitals are too small to form strong covalent bonds with carbon.^{23, 163} Specifically, the mechano-chemical trigger mechanism has been a major argument in favour of a specialised function of corrin systems, based on release of strain energy during catalysis. However, recent experimental¹⁷⁰⁻¹⁷³ and theoretical^{173, 174} results have indicated that such a conformational change is unlikely to drive a catalytic reaction within corrins. Finally, Rovira *et al.* compared the geometric and electronic structure of four-coordinate cobalt complexes with the corrin and porphine by using theoretical calculations.¹⁶⁷ The results show that the excitation energy associated with $d_{x^2-y^2}$ occupation is much higher in corrins than in porphyrins.

Jensen and Ryde have carried out an extensive comparison of the properties of corrins and porphyrins using density functional calculations.¹⁵⁷ During recent years, such methods have successfully been applied to the study of both iron porphyrin species¹⁷⁵⁻¹⁸⁷ and coenzyme B₁₂ models.^{167, 174, 188-192} Theoretical methods have the advantage of being cheap and fast while giving ‘pure’ results (well defined reactions in vacuum). On the other hand, solvation effects and free energies are hard to describe in a consistent way, and the accuracy is limited. This investigation was focused on octahedral M^{II/III} complexes (where M is the metal) with two axial imidazole ligands (as a model of *b* and *c* type cytochromes) or with one imidazole and one methyl ligand (as a model of

methylcobalamin). In addition, five-coordinate M^{II} complexes with imidazole ligand and four-coordinate $M^{I/II}$ complexes without any axial ligands as models of the intermediates in the reaction cycle of coenzyme B_{12} were investigated.¹⁵⁷

As this project is not concerned with density functional theory and how these results are arrived at, we will focus merely on the final conclusions reached in their investigation.

1.8.2.1 Spin-splitting energies

Several authors have suggested that the porphine and corrin ligands were selected to make low-spin (LS) states available for iron and cobalt, because the natural amino acid ligands provide too weak a field to drive iron or cobalt into the LS state.^{7, 168, 193} However, this is probably most important for Co^{II} , for which the vast majority of ligands give rise to a high-spin (HS) state.⁷ Moreover it has been suggested that the porphyrin was selected to keep iron centres close to the crossover point between LS, intermediate-spin (IS) and HS states.¹⁶⁸

In order to confirm these suggestions and to compare the relative strength of the porphine and corrin ligand fields and the intrinsic preferences of iron and cobalt, an investigation was performed to study to differences between the LS, IS and HS states of the octahedral imidazole (Im) and methyl (Me) complexes (on both the M^{II} and M^{III}) and the square-pyramidal imidazole complexes (only M^I), with all four combinations of Fe/Co and Por/Cor.¹⁵⁷

Table 1.5: The energy difference (kJ mol^{-1}) between the low spin and intermediate-spin states of [MCor/PorImMe] complexes.¹⁵⁷

Macrocycle	Fe^{II}	Co^{II}	Fe^{III}	Co^{III}
Porphyrin	39	78	46	82
Corrin	35	*(a)	92	139

*(a) No stable octahedral minimum for the quartet state.

It was found that only one octahedral Im/Me complex was stable in the HS state, [Fe⁺²PorImMe]. All the other complexes lost the imidazole ligand during optimisation. The same applies also to the IS [Co⁺²CorImMe] complex. It is important to note that Co^{II} has seven electrons and therefore does not have a sextet state comparable to the high state quintet state of Fe^{II}. Thus, only the energy difference between the LS and IS states is presented in table 1.5.¹⁵⁷

For these octahedral Im/Me complexes, all combinations of metals and ring systems gave rise to an LS ground state, in accordance with experimental results for the cobalt corrin species.¹⁹⁴ The IS states were 46-139 kJ mol⁻¹ higher in energy for the M^{III} complexes. The difference was approximately 40 kJ mol⁻¹ larger for Co than for Fe and also about 50 kJ mol⁻¹ larger for Cor than for Por. The results in this report confirmed that both Cor and Por gave rise to strong fields and that Fe and Por produced complexes quite close to the spin-crossover point.¹⁵⁷ Although the splitting energies of four- and five-coordinate complexes are reported and interesting, we will not mention the four-coordinate complexes, as they are not relevant to our project. However, overall the spin-splitting energy results indicated that cobalt and corrin favour the LS state, whereas Fe and porphyrin produced a small splitting between the various states.

1.8.2.2 Thermodynamic stability

Considering the *in vivo* abundance of iron porphyrins and cobalt corrins, it is natural to address the relative thermodynamic stability of these complexes. Stability considerations may explain why iron forms biological complexes with porphyrin rather than corrin and why the opposite is true in the case of cobalt. The results from the investigation suggested that the thermodynamic stability favours the two native combinations of ions and ring systems by 8-24 kJ mol⁻¹ over the non-native combinations for all complexes, except the M^{II} with Im/Me complexes. Thus it was reported that there was some intrinsic thermodynamic reason to choose the native combinations.¹⁵⁷

1.8.2.3 Geometries and the size of the ring cavities

It has been suggested that Co^{III} prefers the corrin cavity because it is smaller.^{7, 23, 163, 195} The metal-ligand distances of the optimised Im/Me and Im₂ models were collected in Table 1.6 and 1.7, respectively. The results confirmed suggestions made in earlier studies that the central cavity of the corrin ring is smaller than that of the porphine ring. Therefore, the corrin ring favoured the low-spin states of Co^{III} and Co^{II} , and even the four-coordinate low-spin Co^{I} ion which fits into the corrin ring. Thus, CoCor species are always low spin and all reactants and intermediates in the cobalamin reaction cycles involve small strain in the ring. On the other hand, the cavity in the porphine was too large for low-spin cobalt and iron ions. However, it seemed to be more appropriate for intermediate-spin states. Consequently, intermediate- and high- spin states are available for the FePor species, and they are important for many biochemical reactions.¹⁵⁷

Table 1.6: Metal-ligand bond distances [pm] of the optimised [MCor/PorIm/Me] complexes in their low-spin ground states.

Structure	M-C	M-N _{Im}	M-N _{eq. av}
[Fe ^{II} PorImMe] ⁻	200.5	222.8	201.5
[Fe ^{II} CorImMe] ⁰	201.0	226.0	192.1
[Co ^{II} PorImMe] ⁻	195.2	220.4	202.0
[Co ^{II} CorImMe] ⁰	195.9	225.2	192.8
[Fe ^{III} PorImMe] ⁰	198.9	224.9	201.8
[Fe ^{III} CorImMe] ⁺	199.6	229.0	193.3
[Co ^{III} PorImMe] ⁰	195.7	221.2	200.6
[Co ^{III} CorImMe] ⁺	196.6	225.0	192.0

Table 1.7: Metal-ligand bond distances [pm] of the optimised [MCor/PorIm₂] complexes in their low-spin ground states.

Structure	M-N _{Im1}	M-N _{Im2}	M-N _{eq. av}
[Fe ^{II} PorIm ₂] ⁺	202.4	204.5	204.5
[Fe ^{II} CorIm ₂] ⁰	206.0	209.1	193.5
[Co ^{II} PorIm ₂] ⁺	240.0	240.1	210.2
[Co ^{II} CorIm ₂] ⁰	247.3	251.0	192.1
[Fe ^{III} PorIm ₂] ⁰	201.2	202.6	202.6
[Fe ^{III} CorIm ₂] ⁺	204.7	204.8	193.5
[Co ^{III} PorIm ₂] ⁰	197.9	198.1	200.6
[Co ^{III} CorIm ₂] ⁺	199.5	201.6	192.5

1.8.2.4 Reduction Potential

Another possible reason for the selection of certain combinations of ions and rings is the differences in reduction potential between two metal ions and the two ring systems. Co^{III} in aqueous solution is more easily reduced than iron. However, the results indicated that in tetrapyrrole rings, this tendency was reversed, so that cobalt consistently gave potentials that are 0.1-0.3 eV lower than those for iron. Furthermore, there were also pronounced differences in the reduction potentials of the corrin and porphine rings, in that the former produced higher potentials. It was suggested that this was a result of differing charge on the ring system and therefore strongly dependant on solvation effects. As an effect of these two opposing tendencies, the native FePor and CoCor combinations often produced quite similar reduction potentials at high dielectric constants, but the latter had a higher potential in most solvents.¹⁵⁷

1.8.2.5 Reorganisation energies

The octahedral $[\text{Fe}^{\text{II/III}}\text{Por/CorIm}_2]$ complexes produced much lower inner-sphere reorganisation energies ($8\text{--}9 \text{ kJ mol}^{-1}$) than the corresponding $\text{Co}^{\text{II/III}}$ complexes ($179\text{--}197 \text{ kJ mol}^{-1}$). It was proposed that the reason for this was that the occupation of the dx^2 orbital in the low-spin Co^{II} (d^7), leads to a large difference in the distances to the axial ligands in the two oxidation states.¹⁵⁷ Therefore, it was concluded that the $\text{Co}^{\text{II/III}}$ couple is useless for electron-transfer reactions, whereas the octahedral $\text{Fe}^{\text{II/III}}$ complexes ($d^{5/6}$) formed excellent electron carriers, also in comparison with other metal sites.¹⁵⁷

1.8.2.6 Bond dissociation energies

The homolytic Co-C bond dissociation energy (BDE) for CoCor in coenzyme B_{12} was investigated. Equation 1.6 shows a typical reaction for CoCor in coenzyme B_{12} and it is the energy released in this equation we are determining.



Also the bond dissociation energy (BDE) of the hydrolysis of the six-coordinate Im/Me complexes was investigated, and is shown in Equation 1.7.



It was found that $[\text{Cor/PorImMe}]$ had the largest homolytic Co-C BDE of the studied complexes with a variation of 12 kJ mol^{-1} . It was suggested that this is compensated by the largest resistance towards hydrolysis. In addition, the cobalt complexes were more stable towards hydrolysis than the iron complexes (by approximately 40 kJ mol^{-1}). This is thought to explain why cobalamins are employed in nature for organometallic reactions.¹⁵⁷

The work reported in this article answers many questions and gives several good explanations for why iron is associated with porphine in nature whereas cobalt is

associated with corrin, and also why FePor species are used for electron transfer and oxygen activation whereas CoCor species are involved in organometallic Co-C reactions (methyl transfer and radical formation).¹⁵⁷

1.9 Scope and Objectives of the Research Project

To date, the Bioinorganic Research Group at the University of the Witwatersrand has done extensive research on the chemistry of haempeptides, particularly Fe^{III}-microperoxidase-8 (MP8) and Fe^{III}-microperoxidase-11 (MP11). The group has also done extensive research on the cobalt corrinoid derivative, Vitamin B_{12a}. One of the significant results is the demonstration that Co^{III} in cobalamins is labile. This project aims to explore aspects of the chemistry of *N*-Ac-Co^{III}-microperoxidase-8, a model compound in which Co(III) is coordinated in its equatorial plane by a porphyrin ligand and to compare the results with the extensive data available for vitamin B_{12a} and summarized above.

The specific objectives of the project are:

- Synthesise MP8 using the standard methodology the Wits Bioinorganic Research Group has developed.
- To remove Fe^{III} from MP8 using the reductive demetalation procedure adapted from Primus *et al.*⁷⁹
- To insert Co(III) into the porphyrin by treating with cobaltous acetate; this is followed by aerial oxidation.⁷⁹
- Developing conditions where *N*-Ac-Co^{III}MP8 is monomeric in solution. It has been anticipated that at the very least protection of the peptide *N*-terminus by acetylation will be required.
- Characterise the all the products leading to the synthesis of *N*-Ac-Co^{III}MP8 using a battery of techniques like UV-visible spectroscopy, HPLC (High Performance Liquid Chromatography), ICP-OES (Inductively Coupled Plasma – Optical Emission Spectroscopy), Luminescence spectroscopy and

FAB-MS (Fast Atom Bombardment – Mass Spectroscopy).

- To describe the solution chemistry of *N*-Ac-Co^{III}MP8 by determining the behaviour of Co^{III}-MP8 as a function of pH and ionic strength.
- Determine the feasibility of studying the kinetics and thermodynamics of ligand substitution reactions on Co^{III} ion.

This project will set the foundation for future work in understanding the unique chemistry of the porphyrin and corrin. After determining the Beer's Law behaviour and developing conditions where *N*-Ac-Co^{III}-MP8 is monomeric in solution, we would then be able to begin exploring, the hypothesis that the key to kinetic lability of Co^{III} in the corrin lies in the corrin ligand itself, i.e.; the equatorial ligand imparts lability on the axial coordination site of the metal ion. One way to test this hypothesis is to replace the equatorial macrocycle whilst maintaining the axial coordination site constant. Towards this end, the *N*-Ac-Co^{III}-MP8 fulfils these requirements:

- The metal is Co^{III}.
- The equatorial system features porphyrin rather than corrin.
- The axial ligand (His) is a close match, in so far as electronic effects go, to the axial ligand in B_{12a} (dimethylbenzimidazole).

Although it will take a long time until we fully understand these fundamental evolutionary antiques, therein lies opportunity for endeavours where thermodynamics of ligand coordination, kinetics of substitution processes, molecular modelling, reduction and self-exchange reactions, catalytic studies and various other studies on the solution chemistry of *N*-Ac-Co^{III}MP8 can be unravelled and the results compared to the extensive data available for the cobalt corrinoids. In the long term we should be in a better position to explain the origin of the kinetic lability of Co^{III} in the corrins, and have a better idea about the unique properties this ligand imparts on Co^{III}.

Chapter 2 Materials and Methods

2.1 Materials

Deionised water was produced by a Millipore RO unit, and further purified by reverse osmosis using a Millipore MilliQ Ultra-Pure system (18 M Ω cm). All glassware was washed thoroughly with liquid soap, rinsed with distilled water, rinsed with commercial acetone and dried in an oven set at 100 °C. The reagents used are listed in Table 2.1.

Table 2.1: Materials used in this study.

Reagent	Supplier	Grade
Acetic Acid	BDH	AR
Acetic Anhydride	Merck	GR
Acetone	Merck	CR
Acetonitrile	Agros-Organics	AR
Ammonia	Merck	GR
Ammonium hydrogen carbonate	BDH	AR
Ammonium sulphate	Saarchem	AR
Argon	Afrox	AR
Cobaltous Acetate	Saarchem	AR
Cytochrome c	Seravac	BP
Helium Gas	Afrox	AR
Hydrochloric Acid	Saarchem	GR
Iron(II)chloride	Merck	AR
Liquid Nitrogen	Afrox	
Methanol	BDH	AR
Methyl Ammonium Hydrochloride	Sigma	AR

Reagent	Supplier	Grade
Nitrogen Gas	Afrox	AR
Nitric acid	Saarchem	GR
Pepsin	Sigma	CP
Phosphoric acid	Aldrich	AR
Potassium chloride	Aldrich	AR
Potassium dihydrogen phosphate	Merck	GR
Potassium hydrogen phthalate	Saarchem	GR
Potassium Permanganate	Sigma	AR
Sephadex G50	Sigma	Superfine
Silver chloride	Effective lab supplies Ltd	AR
Sodium chloride	Merck	CP
Sodium cyanide	Merck	GR
Sodium dithionite	Hopkin & Williams Ltd	GR
Sodium hydrogen carbonate	Merck	CP
Trypsin	Sigma	CP
Vitamin B _{12a} (hydroxocobalamin)	Roussel	BP

Key: **AR** = Analytical Reagent

CP = Chemically Pure

BP = Biologically Pure

CR = Commercial Reagent

GR = General Reagent

2.2 General Methods

2.2.1 pH Measurements

pH measurements were performed using a Metrohm 6.05 pH meter and a 6.0234 combination glass electrode. The instrument was calibrated against the standard buffers potassium hydrogen phthalate (pH 4.004), potassium dihydrogen phosphate/dipotassium hydrogen phosphate (pH 6.863) and borax (pH 9.183) at 25.0 °C. The acid and base that were used to adjust the pH were H_3PO_4 and NH_3 , respectively. After calibration, the pH was tested with standards of known pH.

2.2.2 Buffers

The buffers used throughout this work are tabulated in Table 2.2.

Table 2.2: Common Buffers Used in Experiments.

Buffers	$\text{p}K_{\text{a}}$	pH Range
Ammonium hydrogen carbonate	7.8	6.8 – 7.0
CHES	9.3	8.6 – 10.0
MES	6.1	5.5 – 6.7
Tris/HCl	8.1	7.0 – 9.0
MOPS	7.2	6.5 – 7.9
Multi-component buffer (CHES, MES, Tris, MOPS, Potassium hydrogen phthalate)	-	4.0 – 10.0

2.2.3 Concentration and Desalting of Products

The concentrating and desalting of the various products formed during the synthesis of NAc-Co^{III}MP8 was performed using a 50 ml Amicon stirred cell connected to a nitrogen cylinder under *ca.* 70 psi pressure (0.45 μ m, 45 mm). Each sample was concentrated by allowing it to stir until a minimal volume of sample was left in the cell. When the samples were desalted, additional deionised water was added to the concentrated sample and the process repeated until all salts were removed. To test for the presence of salts, 0.5 ml of 0.1 M NaCl was added to the sample initially and the presence of chloride ions in the eluent was monitored using AgNO₃. It was assumed that if all chloride ions were washed out then all other salts present were also washed out or were present in very negligible concentrations. Desalting continued until the eluent test for Cl⁻ ions was negative.

2.2.4 Gel Permeation Chromatography

Gel permeation chromatography separates molecules on the basis of their size. The smaller molecules enter pores (with specified size) in the solid phase, while the larger molecules travel in the mobile phase and are eluted first. Sephadex G50 was used in a 3 x 40 cm column allowing small molecules (between 1 500-30 000 kDa) to pass through the pores in the solid phase and the larger molecules (> 30 000 kDa) to travel in the mobile phase. The resin was degassed before packing. After packing, the column was equilibrated by passing (NH₄)₂CO₃ (0.1 M) at a flow rate of 10 ml h⁻¹ for at least 48 hrs. A Watson-Marlow 101U peristaltic pump was used for elution and the fractions were collected using an ISCO 1850 fraction collector.

2.3 Physical Techniques of Characterisation

2.3.1 High Performance Liquid Chromatography (HPLC)

HPLC was performed using a Phenomenex 5 μ micron (150 X 4.6 mm) C18 reverse phase analytical column, a Spectra-Physics SP8800 ternary gradient pump, a Linear UV/vis 200 detector (set at 410 nm), and a Varian 4290 integrator. 10 μ l samples were injected onto the column using a Rheodyne valve and eluted by a linear gradient elution shown in Table 2.3 below.

Table 2.3: Linear Gradient Elution Program.

Time	% 50 mM Phosphate Buffer/1 mM NaCN	% Deionised Water	% Acetonitrile
0	98	0	2
2	98	0	2
4	75	0	25
6	65	0	35
8	65	0	35
10	75	0	25
12	98	0	2
14	98	0	2

2.3.1 UV-vis Spectrophotometry

All UV-vis spectra were recorded on either a Cary 1E or a Cary 3E spectrophotometer, using quartz cuvettes with a path length of 1 cm. The cell compartment temperature was

kept constant with a water-circulating bath kept at 25.0 °C. The instruments were allowed to warm up for approximately 1 hour prior to ensure optimum performance.

2.3.2 Fast Atom Bombardment – Mass Spectroscopy (FAB-MS)

All products leading to the synthesis of NAc-Co^{III}MP8 were analysed using Fast Atom Bombardment-Mass Spectroscopy (FAB-MS). Figure 2.1 illustrates the proposed mechanism for the formation of molecular ions. FAB has been particularly useful in the analysis of polar biomolecules and natural products like those used in this project.

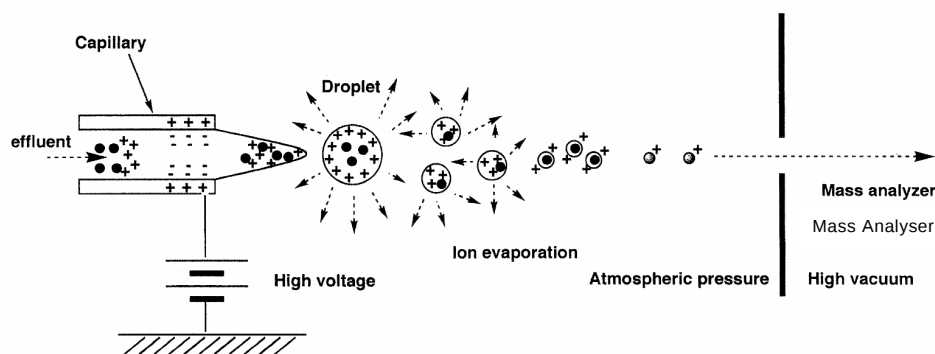


Figure 2.1: Ion formation from an electrospray ionisation source transferred to mass spectrophotometer.¹⁹⁶

The sample preparation involved dissolving the products in a minute amount of a nitro benzoic acid (NBA) matrix. This was then inserted into the VG70-SEQ mass spectrometer with a MASPEC II data system and bombarded with 8-15 keV Cs⁺ ions. The instrumental parameters are outlined in Table 2.4. Following ionisation, the selected positive or negative ions are extracted, accelerated, and then mass analysed. The low resolution FAB mass spectra are characterised by peaks corresponding to matrix cluster ions, analyte ions and ions representing impurities. The observed positive pseudo-molecular ion peaks are typically adducts formed with cations (i.e., [M+H]⁺ and [M+Na]⁺), whereas typical negative pseudo-molecular ions observed are [M-H]⁻.¹⁹⁶

Table 2.4: Instrument Settings - VG70-SEQ by Micromass.

Parameters	Settings
Polarity	Positive
Ionisation	FAB
Regulation	Field
Resolution	1000
Mass Range	3429 amu (7 kV)
Scan Rate	4 secs/decade (External)
Start Mass	2000
End Mass	100

2.3.3 Luminescence Spectroscopy

Solutions of MP8 free base and Co^{III}MP8 of arbitrary concentration were prepared for luminescence spectroscopy analysis. The respective solutions were put into a 1.0 cm pathlength luminescence cuvettes and the emission and excitation spectra were recorded using a Cary Eclipse luminescence spectrophotometer.

2.3.4 Determination of Extinction Coefficient using ICP-OES

Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) is a fast multi-element technique with a dynamic linear range and moderately-low detection limits (~0.2 - 100 ppb). The instrument uses an ICP source to dissociate the sample into its constituent atoms or ions, exciting them to a level where they emit light of a characteristic wavelength. Up to 60 elements can be screened per single sample run of less than one minute and the samples can be analysed in a variety of aqueous or organic matrices.

There is less chemical interference than with Fast Atom Absorption Spectroscopy (FAAS) but some spectral interference is possible and there are some element limitations.¹⁹⁶

In this project a Spectro-Ciros ICP-OES instrument was employed for the determination of extinction coefficient of *N*-AcCo^{III}MP8. It is assumed that the amount of cobalt detected is proportional to the concentration of *N*-AcCo^{III}MP8 once a blank correction is taken into account. *N*Ac-Co^{III}MP8 was accurately weighed out using a six figure Sartorius CP2P balance and transferred quantitatively into a 25 ml A grade volumetric flask kept at 25.0 °C, which was then filled to the mark with distilled water. A set of calibration standards are prepared using a 10 ppm Spectro-multielement stock solution. The calibrating solutions are sprayed into the plasma and standard curves obtained. The samples are then sprayed into plasma and the average concentration of cobalt determined using data from calibration standards and appropriate dilution factors.

2.4 Synthesis of products leading to the formation of *N*Ac-Co^{III}MP8

2.4.1 Preparation of Fe^{III}MP8

The synthesis of Fe^{III}MP8 was carried out according to the procedure developed by Munro and Marques¹⁰⁹ with some modifications and is based on the preparation of ferric MP8 described by Aron and co-workers.¹⁰² Ferric horse heart cytochrome *c* (300 mg) was treated with pepsin (15 mg; activity: 3200 units/mg) in 0.1 M phosphate buffer (10 ml) at pH 2.1 and at 35.0 °C. The reaction mixture was incubated for 1 hr, after which a further 15 mg of pepsin was added. The pH was adjusted to 2.1, and the solution was left for a further 3 hrs. The pH was then raised to 4 with concentrated NH₄OH, at which point the precipitation of MP11 was visible. Solid (NH₄)₂SO₄ was added to the stirred suspension on ice, until no more could be dissolved; saturated (NH₄)₂SO₄ solution was then added dropwise. The reaction mixture was centrifuged for 45 min at 7000-8000 rpm. The supernatant was decanted, and the MP11 pellet was dissolved in ca. 10 ml deionised water. Concentrated NH₄OH was then added to adjust the pH to 8.5 at 35.0 °C. Trypsin (15 mg; activity: 12700 units/mg) was then added and the solution was incubated

overnight under gentle stirring. MP8 was precipitated by lowering the pH to 3.5 with dilute H_3PO_4 . $(\text{NH}_4)_2\text{SO}_4$ was added as before, and the reaction mixture was centrifuged for 45 min at 7000-8000 rpm. The supernatant was decanted and the MP8 pellet was dissolved in 5 ml of deionised water, with dilute NH_4OH to ensure complete dissolution of MP8. The pH was readjusted to 7.00, desalted, and washed using an Amicon stirred cell.

2.4.2 Synthesis of demetalated MP8

The demetalation procedure was adapted from a previously published method developed by Primus and co-workers for the synthesis of Mn^{III} MP8.⁷⁹ During the process, care was taken to avoid direct exposure to light. The reaction was carried out using a vacuum line connected to an Edwards RV12 vacuum pump and an argon cylinder. While the stopcock between the argon line and the reaction vessel was open the following reactants were added to the reaction vessel: Fe^{III} MP8 (10.00 mg, 6.64 μmol); 10.00 ml glacial acetic acid; 166 μl concentrated HCl. The stopcock to the argon line was closed and the reaction mixture frozen with liquid nitrogen. After ensuring that the reactants were thoroughly frozen, the stopcock between the vacuum line and the reaction vessel was open for *ca.* 2-3 minutes and then closed. A Dewar flask containing liquid nitrogen was then removed from under the reaction vessel and the contents were allowed to thaw at room temperature. After the reactants completely thawed, the stopcock to the argon line was opened for about 30 seconds and closed again. The reaction mixture was allowed to undergo two more freeze-thaw cycles followed by purging with argon. After the final freeze-thaw cycle and after the reaction mixture had completely thawed, the reaction vessel was covered in aluminium foil ensuring that the reaction mixture was not exposed to white light. Using a spatula and an argon saturated powder funnel, iron(II)chloride (10.00 mg, 80.16 μmol , 12.07 equiv.) was added to the reaction mixture and was stirred with a magnetic stirrer for about 1 hour at room temperature, resulting in a change of color from red to purple. The foil-covered product was frozen in liquid nitrogen and stored in a freezer set at $-60\text{ }^\circ\text{C}$. The solvent was removed under reduced pressure on a vacuum line attached to an Acatel vacuum pump. The residue was dissolved in deionised

water and immediately re-evaporated in order to completely remove residual HCl. This washing was repeated twice and the final residue was solubilised in 5 ml of 50 mM NH_4HCO_3 (pH 8.00) and desalted with a Sepak C18 cartridge. The demetalated MP8 obtained was then lyophilised using a Virtis-UnitrapII freeze dryer.

2.4.3 Synthesis of Co^{III} MP8

The metalation procedure was adopted from a previously published method developed by Primus and co-workers for the synthesis of Mn^{III} MP8.⁷⁹ Demetalated MP8 was dissolved in 2 ml of water and allowed to reflux in a paraffin oil bath set at 95.0 °C. Cobaltous acetate (2.33 mmol, 2.33 M) was dissolved in 1 ml methanol was prepared before hand. 12 μl cobaltous acetate solution was then added to the reaction mixture containing demetalated MP8 (4.82 μmol). The reaction mixture was allowed to reflux for 3 hours. After the refluxing process the sample could be exposed to white light. The product was then loaded onto the Sepak cartridge and washed with copious amounts of water to remove salts and once all salts were removed the product was eluted with a 1:1 acetonitrile:water solution. The volume of the metalated product obtained was decreased using an Amicon stirred cell and then lyophilised.

2.4.4 Synthesis of $\text{NAc-Co}^{\text{III}}$ MP8

The acetylation procedure was adopted from a previously published method developed by Marques and co-workers for the synthesis of NAc-MP8 .¹⁰⁹ This involved the preparation of a 50 ml of 0.4 M carbonate buffer at pH 9.30. 6 mg of lyophilized Co^{III} MP8 was transferred into a round-bottomed flask. To this 20 ml of the carbonate buffer was added to dissolve the Co^{III} MP8. To aid dissolution approximately 500 μl of a 1 mM $(\text{NH}_4)_2\text{CO}_3$ was added. A 1000 molar excess of acetic anhydride was used to acetylate Co^{III} MP8 (it was assumed that there was a 100% conversion from cytochrome *c* to MP8). The Co^{III} MP8/carbonate solution was cooled to approximately 3.0 °C, and acetic anhydride was slowly pipetted into the reaction flask. Care was taken not to

allow the reaction to become too vigorous. The reaction flask was allowed to stand for 5 min at 3.0 °C after completely adding acetic anhydride. The temperature was set up to 35.0 °C and the solution was allowed to heat at the rate of the bath and allowed to remain 35.0 °C for 3 hours. Finally, the solution was purified and desalted an Amicon cell to ensure that all carbonate and acetate species were removed. The final product obtained was then lyophilised.

2.5 Investigating the Solution Chemistry of $\text{NAc-Co}^{\text{III}}$ MP8

2.5.1 Beer-Lambert Plot of $\text{NAc-Co}^{\text{III}}$ MP8

A concentrated stock solution of $\text{NAc-Co}^{\text{III}}$ MP8 was prepared in distilled water. Small increments of stock $\text{NAc-Co}^{\text{III}}$ MP8 solution were added to a 1.0 cm pathlength cuvette that contained distilled water. The absorbance at 415.05 nm at 25.0 °C for each increment was recorded using a Cary 3E spectrophotometer. After the absorbance reached 2.0 AUFS, 200 μl of the final 1.0 cm pathlength cuvette mixture was transferred to a 0.1 cm pathlength cuvette. Similarly, small increments of stock $\text{NAc-Co}^{\text{III}}$ MP8 solution were added to the 0.1 cm pathlength cuvette. The absorbance at 415.05 nm at 25.0 °C for each increment was again recorded using a Cary 3E spectrophotometer. A Beer-Lambert plot of $\text{NAc-Co}^{\text{III}}$ MP8 in 1.0 M NaOCl_4 was determined using the same methods.

It became clear that there was a discrepancy between the absorbance of solutions in the 1.0 cm and the 0.1 cm pathlength cuvettes, as far as the absorbance of the same solution; when corrected for different pathlengths, was different. We suspect (but have not proved) that this discrepancy arises from the scaling of the cuvettes in the housing of the spectrometer that give pathlengths that are, in effect, different to the actual pathlength of the cuvette. To correct for this, a solution of arbitrary concentration of KMnO_4 was prepared; 100 μl of the KMnO_4 solution was added to a 1.0 cm pathlength cuvette containing 1900 μl distilled water. The solution was mixed carefully using a glass rod. The absorbance at 337.9 nm at 25.0 °C was recorded using a Cary 3E spectrophotometer. This process was then repeated a further four times. 200 μl of each of the five solutions

from the 1.0 cm pathlength cuvettes were transferred to 0.1 cm pathlength cuvette and the absorbance for each of the solutions were recorded at 337.9 nm at 25.0 °C using a Cary 3E spectrophotometer. The mean correction factor for when transferring from the 1.0 cm to 0.1 cm pathlength cuvette was then calculated. Finally the all data collected were corrected accordingly.

2.5.2 Data Analysis

All results were analysed with the computer programme CurveFit¹⁹⁷, which employs a Newton-Raphson procedure.

2.5.3 Determination of Spectroscopic pK_a s of $NAc-Co^{III}MP8$

The titration procedure for the determination of spectroscopic pK_a s of $NAc-Co^{III}MP8$ was adopted from a previously published method developed by Munro and co-workers¹⁰⁹ for the synthesis of $NAc-Fe^{III}MP8$. Spectroscopic pK_a s were determined by titrating 40 ml of a 8.01 μM stock solution of $NAc-Co^{III}MP8$ in a multi-component buffer system containing 1.0 mM each of potassium hydrogen phthalate, MES, MOPS and CHES, and 10 mM Tris as the non-coordinating buffers at a total ionic strength of 0.1 M (KCl used as an ionic adjuster) in a thermostated cell at 25.0 °C. The pH was adjusted from pH 5.5 to 13 for the basic region and from 1.9 to 6.3 for the acid region by diffusion of small volumes of concentrated HCl or NaOH into the solution from drawn out glass capillaries. At each pH, 5 ml of the solution was pumped (using a Watson-Marlow 101U peristaltic pump) into a thermostated 2.0 cm pathlength cuvette in a Cary 3E spectrophotometer and the absorbance at 415.05 nm was recorded before returning the sample to the continuously stirred reaction mixture. The absorbance at 415.05 nm was recorded as a function of solution pH.

The experimental data were fitted using non-linear least-squares program employing a Newton-Raphson procedure to an ionisation isotherm (Equations 2.1 to 2.4), which is relevant when three acid/base equilibria are present.

$$A_T = \frac{A_1*[H^+] + A_2*[H^+]*K_1}{K_1 + [H^+]} \quad (2.1)$$

$$A_T = \frac{A_1*[H^+]^2 + A_2*[H^+]*K_1 + A_3*K_1*K_2}{K_1*K_2 + [H^+]*K_1 + [H^+]^2} \quad (2.2)$$

$$A_T = \frac{A_1*[H^+]^3 + A_2*[H^+]^2*K_1 + A_3*[H^+]*K_1*K_2 + A_4*K_1*K_2*K_3}{K_1*K_2*K_3 + [H^+]*K_1*K_2 + [H^+]^2*K_1 + [H^+]^3} \quad (2.3)$$

$$A_T = \frac{A_1*[H^+]^4 + A_2*[H^+]^3*K_1 + A_3*[H^+]^2*K_1*K_2 + A_4*[H^+]*K_1*K_2*K_3 + A_5*K_1*K_2*K_3*K_4}{K_1*K_2*K_3*K_4 + [H^+]*K_1*K_2*K_3 + [H^+]^2*K_1*K_2 + [H^+]^3*K_1 + [H^+]^4} \quad (2.4)$$

In Equations 2.1 to 2.4, A_T is the absorbance at the absorbance monitoring wavelength, K_1 to K_3 are the acid dissociation constants and A_i is the absorbance of the i^{th} species.

2.5.4 Ligand Binding Studies of Selected *N*-Donor Ligands by *N*Ac-Co^{III}MP8

The equilibrium constants for the coordination of the ligands *N*-methylimidazole and pyridine with *N*Ac-Co^{III}MP8 were determined by the addition of aliquots of stock *N*-methylimidazole solution (0.02012 M) and pyridine solution (0.02008 M) to *N*Ac-Co^{III}MP8, contained in a 1.0 cm pathlength cuvette in a Cary 1E spectrophotometer. The solutions were buffered with MES (1.0 M, pH 5.5); the ionic strength was maintained using KCl (1.0 M) and deionised water was used as the solvent. The temperature of the cell block was maintained at 25.0 °C by a water circulating bath and measured with an electronic thermostat device. The absorbance changes at 420.0 nm were monitored for both reactions, and each absorbance reading was corrected for dilution. The experimental data were fitted using non-linear least-squares program employing a Newton-Raphson procedure to Equations 2.5 and 2.6.

$$A_T = \frac{A_0 + A_1 * K_1 * [L]}{1 + K_1 * [L]} \quad (2.5)$$

$$A_T = \frac{A_0 + A_1 * K_1 * [L] + A_2 * K_1 * K_2 * [L]^2}{1 + K_1 * [L] + K_1 * K_2 * [L]^2} \quad (2.6)$$

In Equations 2.5 and 2.6, A_T is the absorbance at the absorbance monitoring wavelength, K_1 and K_2 are the formation constants, A_0 is the initial absorbance and A_2 is the final absorbance. After the formation constants were determined they were corrected for pH using Equation 2.7.

$$K = K_{obs}(1 + 10^{pK_a - pH}) \quad (2.7)$$

2.5.5 Kinetic Studies of Selected N-Donor Ligands by $N\text{Ac-Co}^{\text{III}}\text{MP8}$

Preliminary spectrochemical investigations were performed using a Cary 1E spectrophotometer. Using the same equipment, the kinetics of the reactions between the ligands *N*-methylimidazole and methylamine with $N\text{Ac-Co}^{\text{III}}\text{MP8}$ were investigated under pseudo-first order conditions. $N\text{Ac-Co}^{\text{III}}\text{MP8}$ concentrations ($< 45 \mu\text{M}$) were prepared and buffered with MES (0.1 M) at pH 7.0 and 8.0 for reactions involving *N*-methylimidazole and methylamine, respectively. The ligand concentrations of *N*-methylimidazole and methylamine ranged from 0.05 to 1.01 M and 0.50 to 2.50 M, respectively. Ligand solutions of *N*-methylimidazole and methylamine were also adjusted quantitatively to the same pH as that of the $N\text{Ac-Co}^{\text{III}}\text{MP8}$ solution by addition of HCl or NaOH, as appropriate. After mixing specific amounts of ligands with $N\text{Ac-Co}^{\text{III}}\text{MP8}$ in a 1.0 cm pathlength cuvette, and immediately inserting this mixture into the spectrophotometer, the absorbance change was monitored at 420.0 nm (the maximum of the Soret band for $N\text{Ac-Co}^{\text{III}}\text{MP8}$ at pH 5.5).

Pseudo-first order rate constants, k_{obs} , were determined by curve-fitting the absorbance versus time traces to Equation 2.8 and 2.9 using non-linear least-squares program employing a Newton-Raphson procedure. After the first order rate constants were determined they were corrected for pH using Equation 2.10.

$$A_T = -A_0 \cdot \exp(-k_{\text{obs}} \cdot t) - A_1 \cdot \exp(-k_{\text{obs}}' \cdot t) + A_0 + A_1 + A_2 \quad (2.8)$$

$$A_T = -A_1 \cdot \exp(-k_{\text{obs}} \cdot t) + (A_0 + A_1) \quad (2.9)$$

$$k = k_{\text{obs}}(1 + 10^{\text{pKa} - \text{pH}}) \quad (2.10)$$

In the equation A_0 , A_1 and k_{obs} are variable parameters. Second order rate constants, k_2 , were obtained from a linear least-square plot of k versus $[L]$.

2.5.6 Kinetic Studies of Selected N-Donor Ligands by B12_a

Similarly, preliminary spectrochemical experiments were performed using a Cary 1E spectrophotometer. Using the same equipment, the kinetics between *N*-methylimidazole and methylamine with B12_a were investigated under pseudo-first order conditions. A stock solution of B12_a (between 50 and 80 μM) was prepared and buffered with MES (0.1 M) at pH 7.0 and 8.0 for the reactions involving *N*-methylimidazole and methylamine, respectively. The ligand concentrations of *N*-methylimidazole and methylamine ranged from 1.06×10^{-4} to 2×10^{-3} M and 2.51×10^{-1} to 2.51 M, respectively. Ligand solutions of *N*-methylimidazole and methylamine were also adjusted quantitatively to the same pH as that of the B12_a solution by addition of HCl or NaOH, as appropriate. After mixing specific amounts of ligands with B12_a in a 1.0 cm pathlength cuvette, and immediately inserting this mixture into the spectrophotometer, the absorbance change for the reaction between *N*-methylimidazole and methylamine with B12_a was monitored at 358.0 and 352.0 nm (the maximum of the π -band). Similarly, the pseudo-first order rate constants, k_{obs} , were determined using Equation 2.9 as above and second order rate constants, k_2 , were obtained from a linear least-square plot of k_1 versus $[L]$.

Chapter 3 Results and Discussion

3.1 Synthesis and Characterisation of products leading to the formation of NAc-Co^{III}MP8

3.1.1 Preparation of Fe^{III}MP8

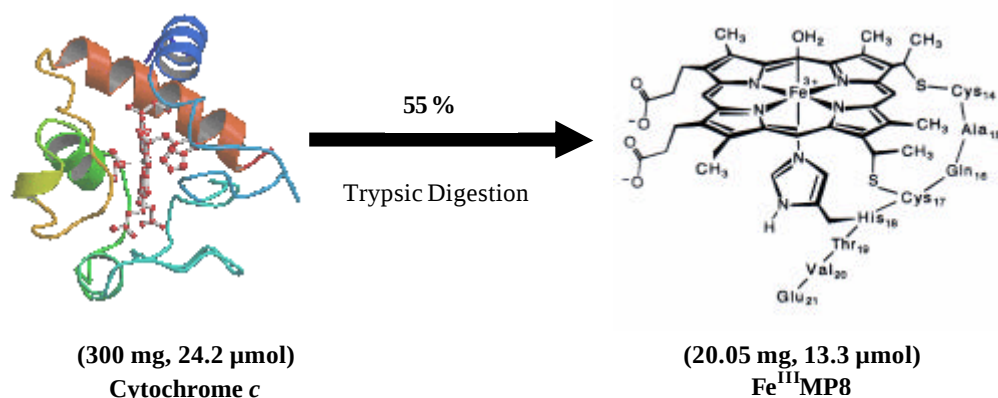


Figure 3.1: Proteolysis of cytochrome *c* to Fe^{III}MP8. The % conversion is calculated from cytochrome *c* to Fe^{III}MP8 and assumes 100 % purity of cyt *c*.

Although Fe^{III}MP8 is commercially available, it is very expensive. Using the procedure developed by Munro and Marques¹⁰⁹ with some modifications, Fe^{III}MP8, the haem-containing octapeptide was successfully prepared. Fe^{III}MP8 was then purified using size exclusion chromatography, where the reaction mixture containing the respective microperoxidases (MP11, MP9, MP8 and MP6) were columned on Sephadex G50 to separate them according to size. HPLC was used as an analytical tool to identify Fe^{III}MP8 fractions that were > 95 % pure (the assumption being that the response factor of all haempeptides at 410 nm is the same) and these fractions were pooled together. Fe^{III}MP8 was produced in 55 % yield as shown in Figure 3.1. Figure 3.2 shows a chromatogram of a fraction of Fe^{III}MP8 that was analysed using HPLC. The pooled fractions were desalted using an Amicon stirred cell and lyophilised. The yield is 5 % higher than that reported by Adams and coworkers.¹⁰² The synthesis of Fe^{III}MP8 by

peptic and tryptic digestion of cytochrome *c* was performed several times with reproducible yields.

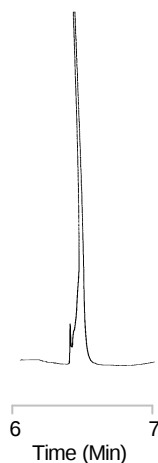


Figure 3.2: HPLC analysis of Fe^{III} MP8 (retention time of 6.45 minutes) with > 95% purity after purification on a column of Sephadex G50.

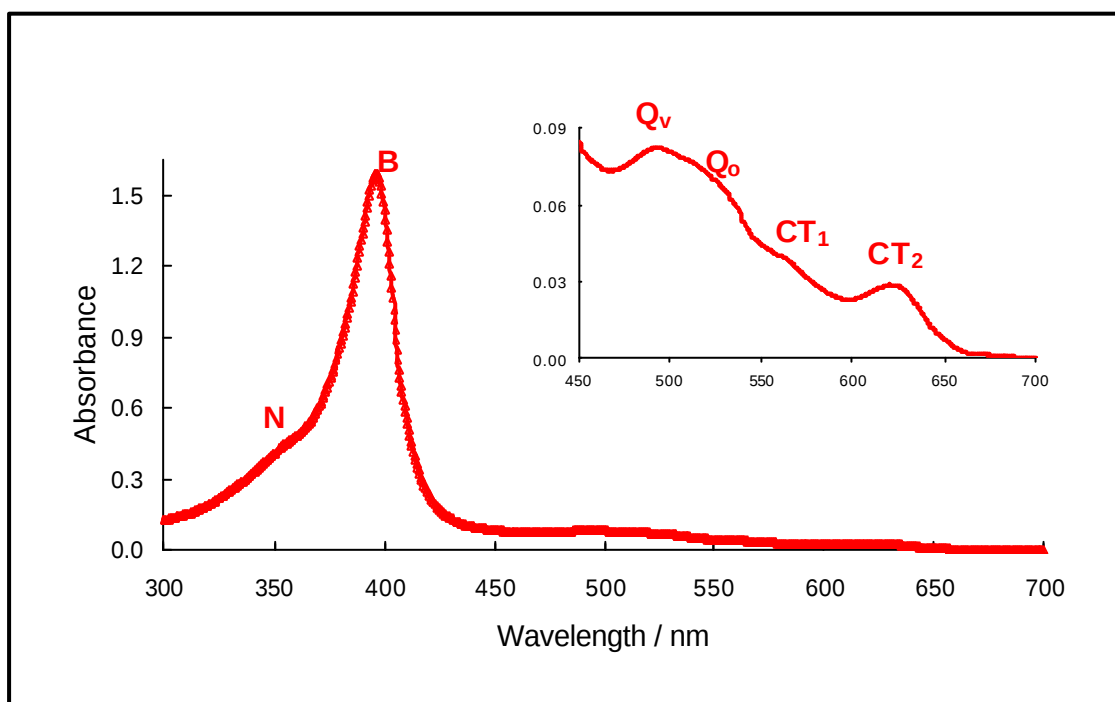


Figure 3.3: UV/visible spectrum of Fe^{III} MP8 in distilled water.

There are six characteristic peaks observed in the electronic spectrum of Fe^{III}MP8 shown in Figure 3.3. The first band occurs at 350 nm and is called the N-band and is assigned to the $a'_{2u}, b_{2u}(p) \rightarrow e_g(p^3)$ transition.¹⁵ The B and Q bands involve $a_{2u}, a_{1u}(p) \rightarrow e_g(p^3)$ transitions. These bands are not purely due to orbital transitions but arise as a result of the mixing of inter-electronic repulsion terms.¹⁵ The Soret band at 395 nm is thought to arise from a combination of transitions to two vibronic components. Usually, a B(1,0) satellite, arising from a transition to the first vibronic state of B electronic state, can be seen to the lower wavelength side of B band (about 20 nm separation between B and B(1,0) bands).^{15, 198} The Q₀ and Q_v bands observed at 491 nm and 530 nm, respectively, are due to different vibrational transitions of the same electronic transition. Q₀ is the (0,0) vibronic component of the $a_{2u}, a_{1u}(p) \rightarrow e_g(p)$ transition, whilst Q_v is the (1,0) vibronic component $a_{2u}, a_{1u}(p) \rightarrow e_g(p)$ transition. Q_v is more intense than Q₀ band as vibronic coupling with the B band enhances its intensity. The Q_v band is thought to arise from the vibrational electronic coupling between the B- and Q-states.^{15, 109, 198, 199} CT₁ represents a charge transfer band due to the spin-allowed porphyrin $a_{2u}'(p) \rightarrow e_g(dp)$ promotion; and the symbol CT₂ represents a charge transfer band due to the spin allowed porphyrin $b_{2u}(p) \rightarrow$ iron $e_g(dp)$ transition. These signals usually occur at 580 nm and 630 nm, respectively; however in this system these bands are observed at 567 nm and 622 nm. The overall trend is characteristic for ferric haem proteins and may be slightly compromised by contributions of both high and low spin components.¹⁵ Other $p \rightarrow p$ transitions at 215 nm and 325 nm do occur in absorption spectra of iron porphyrins but are obscured by the bands for the aromatic peptides, and hence are not observed in the haemproteins.²⁰⁰

FAB-MS has proven to be an extremely useful tool in determining structure of unknown haempeptides with respect to both the haem and the peptide portions, and in characterising the purity of bulk preparations of known peptides such as the microperoxidases. It has also been used to determine proteolytic hydrolysis sites for peptides derived from c-type cytochrome that contain covalently attached haem c.²⁰¹

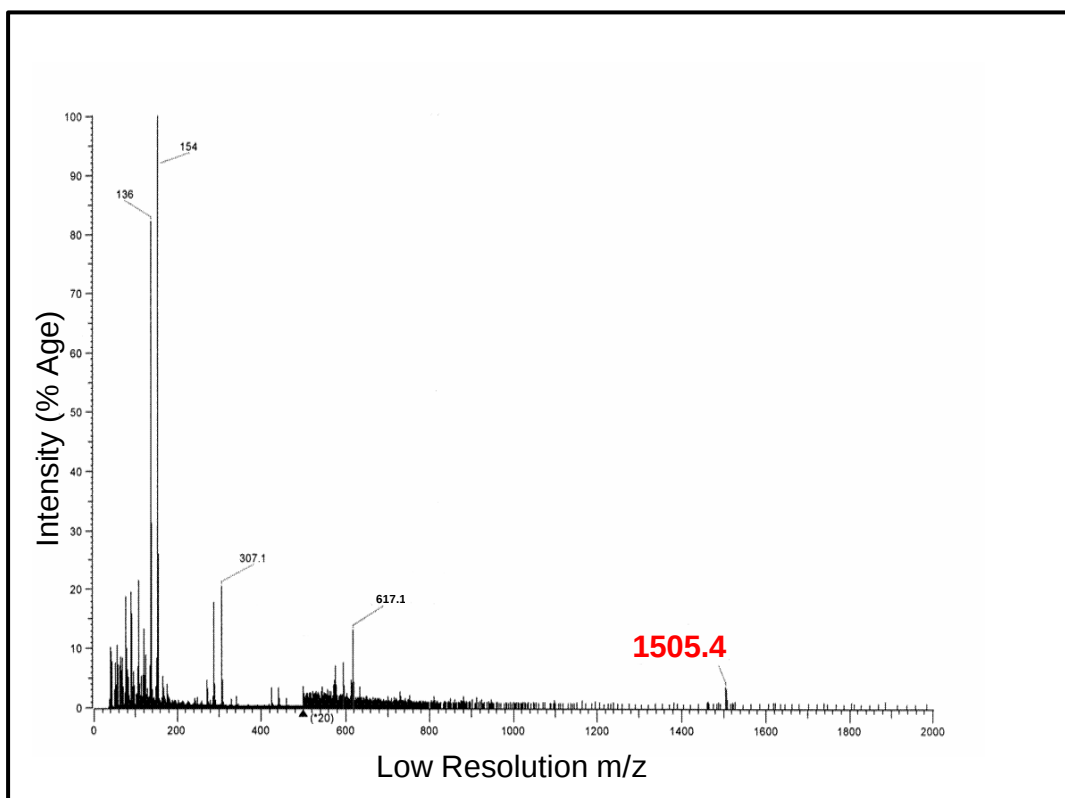


Figure 3.4: FAB-MS spectrum of Fe^{III} MP8 obtained from the proteolytic digestion of horse heart cytochrome *c* in NBA matrix.

A typical low resolution spectrum of the haem-octaepptide is presented in Figure 3.4. The exact mass determinations are in excellent agreement for the haem-octaepptide, Fe^{III} MP8, where the observed m/z is 1505.4 and the calculated value is 1506. An important observation was the presence of a very intense molecular ion at $m/z = 617$ in all Fe^{III} MP8 samples analysed. This ion corresponds to the fragmentation of haempeptide covalent bonds and further dehydrogenation/rearrangement to produce mono-protonated Fe^{III} protoporphyrin IX.²⁰¹ The observed m/z for the mono-protonated Fe^{III} protoporphyrin IX was 617.1 and the calculated 617.2. The above results confirm that Fe^{III} MP8 was prepared successfully with > 95 % purity with reproducible results.

3.1.2 Synthesis of demetalated MP8

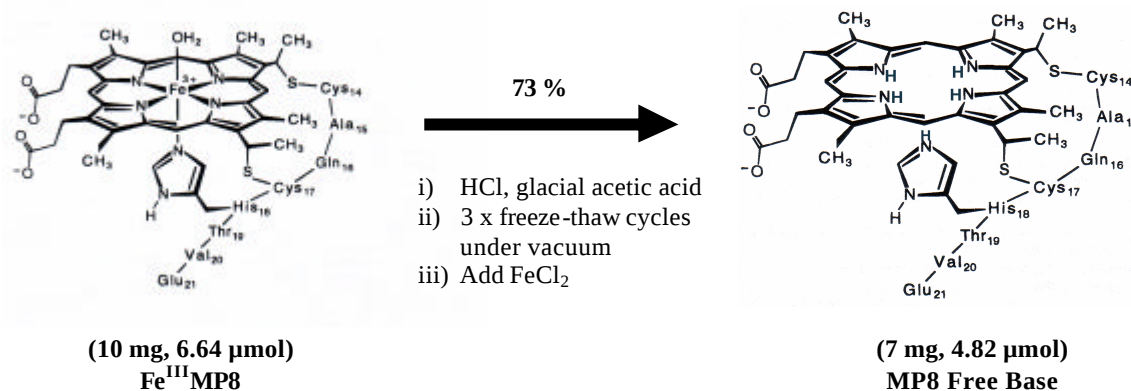


Figure 3.5: Reductive demetalation of Fe^{III}MP8 to MP8 Free Base. The % conversion is calculated from Fe^{III}MP8 to MP8 Free Base and assumes Fe^{III}MP8 is 100 % pure.

The method of preparation of MP8 free base was adopted from the procedure of Primus and co-workers which involves removing the central metal Fe^{III} ion by reductive demetalation.⁷⁹ The demetalation process was successful as the expected colour change from red to purple was observed⁷⁹ and MP8 free base was synthesised in 73 % yield shown in Figure 3.5.

The mechanism of reductive demetalation has not been clarified. We suggest that in acidic conditions as those provided, the two central nitrogen atoms are protonated competitively,²¹ and Fe^{III} ion is reduced to form Fe^{II} ion. It has been suggested that Fe^{II} ion is less tightly bound to a porphyrin than Fe^{III} ion; this can be attributed to Fe^{III} having a higher charge and therefore bonding more tightly to the highly electron dense porphyrin system. Thus, the Fe^{II} is thought to be forced out of the porphyrin macrocycle by competition from H⁺.

After the MP8 free base was desalted using Sepak cartridges, it was analysed using HPLC. The HPLC chromatogram is shown in Figure 3.6 and its UV-vis spectrum is shown in Figure 3.7.



Figure 3.6: HPLC chromatogram of MP8 free base (retention time of 5.99 minutes) with 81 % purity.

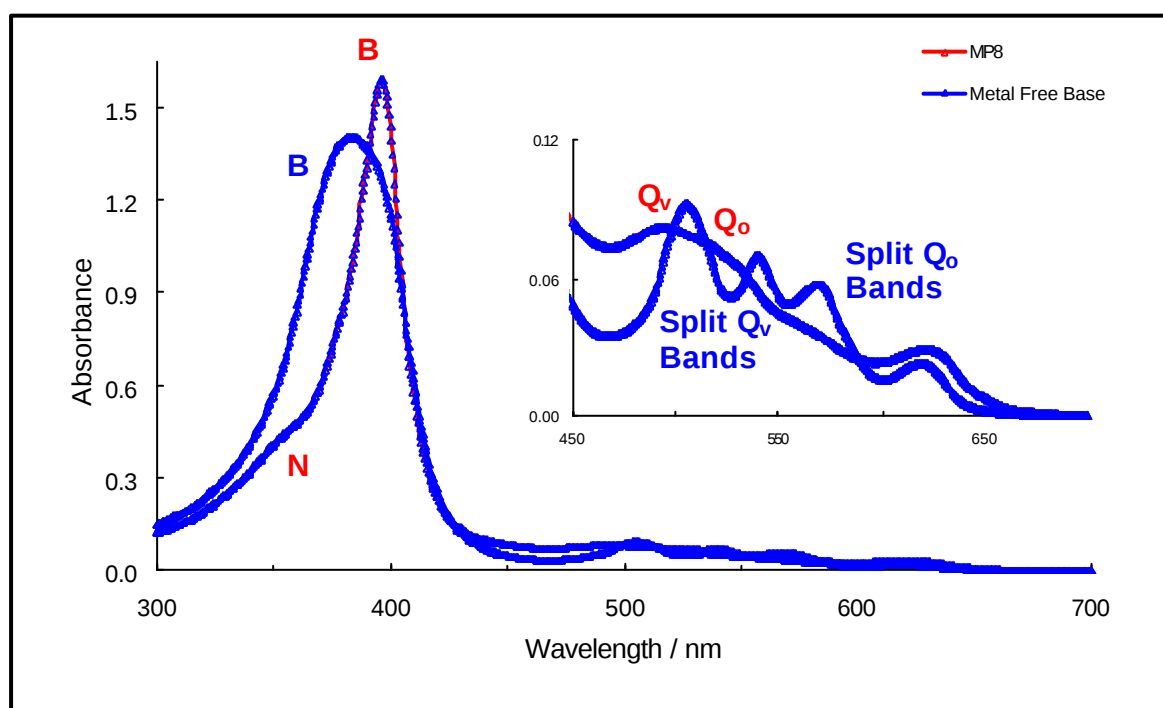


Figure 3.7: UV/visible spectrum of MP8 free base in 50 % methanol/water solution and Fe^{III}MP8 in water.

In demetalated porphyrins (D_{2h}), there are four bands in the visible region and one in the ultraviolet region (Soret).¹⁹⁸ These absorption bands for metal-free MP8 are observed at 380 nm, 503 nm, 538 nm, 568 nm and 618 nm as shown in Figure 3.7. The intense Soret band described earlier occurs at 380 nm. The Q_v band is split and the resulting bands occur at 503 nm and 538 nm, respectively. Also, the Q_o band is split and the resulting bands occur at 564 nm and 616 nm, respectively. When comparing the spectra of MP8 and MP8 free base we do not observe charge transfer bands since for a complex to demonstrate charge-transfer behaviour, one of its components must have electron donating properties and another component must be able to accept electrons. Since there is no metal present no charge transfer band will be observed.²¹ In addition, the N-band disappears completely which is a direct result of metal absence.

The UV/visible data obtained could not be compared with data obtained by Primus *et al.*⁷⁹ since their data were obtained under acidic conditions. Primus *et al.* found the Soret band at 403 nm and other bands at 552 nm and 595 nm which is typical for a free base porphyrin at low pH. In our results, the UV/visible analysis data were obtained under neutral conditions. However, our data can be compared to Low *et al.*²⁰² who found a very broad Soret band with a maximum at 383 nm as well as the other bands at 508 nm, 542 nm, 574 nm and 623 nm which are characteristic of free base porphyrins. Thus, the results obtained for UV/visible analysis are consistent with the characteristic peaks for a free base porphyrin, confirming that the demetalation procedure was successful. Furthermore, the retention time of 5.99 minutes observed for the free base porphyrin was significantly different from the observed retention time of 6.45 minutes for Fe^{III} MP8. In addition, the 81% purity detected by HPLC showed that the sample is relatively pure.

However, the overlap of multiple peaks in the chromatogram (Figure 3.6) means that the sample is relatively pure and that this technique is merely an assessment of purity is very much an estimate.

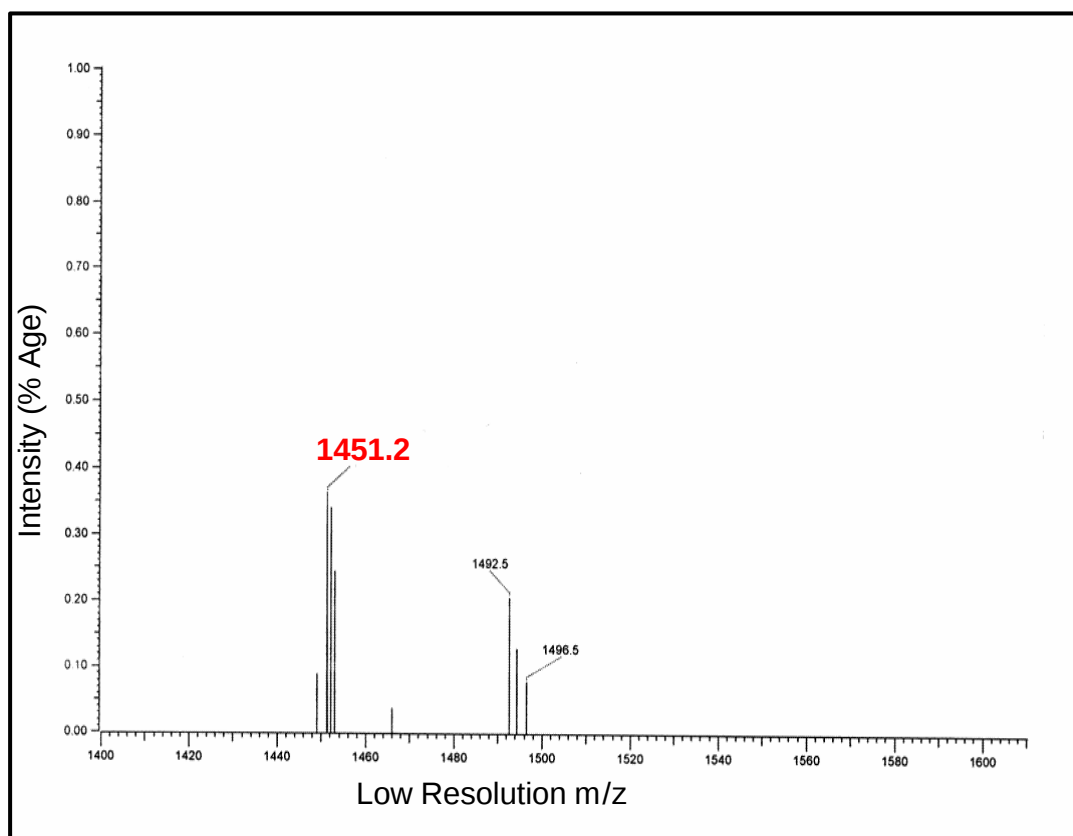


Figure 3.8: Enlarged view of FAB-MS MP8 Free Base in NBA Matrix.

The exact mass determinations for the MP8 free base shown in Figure 3.8 were also excellent and in agreement where the observed m/z was 1451.2 and the calculated 1451. We propose that the signal observed at $m/z = 1492.5$ are traces of calcium ions since the mass difference between this signal and the free base is ~ 41 m/z units. However, the other signal where $m/z = 1496.5$ cannot be explained.

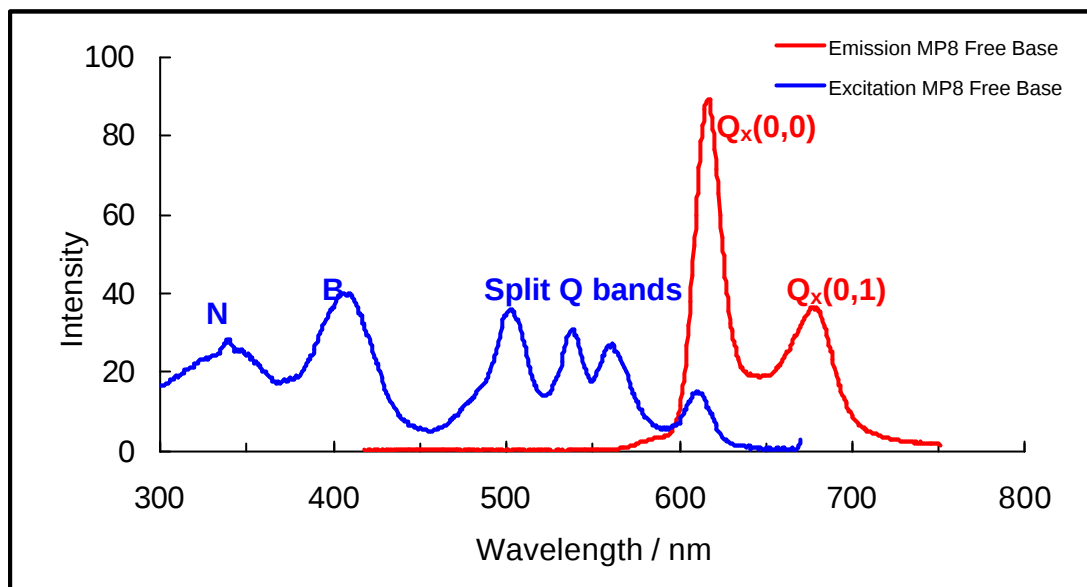


Figure 3.9: Luminescence Spectra of MP8 free base.

The excitation spectrum of MP8 free base drawn in blue on Figure 3.9 was recorded from 300 nm to 670 nm with emission $\lambda = 677$ nm. There were six bands observed, four in the visible region and two bands in the ultraviolet region. The bands at 343 nm and 410 nm are characteristic of the N-band and Soret band, respectively, while, the bands at 505 nm, 541 nm, 565 nm and 614 nm are the same split Q-bands we mentioned earlier. The emission spectra of MP8 free base was recorded from 418.5 nm to 750 nm with excitation $\lambda = 398.5$ nm. There are two bands observed in the visible region at 617.5 nm and 680 nm are characteristic of the transient triplet-triplet absorption observed in free base porphyrins and are assigned $Q_x(0,0)$ and $Q_x(0,1)$, respectively.²¹ The appearance of the Q_x bands in the emission spectrum and disappearance of the split Q bands from the excitation spectrum provide more evidence that demetalation was successful. Metalated porphyrins have no significant luminescence spectra because of rapid quenching of the excited state by the central metal.

3.1.3 Synthesis of Co^{III}MP8

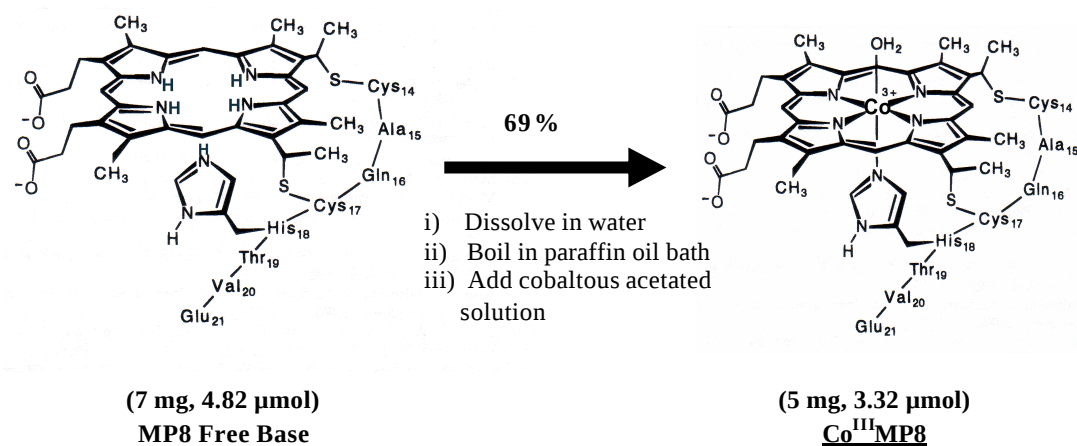


Figure 3.10: Metalation of MP8 Free Base to Co^{III}MP8. The % conversion calculated from MP8 Free Base to Co^{III}MP8 assuming that the metal-free MP8 is 100 % pure.

The metalation procedure was also adapted from the report by Primus and coworkers.⁷⁹ However, in this project cobalt was inserted into the porphyrin instead of manganese, so as to mimic the cobalt in the vitamin B_{12a} environment. The metalation process was successful and Co^{III}MP8 was synthesised in 69 % yield shown in Figure 3.10.



Figure 3.11: HPLC chromatogram of Co^{III}MP8 (retention time of 6.82 minutes) with 79 % purity.

The HPLC chromatogram of $\text{Co}^{\text{III}}\text{MP8}$ is shown in Figure 3.11 and its UV-vis spectrum is shown in Figure 3.12. When comparing the HPLC retention times of $\text{Co}^{\text{III}}\text{MP8}$ with MP8 free base, we find that the retention time of $\text{Co}^{\text{III}}\text{MP8}$ is 6.82 minutes and is significantly different from the retention time of MP8 free base which is 5.99 minutes. This shows that when the metal is inserted into the MP8 free base, it moves slightly slower on the column due to the increased polarity of the newly inserted metal ion.

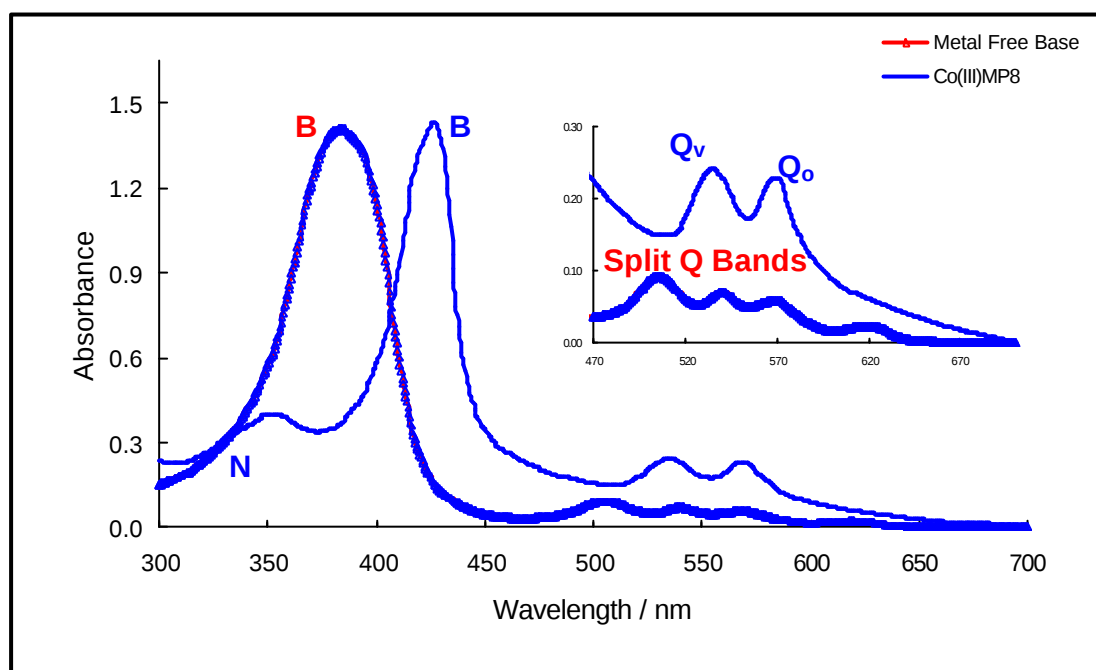


Figure 3.12: UV/visible spectrum of MP8 free base $\text{Co}^{\text{III}}\text{MP8}$ in 0.001 M

$\text{NH}_4(\text{CO}_3)_2$ buffer and MP8 Free Base in 50 % methanol/water.

Four distinct absorption bands are observed at 350 nm, 425 nm, 533 nm and 567 nm for $\text{Co}^{\text{III}}\text{MP8}$ as shown in Figure 3.12. The characteristic N-band and Soret band occurs at 350 nm and 425 nm, respectively. The Q_v band occurs at 533 nm and the Q_o band occurs at 567 nm. Upon insertion of the cobalt into the MP8 free base, we observe the band position of the Soret for the MP8 free base shifting from 380 nm to 425 nm. Furthermore, the four split Q-bands that were formed in the MP8 free base, merged to form two new Q-bands. The spectrum shows that there is no charge transfer band observed for Co^{III} . Although there are restricted data available for Co^{III} , it can be

compared to another d^6 ion like Fe^{II} , although the two species are not comparable given that Fe^{II} is high spin whereas the Co^{III} system involves a low spin metal ion. The ligand to metal charge transfer in a low spin d^6 at 476 nm is due to the transition, $a_{2u} (?) \rightarrow a_{1g} (dz^2)$ and another at 320 nm is due to, $a'_{2u} (?) \rightarrow b_{2u} (?)$. However, for Fe^{II} in the high spin state the metal to ligand charge transfer band occurs between 758 nm and 930 nm which involves the transition, $e_g(d?) \rightarrow e_g(?^*)$.²⁰³

From the above spectra it can be clearly seen that there is a shift in peak positions which is due to the presence or absence of the central Co^{III} ion. Low *et al.*²⁰² performed a similar study where Mn^{III} was inserted into MP8 free base utilising a reductive demetalation procedure involving HF. There were split Soret bands observed which were attributed to inequivalent interactions between the porphyrin p_x and p_y LUMOs and dp electrons of manganese ion. However, in our study we do not observe this characteristic split Soret bands.

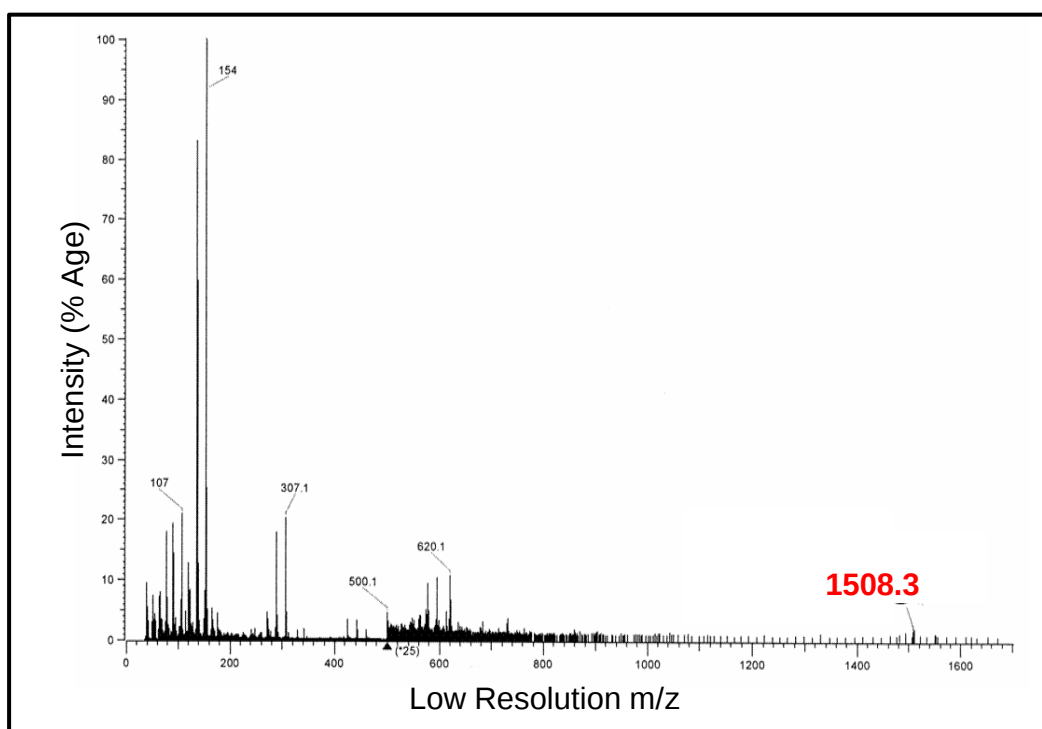


Figure 3.13: FAB-MS spectrum of Co^{III} MP8 in NBA matrix.

In addition, FAB-MS was performed on Co^{III} MP8. The exact mass determinations for Co^{III} MP8 shown in Figure 3.13 were also excellent and in agreement, where the observed m/z was 1508.3 and the calculated 1508. As this was the first time that Co^{III} MP8 was synthesised there were no comparative data available.

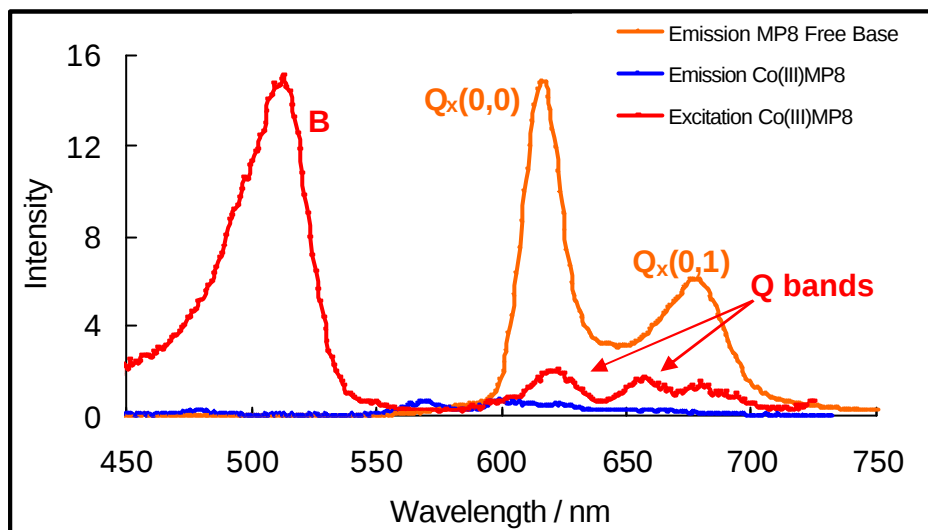


Figure 3.14: Excitation and emissionspectra of Co^{III} MP8 and emission spectrum of MP8 free base.

The excitation spectrum of Co^{III} MP8 shown in red on Figure 3.14 was recorded from 300 nm to 607 nm with emission $\lambda = 617$ nm. There were four bands observed, one in the ultraviolet region and three bands visible in the region. The band at 395 nm is the characteristic Soret band. We expected only two bands in the visible region at 530 nm and 566 nm, which are characteristic of the Q_0 and Q_v bands; however three bands are observed at 505 nm, 541 nm and 564 nm. This shows that the reaction did not go to completion and that not all the free base porphyrin molecules were not metalated. The emission spectra of Co^{III} MP8 was recorded from 436 nm to 750 nm and shows that there are no intense bands formed and that the transient triplet-triplet absorption observed for free base porphyrins is quenched significantly.²¹ This provides more evidence that metalation was successful. The fact that the Q_x bands do not disappear completely shows that there are trace amounts of free base porphyrins in solution that have not been metalated.

3.1.4 Synthesis of NAc-Co^{III}MP8

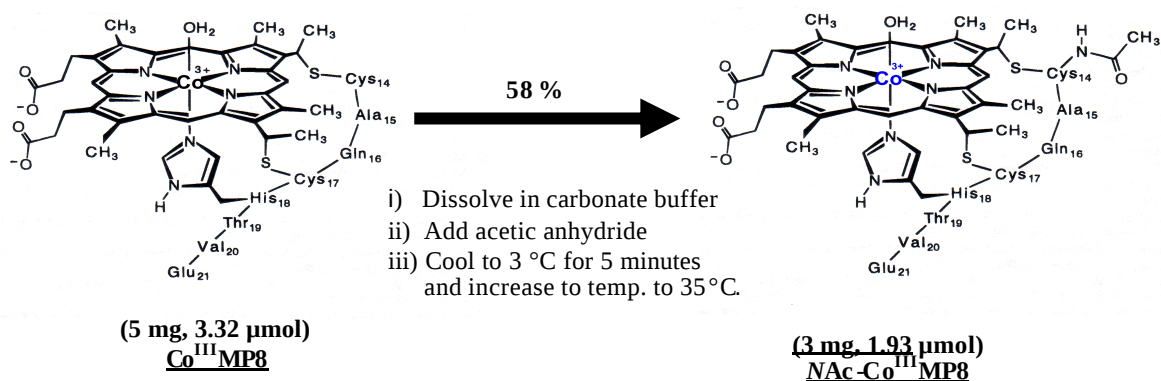


Figure 3.15: Acetylation of Co^{III}MP8 to NAcCo^{III}MP8. The % conversion is calculated from Co^{III}MP8 to NAcCo^{III}MP8 assuming Co^{III}MP8 is 100 % pure.

Acetylation of Co^{III}MP8 was carried out according to methods developed by Munro and Marques.¹⁰⁹ Figure 3.15 shows a schematic of the reaction performed according to the above mentioned conditions. The acetylation process was successful and NAcCo^{III}MP8 was synthesised in 58 % yield. However, the acetylation did not go to completion and some of the unreacted Co^{III}MP8 remained. The two characteristic peaks shown in Figure 3.16 is indicative that the acetylation did not go to completion.



Figure 3.16: HPLC chromatogram of Co^{III}MP8 (6.59 minutes) and NAc-Co^{III}MP8 (5.99 minutes).

The two major peaks observed at 5.99 minutes and 6.59 minutes have a percentage purity of 66 and 27 %, respectively. When comparing these HPLC results to the retention time observed for Co^{III} MP8 (6.82 minutes) in Figure 3.11, we find that the peak observed at a retention time of 6.59 minutes is the closest match. Thus if we assume the above to be correct, then by elimination the peak that has the retention time of 5.99 minutes is due to $\text{NAC-Co}^{\text{III}}$ MP8 and is 66 % pure.

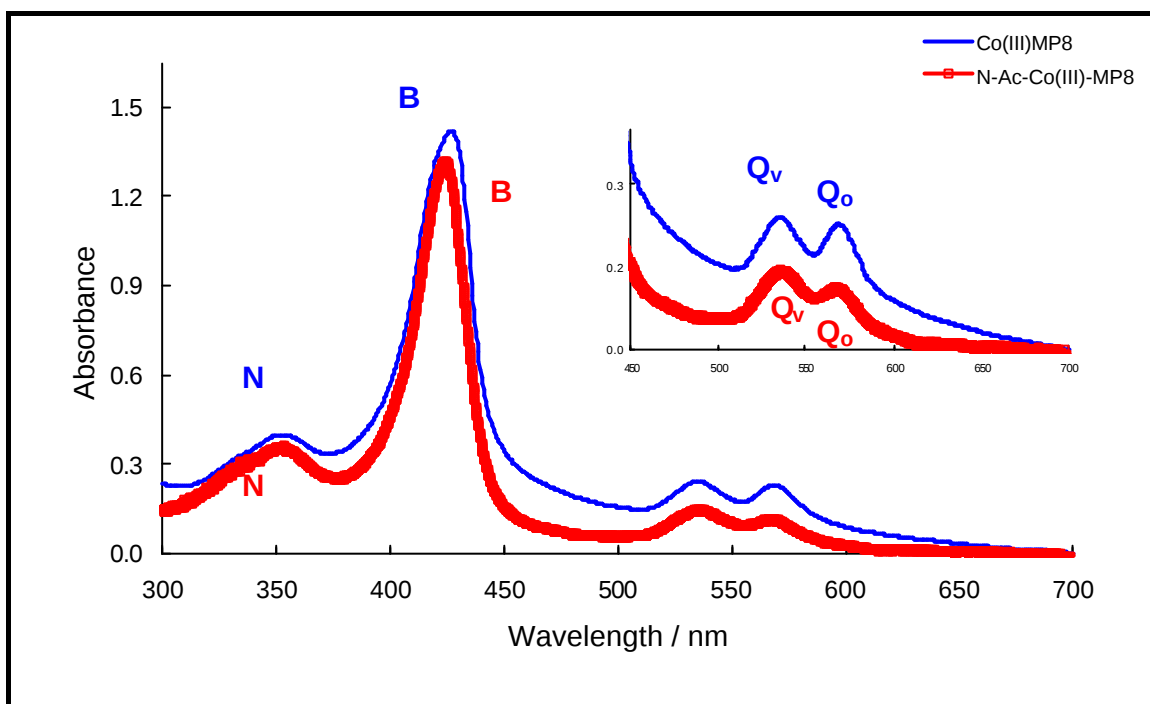


Figure 3.17: UV/visible spectrum of NACo^{III} MP8 and Co^{III} MP8 in 0.001 M ammonium carbonate buffer.

Four distinct absorption bands are observed at 350 nm, 425 nm, 533 nm and 567 nm for NACo^{III} MP8 as shown in Figure 3.17 which corresponds to those earlier described in Figure 3.12. Although the Soret seems to be slightly shifted by about 4 nm when going from Co^{III} MP8 to NACo^{III} MP8, the above spectra show peak positions that were expected and confirm that acetylation has no direct effect on the porphyrin electronic structure itself.

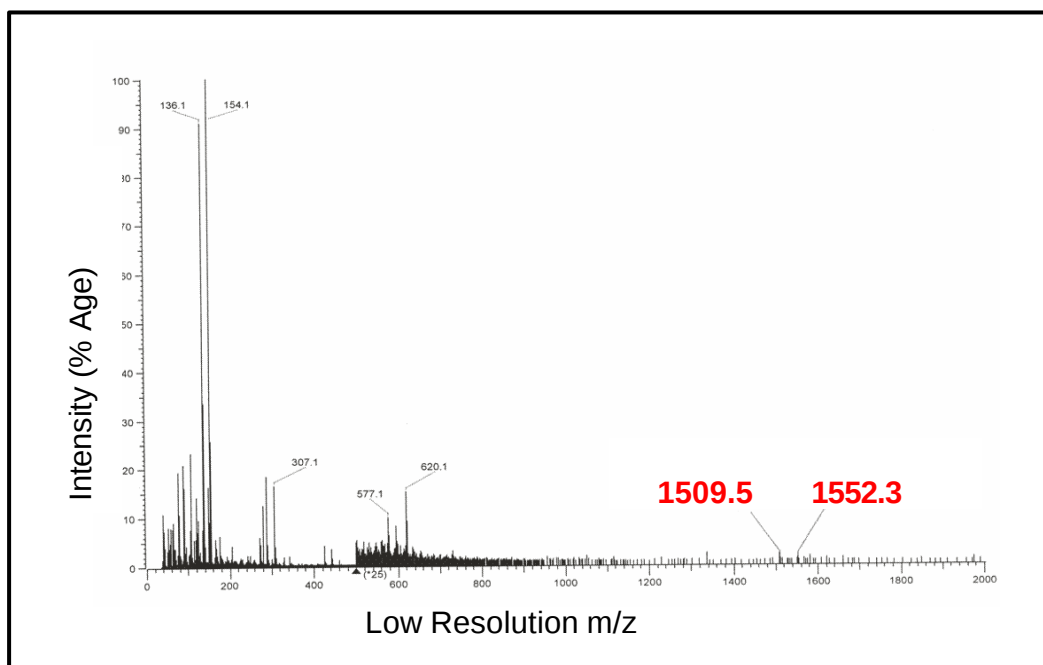


Figure 3.18: FAB-MS spectrum of $\text{NAcCo}^{\text{III}}\text{MP8}$ in NBA matrix.

Furthermore, FAB-MS results gave m/z values expected for the molecular ions formed of $\text{Co}^{\text{III}}\text{MP8}$ and $\text{NAcCo}^{\text{III}}\text{MP8}$ as shown in Figure 3.18. The observed m/z for $\text{Co}^{\text{III}}\text{MP8}$ and $\text{NAcCo}^{\text{III}}\text{MP8}$ were 1509.5 and 1552.3 and the calculated 1508 and 1552, respectively. The molecular ions formed have low intensities, and is due to the small concentration of $\text{NAcCo}^{\text{III}}\text{MP8}$ in the matrix.

3.2 Solution Chemistry of NAc-Co^{III}MP8

3.2.1 Determination of the Extinction Coefficients of NAc-Co^{III}MP8

The extinction coefficients (or molar absorption coefficients), ϵ , were determined using ICP-OES and UV-vis spectroscopy. ICP-OES was used to determine the concentration of Co^{III} ions in 0.210 mg NAc-Co^{III}MP8, which was dissolved in 25 ml of 20 % acetonitrile/water (v/v) and further diluted 10 times to fall within the range of the ICP-OES multi-element calibration standards. Table 3.1 shows the mean concentration obtained for cobalt ions detected during ICP-OES analysis. Once the blank was subtracted, it was assumed that the concentration of Co^{III} ions detected by ICP-OES was equivalent to the concentration of NAc-Co^{III}MP8 in solution. The emission of Co^{III} ions were detected at 237.8 nm.

Table 3.1: Summary of ICP-OES results for NAc-Co^{III}MP8 .

Statistical Values	Co_237.8 / ppm
Mean Concentration, $\bar{x}$	0.0236
Std. Deviation, s	0.0004
Relative Std. Deviation, s_r	1.5 %

The sample was analysed and the mean concentration observed was 0.0236 ppm with a relative standard deviation of 1.5 %. After taking the dilution factor into account, it was calculated that the expected $[\text{Co}^{\text{III}}] = 0.00319 \text{ mg l}^{-1}$ and the observed $[\text{Co}^{\text{III}}] = 0.0236 \text{ mg l}^{-1}$. Thus, the % cobalt in solution was 73.98 %. This suggests that there is 73.98 % NAc-Co^{III}MP8 in 25 ml solution. This is expected as the starting cytochrome *c* was only *ca.* 80 % pure (from manufacturer's specifications). The same solution of NAc-Co^{III}MP8 was then put into a 1.0 cm pathlength curvette and using a UV-vis spectrophotometer, the extinction coefficients were determined from Beer's law ($\epsilon = bc/A$, where ϵ represents the molar extinction coefficient; b represents the pathlength of the cell; c represents the concentration of the solution and A represents the absorbance recorded for that particular solution). Table 3.2 summarises the results obtained using Beer's Law.

Table 3.2: Calculation of Extinction Coefficients using Beer's Law.

?	Average	Average ϵ	Std.	Relative Std.
(nm)	Absorbance	($\times 10^5$) / $M^{-1} \text{ cm}^{-1}$	Deviation	Deviation
560.8	0.0289	0.139	0.019	1.37
527.9	0.0286	0.137	0.017	1.27
415.1	0.3350	1.61	0.052	0.32
347.7	0.0617	0.296	0.035	1.18
337.9	0.0560	0.268	0.035	1.30

3.2.2 Beer-Lambert Behaviour of NAc-Co^{III}MP8

The microperoxidases have been used as biomimetic models for haemproteins for many years, although many studies have been complicated by their tendency to aggregate. An effective method for accessing concentration-dependant aggregation phenomena in solutions of haemproteins is to determine the degree of adherence of the solution to Beer's law over the solute concentration range of interest.²⁰⁴ Deviation from Beer's law signals aggregation of the absorbing species, while a single set of isosbestic points reflect a simple equilibrium between two species.²⁰⁵ Marques *et. al*¹⁰² carried out a Beer's law study of MP8 in aqueous phosphate buffer solution (pH 7.00, $\mu = 0.1 \text{ M}$, 25 °C) and revealed the existence of a simple monomer/dimer equilibrium (dimerisation constant $K_D = (1.17 \pm 0.02) \times 10^5 \text{ M}^{-1}$) below an MP8 concentration of $8.08 \times 10^{-6} \text{ M}$ but higher aggregates at concentrations above this. Since low-spin MP8 was obtained at highest concentrations, this study suggested the likelihood of an aggregation mechanism involving intermolecular coordination by Cys-14 α -amino group of MP8.¹⁰² Marques *et al.*¹⁰⁹ performed a similar study on NAc-MP8 in aqueous phosphate buffer solution (pH 7.00, $\mu = 0.5 \text{ M}$, 25 °C) and found that the Soret absorbance varies linearly with concentration up to $\sim 3 \times 10^{-5} \text{ M}$, hereafter the slope decreases. The limiting slope of NAc-MP8 was reported to be $1.55 \pm 0.01 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, and was in agreement with that

obtained for monomeric MP8 ($1.57 \pm 0.01 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$). It is well established that the observed departure from linearity is the consequence of a simple dimerisation equilibrium (Equation 3.1, where M and D are monomeric and dimeric forms of NAc-MP8, respectively).^{204, 205}

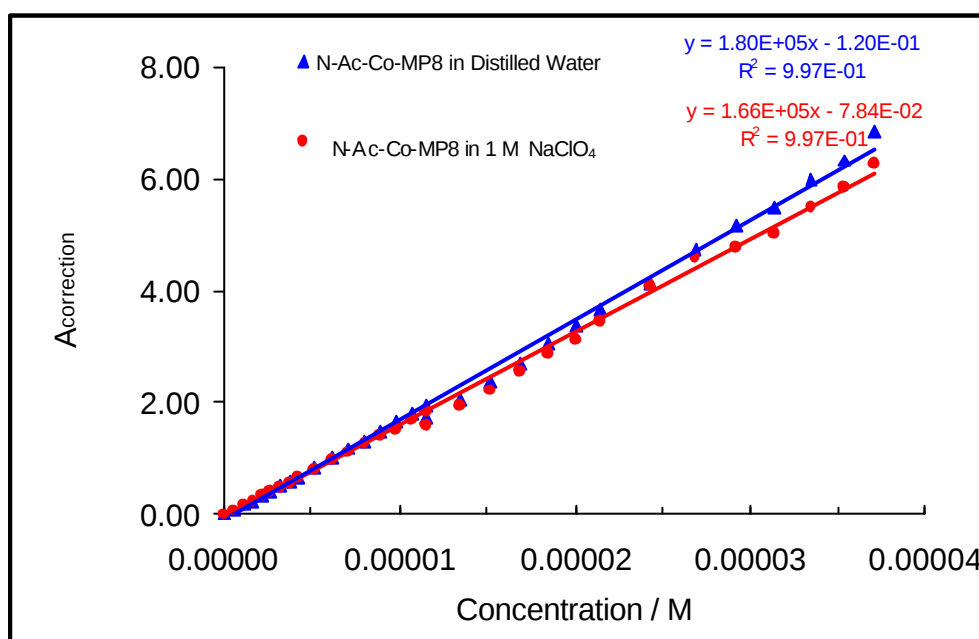
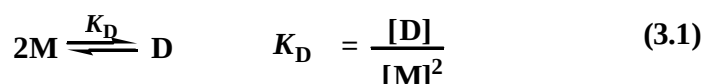


Figure 3.19: Beer's law plot for an aqueous solution of $\text{NAcCo}^{\text{III}}\text{MP8}$ (pH 7.00, 25 °C) in distilled water and 1.0 M NaClO_4 .

The limiting slope for aqueous solution of $\text{NAcCo}^{\text{III}}\text{MP8}$ (pH 7.00, 25 °C) in distilled water and 1.0 M NaClO_4 was $1.80 \pm 0.01 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and $1.66 \pm 0.01 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. These values were slightly higher than that obtained for MP8 and NAc-MP8 which were mentioned earlier and undoubtedly a consequence of the central metal being changed from iron to cobalt. When the ionic strength of the $\text{NAcCo}^{\text{III}}\text{MP8}$ solution was increased to 1.0 M using NaClO_4 there was no deviation from linearity, but there was a small decrease in the limiting slope. Figure 3.19 shows that both solutions of $\text{NAcCo}^{\text{III}}\text{MP8}$ obey Beer's law over the concentration range monitored. Thus, all

$\text{NACo}^{\text{III}}\text{MP8}$ solutions used for future experiments should be carried out with $[\text{NACo}^{\text{III}}\text{MP8}] < 40 \mu\text{M}$ to avoid any possible aggregation. This is more than sufficient for conventional UV-vis spectroscopic work.

3.2.3 Determination of Spectroscopic pK_a s of $\text{NACo}^{\text{III}}\text{MP8}$

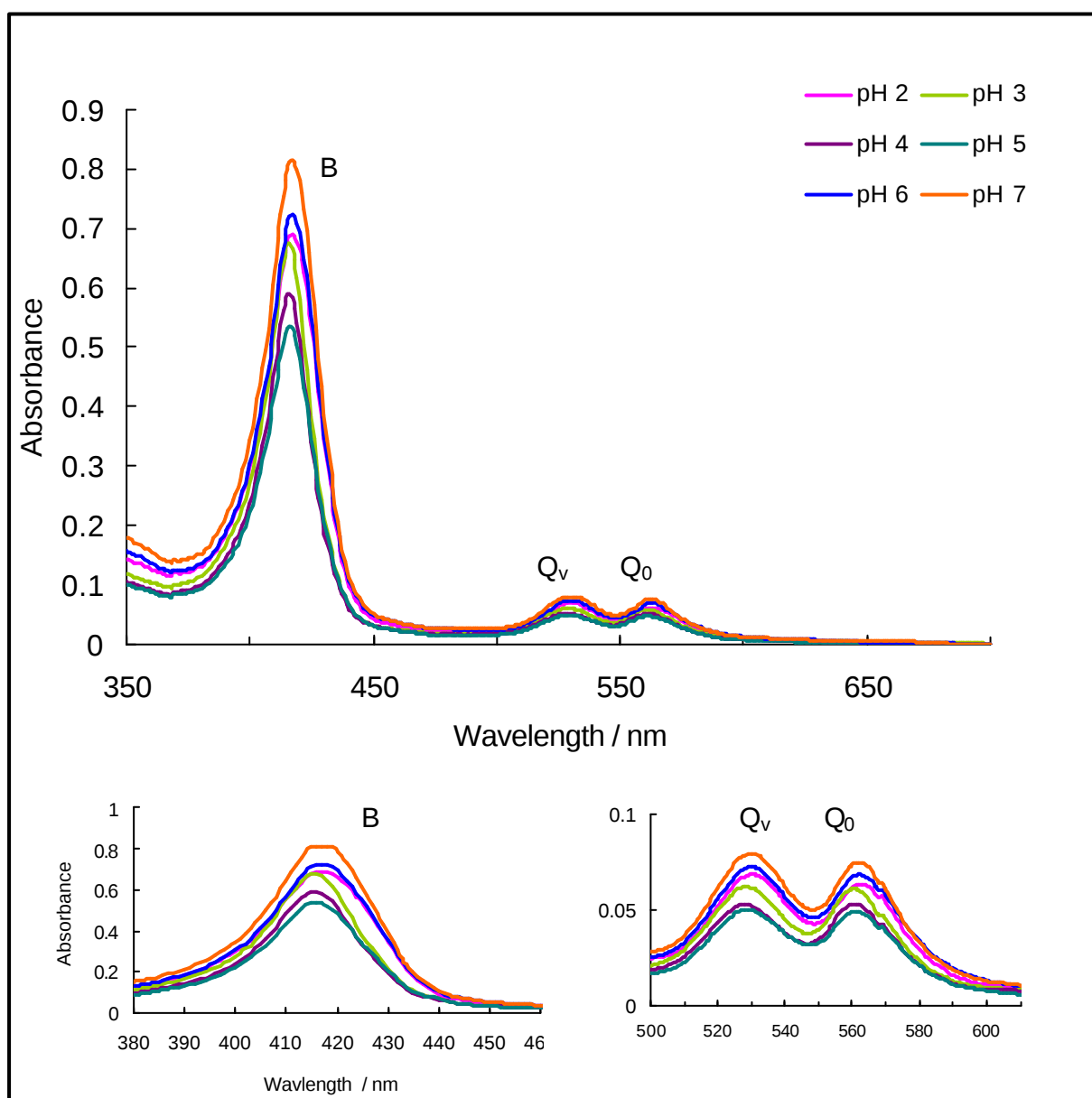


Figure 3.20: pH dependence of $\text{NACo}^{\text{III}}\text{MP8}$ in acidic region.

The spectroscopic pK_a s of $NAc-Co^{III}MP8$ were determined using UV-vis spectroscopy. The pH dependence of the electronic spectrum of $NAcCo^{III}MP8$ in an aqueous multicomponent buffer in the acidic region shows a shift of the Soret (B) band from $\lambda_{max, pH2} = 417.0$ nm to $\lambda_{max, pH6} = 415.1$ nm as shown in Figure 3.20. This change in wavelength is also accompanied by increase in the initial intensity by 5.40 %. Although the peak position observed for the Q bands did not change, there were changes in intensity. When changing from pH 2 to 6, the initial intensity Q_v and Q_0 bands increased by 5.79 % and 7.93 %, respectively.

Firstly, the experimental data was fitted to an ionisation isotherm involving one spectroscopic pK_a (Equation 2.1). The fit was reasonable with an r^2 value of 99.52 % and pK_a value of 3.68 ± 0.02 . The same data were then fitted to an ionisation isotherm involving two spectroscopic pK_a s (Equation 2.2). The fit was marginally better with an r^2 value of 99.55 %. The values of the spectroscopic pK_a s determined were 3.4 ± 0.5 and 4.2 ± 0.7 . The experimental data were fitted to an ionisation isotherm involving three consecutive equilibria (Equation 2.3). The r^2 value again improved with a fit of 99.61 %. The values of the three spectroscopic pK_a s obtained from the theoretical curve fitting the data (Figure 3.21) were $pK_1 = 2.0 \pm 0.3$, $pK_2 = 2.8 \pm 0.1$ and $pK_3 = 3.91 \pm 0.03$. Finally, the experimental data were fitted to an ionisation isotherm with four consecutive equilibria (Equation 2.4). The r^2 value was excellent with a fit of 99.86 %. The values of the four spectroscopic pK_a s were 1.5 ± 1.8 , 3.3 ± 0.1 , 4.7 ± 0.2 and 6.8 ± 1.6 . The r^2 value of the fit involving three pK_a s is better than the fit involving one and two pK_a s but the r^2 value of the fit involving four pK_a s is better than the fit involving three pK_a s. However, some values obtained for four pK_a fit are unrealistic since it produces values that are beyond the pH range under which we are working (1.5 ± 1.8 and 6.8 ± 1.6) with significant error. In addition, glass electrode potentiometry cannot be used below pH of about 2 because of the non-Nernstian response. Therefore, we conclude that the most reliable fit was observed for the isotherm with three consecutive equilibria (Figure 3.21).

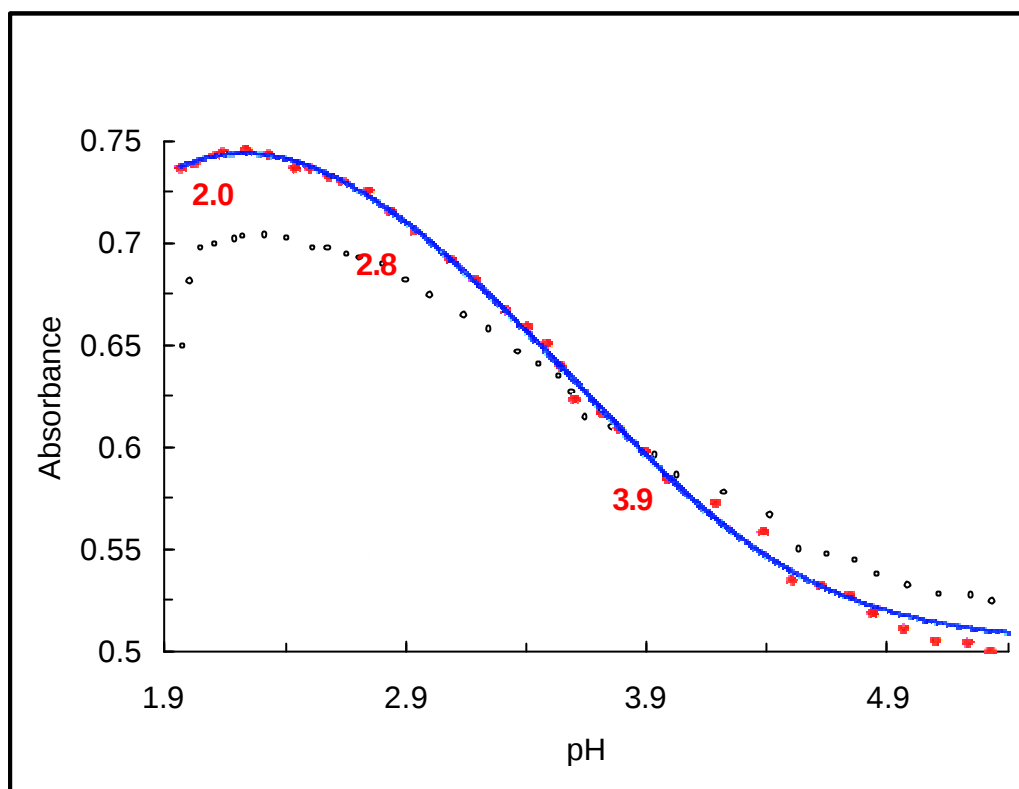


Figure 3.21: Variation of the Soret absorbance (415.05 nm) with pH titration of $\text{NAcCo}^{\text{III}}\text{MP8}$ in acidic region (pH 2 – 6) with NaOH.

The pH dependence of the electronic spectrum of $\text{NAcCo}^{\text{III}}\text{MP8}$ in aqueous multicomponent buffer in the basic region shows a shift of the Soret (B) band from $\lambda_{\text{max, pH7}} = 414.1 \text{ nm}$ to $\lambda_{\text{max, pH12}} = 419.1 \text{ nm}$. This change in wavelength is also accompanied by increase in the initial intensity by 49.34 %. The λ_{max} of Q_v and Q_0 observed at pH 7 were 529 nm and 561 nm, respectively. However, λ_{max} of Q_v and Q_0 at observed at pH 12 was 533 nm and 566 nm, respectively. Thus there was an overall shift in wavelength. Similarly, this change in wavelength is accompanied by an initial increase in intensity of Q_v and Q_0 bands by 50.3 % and 30.6 %, respectively.

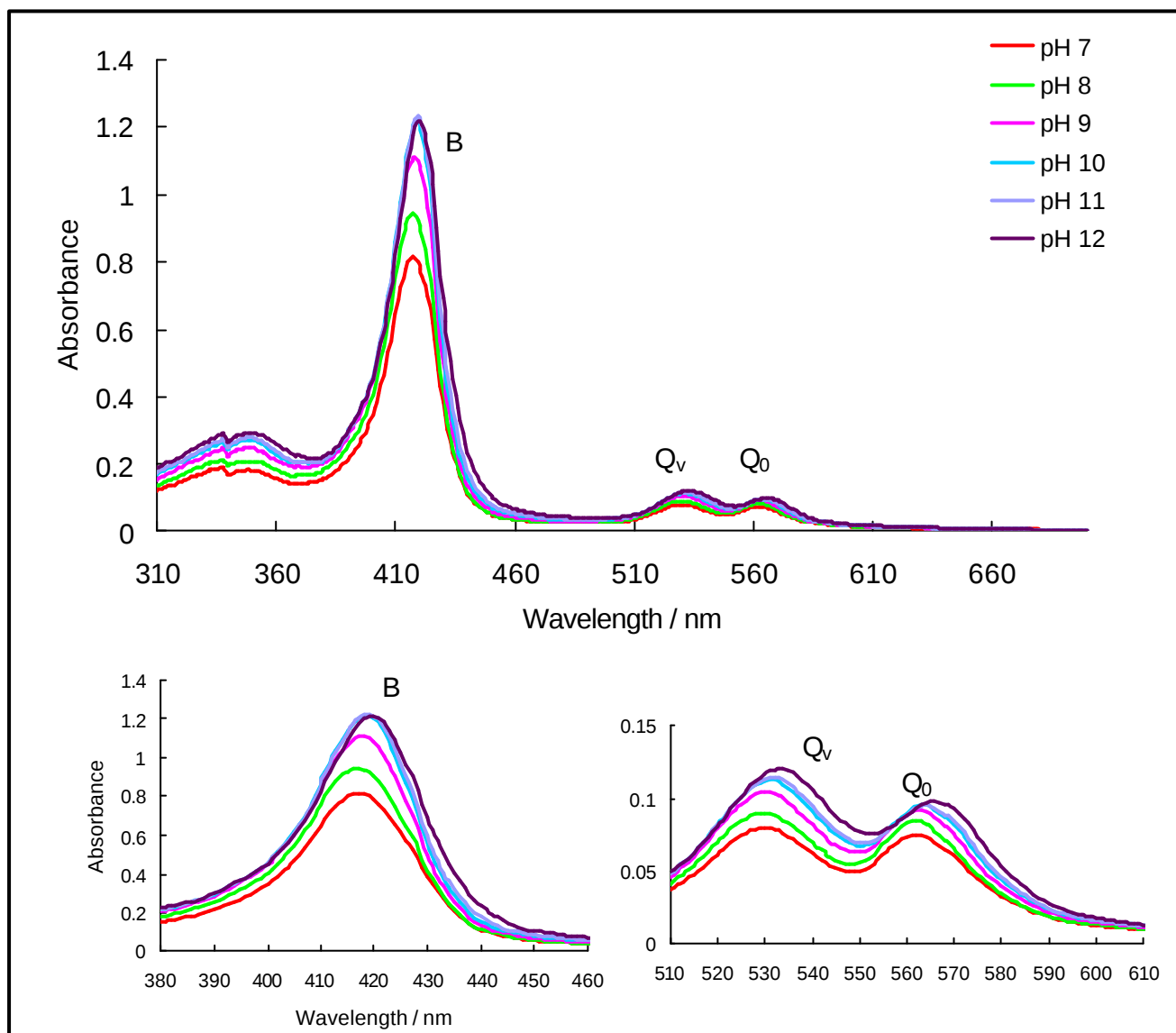


Figure 3.22: pH dependence of $\text{NAcCo}^{\text{III}}\text{MP8}$ in basic region.

The data were fitted for one, two, three and four spectroscopic pK_a s. Firstly, the experimental data were fitted to an ionisation isotherm with one spectroscopic pK_a (Equation 2.1). The fit was poor with an r^2 value of 90.67 % and pK_a value of 8.02 ± 0.09 . The same data were fitted to an ionisation isotherm with two spectroscopic pK_a s (Equation 2.2). The fit was slightly better with an r^2 value of 91.23 %. The values of the spectroscopic pK_a s determined were 7.27 ± 0.09 and 8.51 ± 0.01 . Also, the experimental

data were fitted to an ionisation isotherm (Equation 2.3) consisting of three consecutive equilibria. The values of the three spectroscopic pK_a s obtained from the theoretical curve fitting the data were $pK_4 = 7.47 \pm 0.03$, $pK_5 = 9.19 \pm 0.04$ and $pK_6 = 12.07 \pm 0.03$. The r^2 squared value was excellent with a fit of 99.88%. Finally, the experimental data were fitted to an ionisation isotherm containing four consecutive equilibria (Equation 2.4). The r^2 value was excellent with a fit of 99.92 %. The values of the four spectroscopic pK_a s were 7.49 ± 0.03 , 9.24 ± 0.01 , 11.8 ± 0.8 and 13.5 ± 0.8 . The r^2 value of the fit involving three pK_a s is better than the fits involving one and two pK_a s but the r^2 value of the fit involving four pK_a s is better than the fit involving three pK_a s. However, the fourth pK_a (13.5 ± 0.8) is unreliable as glass electrodes typically display Nernstian behaviour up to pH values of between 12 and 13. Moreover this pK_a is not observed in other MP8 studies.^{102, 109} Therefore, the most reliable fit was observed for the isotherm containing three consecutive equilibria (Figure 3.23).

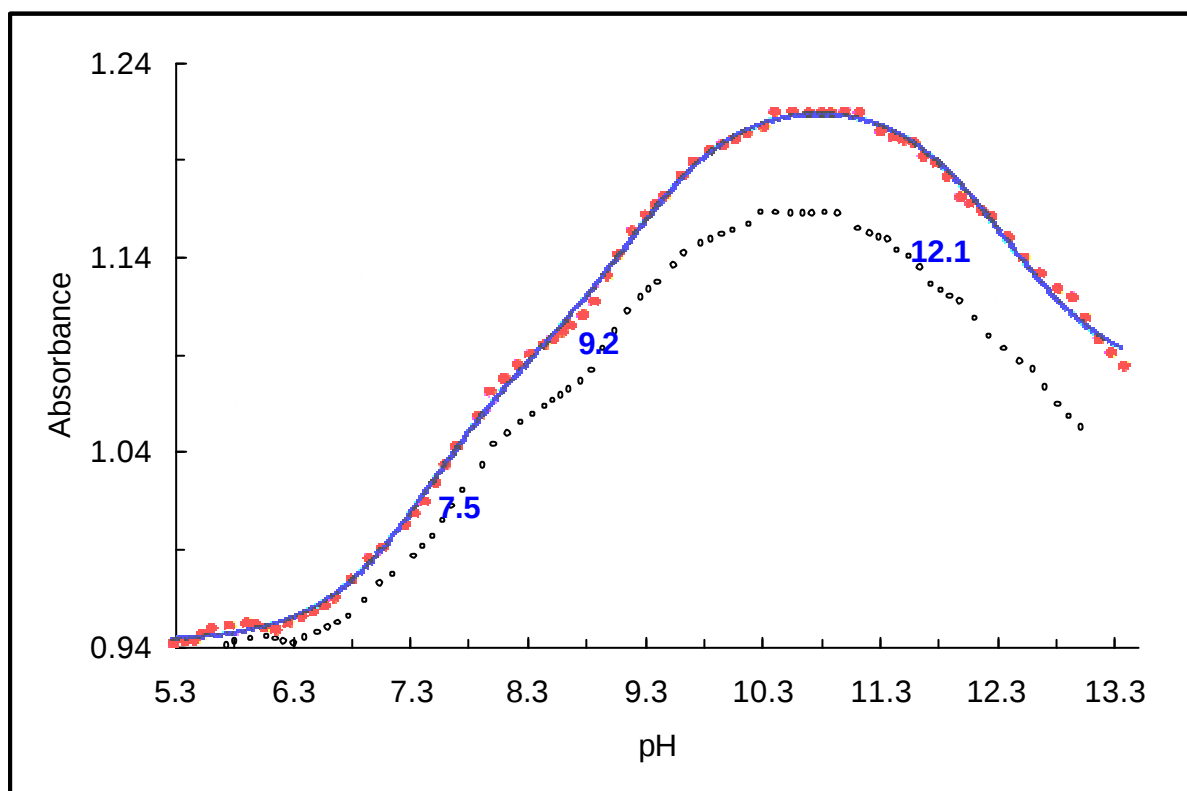


Figure 3.23: Variation of the Soret absorbance (415.05 nm) with pH titration of $NAcCo^{III}MP8$ in basic region (pH 6-13) with NaOH.

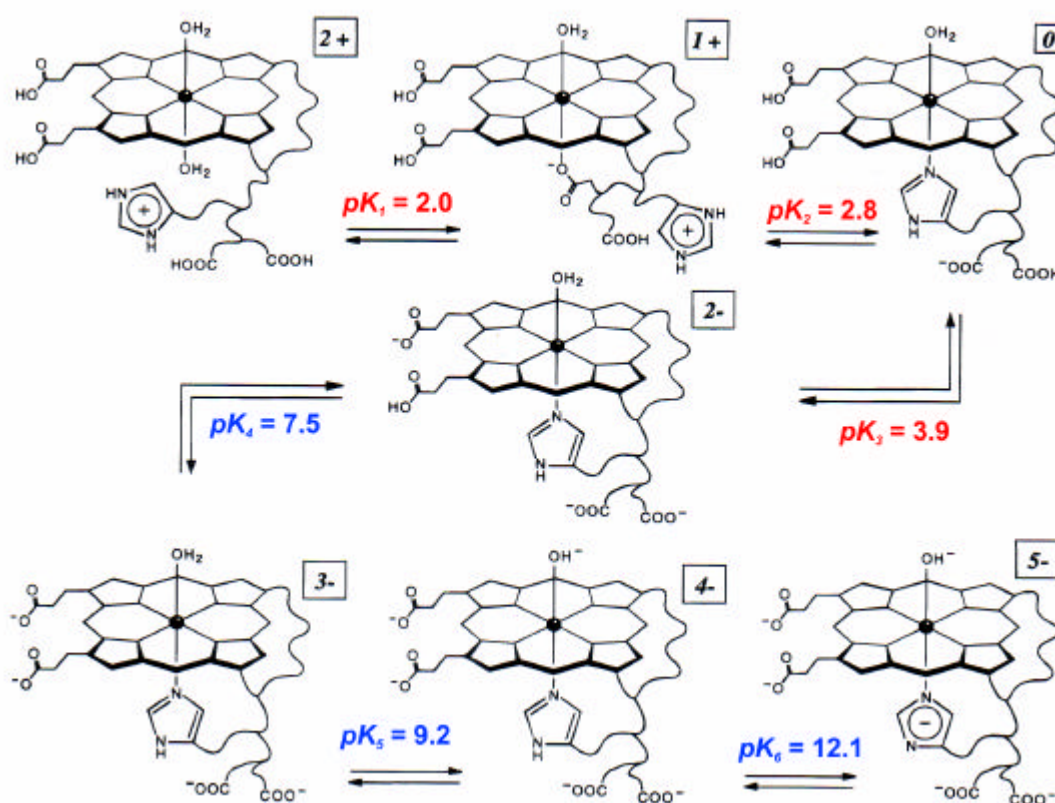


Figure 3.24: Species distribution as a function of pH at 25 °C for $\text{NAcCo}^{\text{III}}\text{MP8}$ in an aqueous multicomponent buffer system.

The interpretation of all the spectroscopic pK_a s is summarised in Figure 3.24. At pH < 1.5, we propose, largely by analogy with the Fe^{III} system, that $\text{NAcCo}^{\text{III}}\text{MP8}$ exists as a six-coordinate diaqua complex and that the first transition ($pK_1 = 2.0 \pm 0.3$) involves coordination of the *c*-terminal carboxylate group (Glu-21) of the appended polypeptide. It is known that the pK_a of the *c*-terminal carboxylic acid group in proteins is 2.13.²⁰⁶ In the present case, the interaction with Co^{III} -haem centre (with the a residual charge of +1) does not appear to depress this pK_a significantly.¹⁰⁹ The second acid range transition ($pK_2 = 2.8 \pm 0.1$) for $\text{NAcCo}^{\text{III}}\text{MP8}$ probably involves the deprotonation of the cationic His-18 and concomitant replacement of the *c*-terminal carboxylate by the neutral heterocyclic base. The suggestion that the pK_a at 2.8 reflects the coordination of His-18 is consistent with ^1H NMR and other studies on several of the cytochromes c ,²⁰⁷⁻²¹³ as well as studies on model haempeptides.⁹⁵ In Figure 3.24 we have assigned pK_3 (3.91 ± 0.03) to the ionisation of one of the haem propanoic acid groups. This is an assumption

based on the pK_a (4.31) for this group in the free amino acid,²¹⁴ which is very close to the value assigned to pK_3 . The second haem propanoic acid group is assigned to the ionization with $pK_4 = 7.47 \pm 0.03$. It is assumed that the pK_a for this is raised relative to that of the free propanoic acid (4.69 ± 0.03 , 25 °C, 0.1 M)²¹⁴ because of the electronic repulsion between its conjugate base and the first propanoate group. It is important to note that *c*-terminal *n*-propylamide NAcMP8 also shows two haem propanoic pK_a s in this region at 4.95 ± 0.07 , and 6.1 ± 0.3 , respectively.¹⁰⁹ Indeed, many NMR and redox studies on a variety of haemproteins have shown that the ionisation of one haem propanoic acid group occurs in the pH range 4.5–7.5, while that of the second, also falling within this range, is frequently observed.^{28, 215–221}

pK_5 (9.19 ± 0.04) was assigned to the ionisation of the cobalt-bound water molecule. Table 3.3 shows the pK_a values of other metal-bound water molecules; however the central metal in these cases is iron. Comparing pK_5 with the tabulated values shown below we notice that there is no significant difference between their values although the central metal has changed.

Table 3.3: Ionisation of iron-bound water (pK_{H_2O}) in various haemproteins.

Ferrihaemprotein/Model	$pK_{(H_2O)}$
horse Hb ²²²	8.86 ± 0.02
horse Mb ²²²	9.04 ± 0.03
sperm whale Mb component IV ²²³	8.93
sperm whale Mb component IIIA ²²³	9.03
sperm whale Mb component II ²²³	9.07
NAcMP8 ¹⁰⁹	9.59 ± 0.01

To confirm that pK_5 (9.19 ± 0.04) was assigned correctly to the ionisation of the cobalt-bound water molecule an investigation was carried out. Using simple ligand substitution, pyridine was used to mask the ionisation of the cobalt-bound water molecule attached to the axial position of the cobalt porphyrin. Figure 3.25 shows the change in absorbance at 415.1 nm as a function of pH for an aqueous solution of $NaCo^{III}MP8$ with pyridine at 25 °C ($\mu = 0.1$ M KCl).

As before, the data were fitted for one, two, three and four spectroscopic pK_a s using Equations 2.1 to 2.4, respectively. Firstly, the experimental data were fitted to an ionisation isotherm consisting of one spectroscopic pK_a . The fit was poor with an r^2 value of 75.25 % and pK_a value of 7.7 ± 0.2 . The data were then fitted to an ionisation isotherm with two spectroscopic pK_a s. The fit was good with an r^2 value of 98.47 %. The values of the spectroscopic pK_a s determined were 8.06 ± 0.04 and 11.97 ± 0.08 . The experimental data were fitted to an ionisation isotherm (Equation 2.1) with three consecutive equilibria. The values of the three spectroscopic pK_a s obtained from the theoretical curve fitting the data were $pK_4 = 7.61 \pm 0.06$, $pK_5 = 9.16 \pm 0.04$ and $pK_6 = 11.78 \pm 0.03$. The r^2 squared value was excellent with a fit of 99.72 %. Finally, the experimental data were fitted to an ionisation isotherm of four consecutive equilibria. The r^2 value was excellent with a fit of 99.77 %. The values of the four spectroscopic pK_a s were 7.4 ± 0.2 , 8.5 ± 0.3 , 9.9 ± 0.4 and 11.73 ± 0.05 . The r^2 value of the fit involving three pK_a s is better than the fits involving one and two pK_a s but the r^2 value of the fit involving four pK_a s is slightly better than the fit involving three pK_a s. Using pyridine to bind to the axial position of $NaCo^{III}MP8$, so as to mask the ionisation the water molecule was not successful. Comparing the fitted data for three spectroscopic pK_a s in the basic region for $NaCo^{III}MP8$ with pyridine and without pyridine, we found that there was no significant change in the value of the pK_a s.

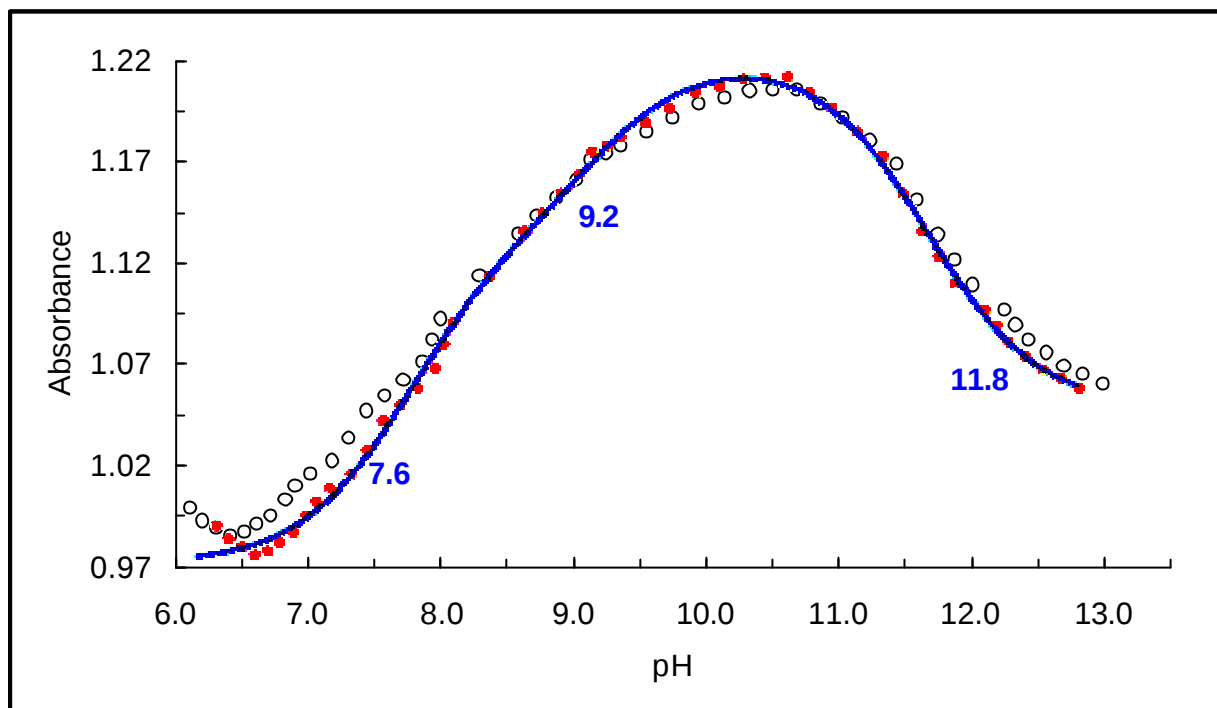


Figure 3.25: Variation of the Soret absorbance (415.05 nm) with pH titration of $\text{NAcCo}^{\text{III}}\text{MP8}$ with pyridine in basic region (pH 6-13) with NaOH.

Finally, we attribute pK_a (12.07 ± 0.03) to the ionisation of the coordinated histidine *trans* to the OH^- to form the histidinate complex ($\text{His}^- \text{-Co}^{\text{III}} \text{-OH}^-$). The pK_a values of the free histidine and imidazole are 14.4 and 14.2, respectively,²²⁴ while coordination of the histidine to the transition metals such as Co^{II} and Cu^{II} reduces the value of these pK_a s to 12.5 and 11.7, in each case.²²⁵

In a similar study, Marques *et. al* reported six spectroscopic pK_a s in the acidic and basic regions for $\text{NAc-Fe}^{\text{III}}\text{MP8}$; $\text{pK}_1 = 2.1 \pm 0.3$, $\text{pK}_2 = 3.12 \pm 0.07$ and $\text{pK}_3 = 4.95 \pm 0.07$, $\text{pK}_4 = 6.1 \pm 0.3$, $\text{pK}_5 = 9.59 \pm 0.01$ and $\text{pK}_6 = 12.71 \pm 0.05$.¹⁰⁹ When their data are compared to our data, it is clear that the results are quite similar in many instances.

Also of interest is the pK_a of water coordinated to Co^{III} in aquacobalamin. That pK_a is reported to be 8.09.¹⁵⁵ Robinson²²⁶ and coworkers have reported the pK_a of aquacobalamin to be 7.8. However this study was performed in 0.5 M KCl and the Cl

ion may coordinate to the cobalt centre and affect results. Although the authors have suggested that the electrolyte has no effect on electrochemical potentials used in their work. We have decided to use the pK_a value of 8.09 for comparative purposes. The ionisation of the metal-bound water molecule for $NAcCo^{III}MP8$ and aquacobalamin differs by 1.1 pH units. This difference is significant and may be attributed to the environmental surroundings of the central cobalt ion, namely the porphine and corrin macrocyclic environments.

It has been well established that the corrin cavity for cobalamin is much smaller than the porphyrin cavity in microperoxidase.^{7, 23, 163, 167, 195} Although the formal charge of cobalt in the porphyrin is +3, the actual charge of the metal depends on the extent the ligands donate electron density to the electron deficient cobalt centre. Now, if we assume to a first approximation that the electronic properties of histidine (in $NAcCo^{III}MP8$) and dimethylbenzimidazole (in B_{12a}) have the same σ donor/ π acceptor abilities toward the cobalt centre, then the only variable is the difference in the donor ability of corrin and porphine macrocycles. In cobalt corrinoids like B_{12a} , Co^{III} centre is in close proximity to the electron rich corrin macrocycle and becomes more electron rich. This leads to a decrease the electron density on the coordinated water molecule and consequently increases the ionisation of the axial water molecule (high K_a) and small pK_a values. In $NAcCo^{III}MP8$, the Co^{III} centre is not in close proximity to the electron rich porphine macrocycle (due to the cavity being larger and more rigid) and does not significantly change the electron density on the coordinated water molecule. This results in smaller ionisation constants being observed (larger pK_a values). Hence, the results show that the corrin moiety is a better electron donor toward Co^{III} , than the porphine moiety.

3.2.4 Ligand Binding Studies of Selected N-Donor Ligands by $NAc-Co^{III}MP8$

Solutions of $NAc-Co^{III}MP8$ in the presence of varying concentrations of ligand were allowed to equilibrate overnight in a water bath where the temperature was maintained at 25.0 ± 0.1 °C. Since Co^{III} in $NAc-Co^{III}MP8$ is likely to be inert, the ligand had at least 16

hours in which to bind to $\text{NAC-Co}^{\text{III}}\text{MP8}$. It was our intention to study the thermodynamic rather than the kinetic product. Consequently, the longer equilibrium had no effect on the final form. Absorbance measurements were taken for each solution of $\text{NAC-Co}^{\text{III}}\text{MP8}$ with the corresponding ligand and these values were plotted against ligand concentration. Table 3.4 shows the recorded absorbance measurements for the reaction of $\text{NAC-Co}^{\text{III}}\text{MP8}$ with varying concentration of *N*-methylimidazole.

Table 3.4: Absorbance measurements for the reaction of $\text{NAC-Co}^{\text{III}}\text{MP8}$ with varying concentration of *N*-methylimidazole. (pH 5.45, $\mu = 1.0 \text{ M}$, $25 \text{ }^{\circ}\text{C}$)

Absorbance at 420 nm	[<i>N</i> -methylimidazole]
0.3535	0.000
0.3725	0.000112
0.3770	0.000335
0.3824	0.000668
0.3855	0.00111
0.3883	0.00167
0.3903	0.00277
0.3922	0.00388
0.3935	0.00498
0.3959	0.00719
0.3980	0.00883
0.4010	0.0127
0.4028	0.0149
0.4046	0.0182
0.4059	0.0214
0.4075	0.0236
0.4103	0.0290
0.4102	0.0344

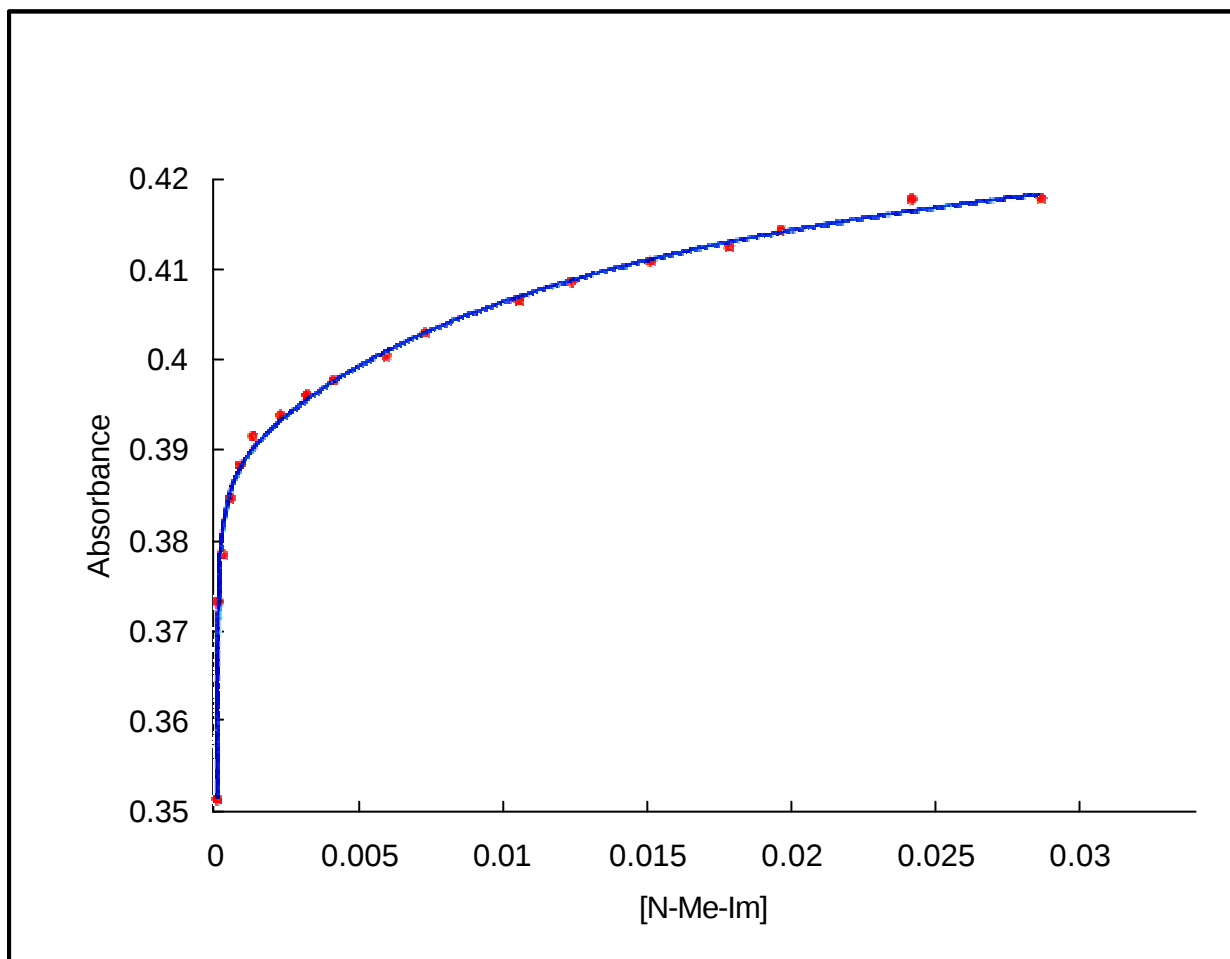


Figure 3.26: Plot of the titration data for the binding of *N*-methylimidazole to *NAc-Co^{III}MP8* at 420 nm. (25 °C, $\mu = 1.0$ M)

The formation constants between *NAc-Co^{III}MP8* and *N*-methylimidazole were determined using Equations 2.5 and 2.6. The plot of the titration data for the binding of *N*-methylimidazole to *NAc-Co^{III}MP8* is illustrated in Figure 3.26. The data obtained were initially fitted to account for a single formation constant (Equation 2.5). $K_{1\text{obs}}$ was determined to be $817 \pm 264 \text{ M}^{-1}$. The fit was poor, with an r^2 value of 91.68 %.

The pK_a of *N*-methylimidazole is 7.21. The final pH of the *NAc-Co^{III}MP8* solution with *N*-methylimidazole was 5.396. The concentration of ligand in solution is dependent on

the pH. Therefore K_{obs} values had to be converted into pH-independent formation constants using Equation 2.7. After correcting for pH, $\log K_1$ was found to be 4.7 ± 0.9 .

We then proceeded to fit two formation constants using Equation 2.6 and the results were excellent with an r^2 value of 99.70 %. We propose that the second formation constant, K_2 , is due to the formation of an outer-sphere complex, which eventually leads to a bisubstituted complex shown below in Figure 3.27 (where $K_2 = K_{\text{OS}} \cdot K_2'$). Attempts have been made to rationalise the nature of the outer-sphere complex formed in aquacobalamin and the incoming ligand;²²⁷ it was suggested that the complex was formed by p-p interactions between the corrin ring (or functional groups on the corrin, presumably the amide side chains) and the ligand. This can be extrapolated to $\text{NAC-Co}^{\text{III}}\text{MP8}$ since it is a model for aquacobalamin. $K_{1\text{obs}}$ and $K_{2\text{obs}}'$ were determined to be 10539 ± 1704 and 50 ± 13 . Similarly, the data were corrected for pH using Equation 2.7. $\log K_1$ and $\log K_2$ were calculated to be 5.8 ± 0.9 and 3.6 ± 0.8 , respectively.

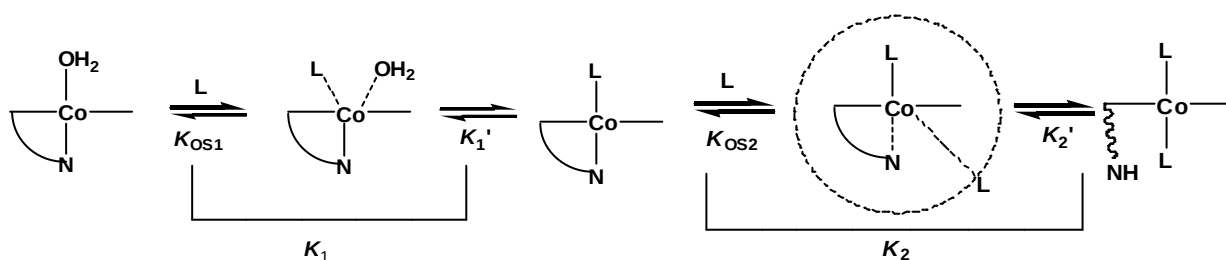


Figure 3.27: Proposed ligand substitution for $\text{NAc-Co}^{\text{III}}\text{MP8}$.

As before, absorbance measurements were taken for the reaction of each solution of $\text{NAc-Co}^{\text{III}}\text{MP8}$ with varying concentration of pyridine (Table 3.5). The formation constants between $\text{NAc-Co}^{\text{III}}\text{MP8}$ and pyridine were determined using Equations 2.5 and 2.6. The data obtained was initially fitted to account for only one formation constant (Equation 2.5). $K_{1\text{obs}}$ was determined to be $13 \pm 2 \times 10^4 \text{ M}^{-1}$. The fit was reasonable with an r^2 value of 98.64 %. The pK_a of pyridine is 5.19. The final pH of the $\text{NAc-Co}^{\text{III}}\text{MP8}$ solution with *N*-methylimidazole was 5.436. After correcting for pH using Equation 2.7, $\log K_1$ was found to be 4.3 ± 0.8 .

Table 3.5: Absorbance measurements for the reaction of NAc-Co^{III}MP8 with varying concentration of pyridine. (pH 5.44, $\mu = 1.0$ M, 25 °C)

Absorbance at 420 nm	[Pyridine]
0.3606	0.000
0.3684	0.0000112
0.3849	0.0000445
0.3925	0.000111
0.3956	0.000166
0.3992	0.000221
0.4013	0.000331
0.405	0.000661
0.4105	0.00186
0.4112	0.00240
0.4127	0.00349
0.4159	0.00676
0.4187	0.0133
0.4207	0.0186

We then proceeded to fit two formation constants using Equation 2.6. The fit was excellent with an r^2 value of 99.84% as shown in Figure 3.28. $K_{1\text{obs}}$ and $K_{2\text{obs}}$ were determined to be $2.3 + 0.3 \times 10^4 \text{ M}^{-1}$ and $2.4 + 0.8 \times 10^2 \text{ M}^{-1}$. As before, the data were corrected for pH using Equation 2.7. $\log K_1$ and $\log K_2$ were calculated to be 4.6 ± 0.8 and 2.6 ± 0.8 , respectively.

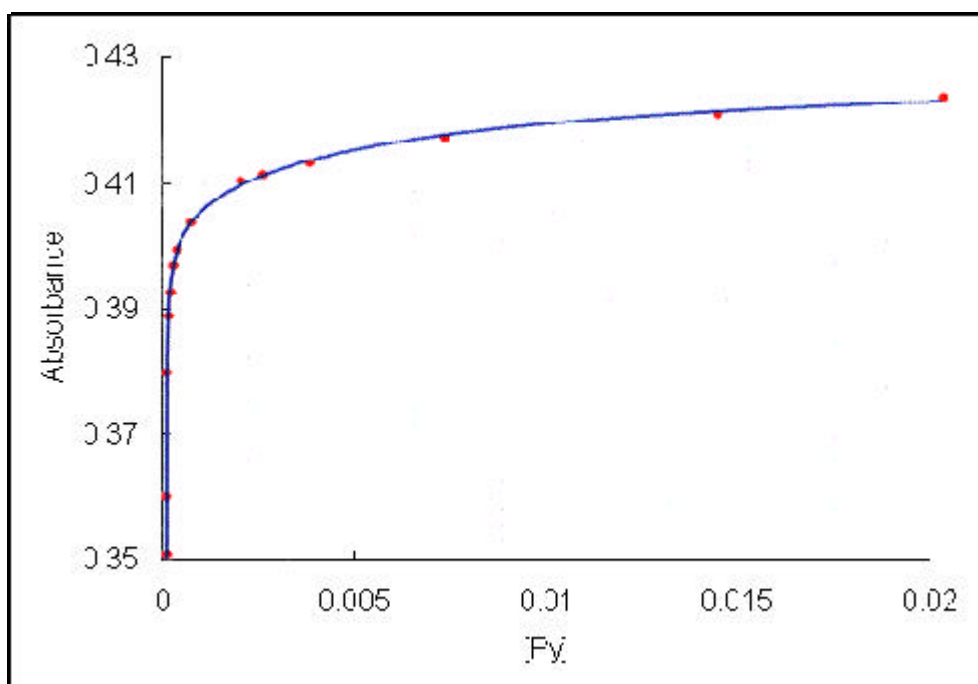


Figure 3.28: Titration data for the binding of Pyridine to $\text{NAc-Co}^{\text{III}}\text{MP8}$ at 420 nm.
(25 °C, $\mu = 1.0 \text{ M}$)

Table 3.6: Formation constants for $\text{NAc-Fe}^{\text{III}}\text{MP8}$, $\text{NAc-Co}^{\text{III}}\text{MP8}$ and $\text{B}_{12\text{a}}$.

Formation Constants	$\text{NAc-Fe}^{\text{III}}\text{MP8}$	$\text{NAc-Co}^{\text{III}}\text{MP8}$	$\text{B}_{12\text{a}}$
<i>N</i>-methylimidazole			
$\log K_1$	4.17 ± 0.01	5.8 ± 0.9	4.63 ± 0.05
$\log K_2$	-	3.6 ± 0.8	-
Pyridine			
$\log K_1$	2.62	4.6 ± 0.8	1.23 ± 0.07
$\log K_2$	-	2.6 ± 0.8	

Munro *et al.*²²⁸ reported the $\log K_1$ for the reaction between *N*-methylimidazole with $\text{NAc-Fe}^{\text{III}}\text{MP8}$ to be 4.17 ± 0.01 . Marques *et al.*¹⁴⁴ reported the $\log K_1$ for the reaction between *N*-methylimidazole with aquacobalamin to be 4.63 ± 0.05 . These values of $\log K_1$ for $\text{NAc-Co}^{\text{III}}\text{MP8}$ with *N*-methylimidazole are significantly higher than those reported for $\text{NAc-Fe}^{\text{III}}\text{MP8}$ and $\text{B}_{12\text{a}}$. Munro *et al.*²²⁸ reported the $\log K_1$ for the reaction between

pyridine with $\text{NAC-Fe}^{\text{III}}\text{MP8}$ to be 2.62. Knapton *et al.*²²⁹ reported the $\log K_1$ for the reaction between pyridine with aquacobalamin to be 1.23 ± 0.07 . Similarly, the values of $\log K_1$ for $\text{NAC-Co}^{\text{III}}\text{MP8}$ with pyridine are significantly higher than those reported for $\text{NAC-Fe}^{\text{III}}\text{MP8}$ and $\text{B}_{12\text{a}}$.

Although $\log K_2$ is not reported in the above studies, the formation of a bis-complex is well established in MP8 chemistry. Munro and coworkers found that in their ligand binding studies of $\text{NAC-Fe}^{\text{III}}\text{MP8}$ with pyridine and other amines, there were multiple equilibria observed.²²⁸ They also observed that there was a clear relationship between the basicity of the incoming ligand (as measured by its pK_a) and the $\log K$ for the binding of that ligand by $\text{NAC-Fe}^{\text{III}}\text{MP8}$. Thus, the affinity of the ligand toward Fe(III) in an iron porphyrin parallels its affinity toward the proton. This was evident for primary amines and pyridines. A similar observation was made by Pratt *et al.*²³⁰ In B_{12} chemistry precedents also exist for substitution of axial base by an exogenous ligand.²²⁹ Marques and coworkers have made an outstanding contribution and have performed numerous studies on aquacobalamin with a variety of ligands which all show that only the axial base was replaced and that ligand substitution proceeds through a dissociative interchange mechanism.^{143-146, 148-155}

In Table 3.6 we see that the value of formation constants formed between the selected ligands and $\text{NAC-Co}^{\text{III}}\text{MP8}$ are higher than those formed for $\text{NAC-Fe}^{\text{III}}\text{MP8}$ and $\text{B}_{12\text{a}}$. It has already been established that the porphyrin cavity is larger and less flexible than the corrin cavity and that the Co(III) ion is smaller than Fe(III) ion.^{7, 23} In $\text{NAC-Co}^{\text{III}}\text{MP8}$ we have a small Co(III) ion in a large porphyrin cavity. This results in weaker bonds being formed between cobalt and the porphyrin and the axial and proximal residues with the metal centre (due to longer bond distances). This consequently leads to bis-substitution in $\text{NAC-Co}^{\text{III}}\text{MP8}$. However, in $\text{NAC-Fe}^{\text{III}}\text{MP8}$ and $\text{B}_{12\text{a}}$ we observe only mono-substitution, which strongly suggests that the Co(III)-His bond in $\text{NAC-Co}^{\text{III}}\text{MP8}$ is weaker than the same bond in $\text{B}_{12\text{a}}$ and the Fe(III)-His bond in $\text{NAC-Fe}^{\text{III}}\text{MP8}$. This result is unexpected and clearly merits further, and more detailed investigation. It was not the principal focus of this investigation which was merely to show that investigations are

feasible or not. Overall, it is not clear that as to whether the size of the macrocyclic cavity and the identity of the central metal play an important role in the chemistry that occurs at the metal centre.

3.2.5 Kinetic Studies of the Reaction of Selected N-Donor Ligands with $\text{NAC-Co}^{\text{III}}\text{MP8}$

The pseudo-first order rate constants were determined for the reaction between $\text{NAC-Co}^{\text{III}}\text{MP8}$ and *N*-methylimidazole (NMI) and methylamine (MeNH_2) at pH 7.063 and 8.011, respectively. As mentioned earlier, the pseudo-first order rate constants, k_{obs} , were determined by curve fitting the absorbance versus time traces to Equation 2.8 using non-linear least-squares program employing a Newton-Raphson procedure. It has been well established that the concentration of ligand in solution is dependent on the pH. Consequently, k_{obs} values had to be converted into pH-independent rate constants using Equation 3.2.

$$k_{\text{corrected}} = k_{\text{obs}}(1 + 10^{\text{pK}_a - \text{pH}}) \quad (3.2)$$

In the reaction between $\text{NAC-Co}^{\text{III}}\text{MP8}$ with *N*-methylimidazole we initially noticed that the data obtained did not follow convention pseudo-first order kinetics; instead there was some evidence for biphasic kinetics. Table 3.4 below shows the data obtained when these data was fitted to Equation 2.8. The data show that there are two rate constants; we proposed the first rate constant, k_1 , to be the rate at which the first incoming ligand displaces water and the second, k_1' , to be the rate constant for another incoming ligand which displaces the histidine to form a bis-complex.

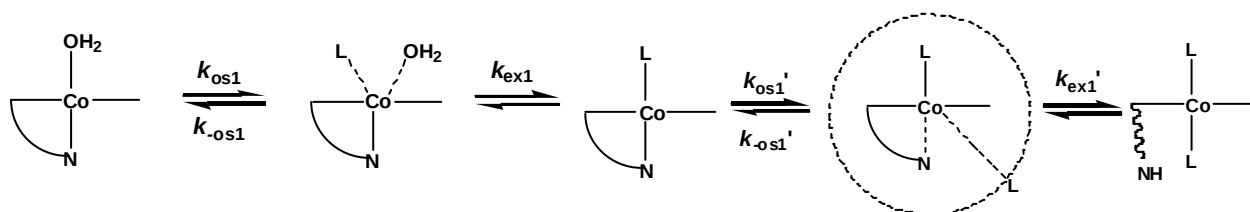


Figure 3.29: Proposed kinetics of ligand substitution for $\text{NAC-Co}^{\text{III}}\text{MP8}$.

In Figure 3.29, k_{os1} and k_{os1}' represents the rate of formation of the outersphere complexes and, k_{ex1} and k_{ex1}' represents the exchange rate constants for the first and second ligand substitution, respectively. The derivation of Equations 3.3 and 3.4 is presented in the Appendix E.

$$k_1 = K_{os1} * k_{ex1} \quad (3.3)$$

$$k_1' = K_{os1}' * k_{ex1}' \quad (3.4)$$

Table 3.7: Kinetics of $NAC-Co^{III}MP8$ with NMI.

[NMI] / M	k_{1obs} / s^{-1}	k_1 / s^{-1*}	k_{1obs}' / s^{-1}	k_1' / s^{-1*}
0.0503	2.05E-04	4.92E-04	4.42E-06	1.06E-05
0.1006	3.21E-04	7.70E-04	2.38E-05	5.74E-05
0.20121	2.87E-04	6.89E-04	4.86E-05	1.17E-04
0.5030	1.93E-04	4.63E-04	2.74E-05	6.59E-05
0.7604	2.60E-04	6.25E-04	5.04E-05	1.21E-04
0.8048	2.07E-04	4.98E-04	2.79E-05	6.71E-05
1.0060	2.40E-04	5.76E-04	2.32E-05	5.59E-05

* Corrected for pH using Equation 3.2

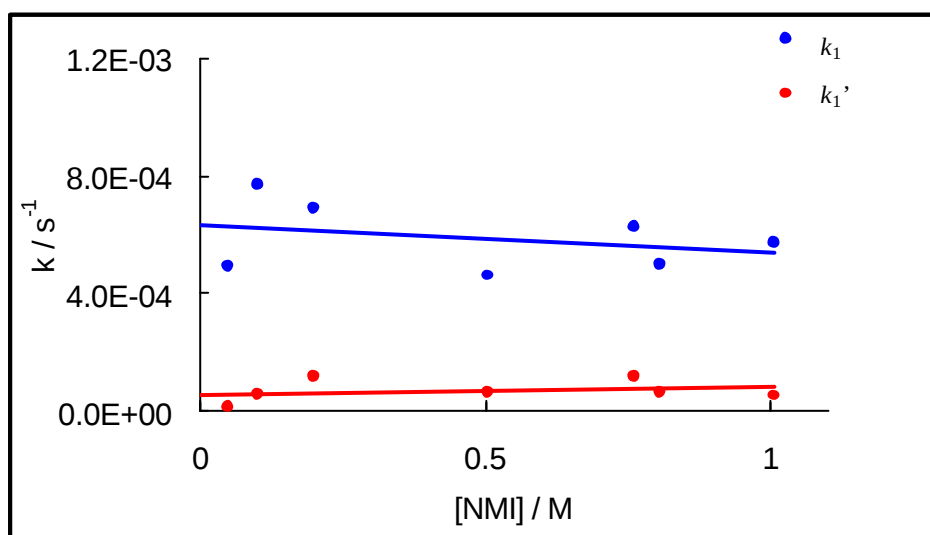


Figure 3.30: Plot of [NMI] against the observed rate constants for the reaction with $NAC-Co^{III}MP8$.

The plot of k versus $[NMI]$ in Figure 3.30 shows that the rate at which ligand substitution occurs is independent of the concentration of the incoming ligand within experimental error.

The results can be explained if the mechanism proceeds through a purely dissociative mechanism, i.e., if the rate of the reaction is controlled by the rate at which, firstly, the water molecule dissociates from the Co^{III} centre and, secondly, the histidine dissociates from the metal. Hence, Figure 3.29 should be modified as shown in Figure 3.31.

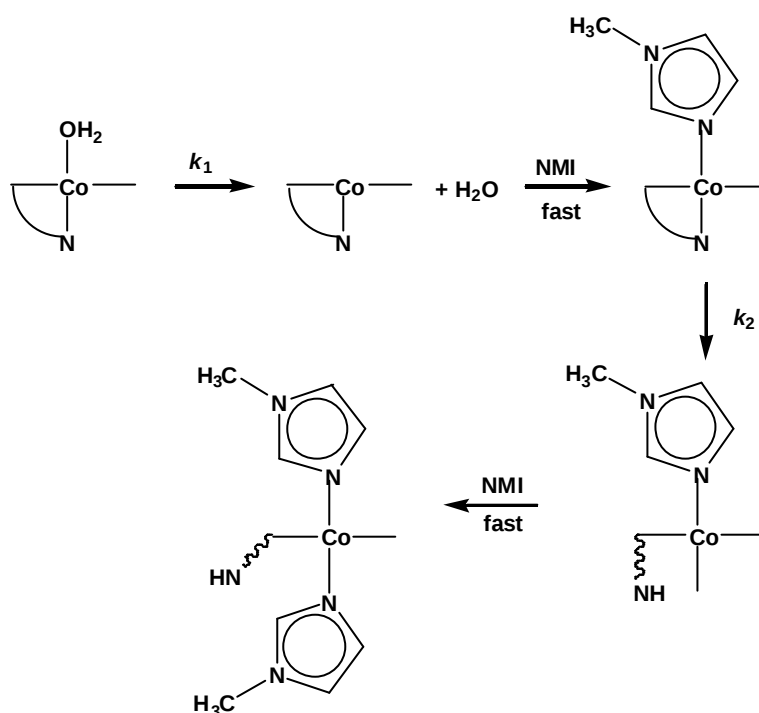


Figure 3.31: Proposed kinetics of ligand substitution of NMI with $\text{NAc-Co}^{\text{III}}$ MP8.

This reaction depicts a dissociative mechanism (D) since there is no nucleophilic participation of the incoming ligand in the transition state.^{147, 155} The second order rate constant, k_2 , cannot be determined since the rate at which the reaction occurs is independent of *N*-methylimidazole concentration.

Similarly, Table 3.8 below shows the two rate constants obtained when the data for the reaction between $\text{NAc-Co}^{\text{III}}$ MP8 with methylamine. The $\text{p}K_a$ of methylamine is 10.941.

As before the k_{obs} values had to be converted into pH-independent rate constants using Equation 3.2.

Table 3.8: Kinetics of $\text{NAC-Co}^{\text{III}}\text{MP8}$ with MeNH_2 .

$[\text{MeNH}_2] / \text{M}$	$k_{1\text{obs}} / \text{s}^{-1}$	$k_1 / \text{s}^{-1} *$	$k_{1\text{obs}}' / \text{s}^{-1}$	$k_1' / \text{s}^{-1} *$
0.501	9.79E-03	8.34	4.47E-02	38.09
1.001	1.56E-02	13.29	6.05E-02	51.55
1.502	2.26E-02	19.26	4.07E-01	346.82
1.753	3.27E-02	27.86	1.62E-01	138.05
2.003	2.44E-02	20.79	4.38E-01	337.24
2.253	4.78E-02	40.73	3.99E-01	340.00
2.504	3.58E-02	30.51	4.29E-01	365.57

* Corrected for pH using Equation 3.2

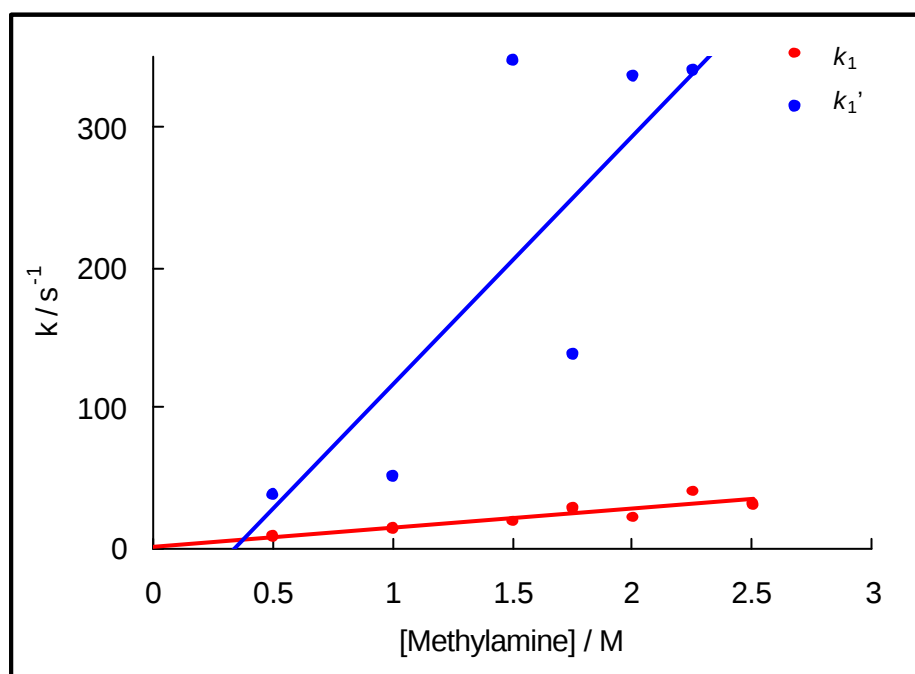


Figure 3.32: Plot of $[\text{MeNH}_2]$ against the observed rate constants for the reaction with $\text{NAC-Co}^{\text{III}}\text{MP8}$.

The plot of k versus $[\text{MeNH}_2]$ in Figure 3.32 for the rate constants, k_1 and k_1' , are dependent on the concentration and participation of the incoming ligand and we propose that it involves the displacement of water and histidine residue, respectively. Thus a dissociative interchange (I_d) mechanism may be operating in both cases.¹⁵⁵ We propose that the displacement of water and histidine by methylamine involves an interchange mechanism (I_d), where the bond forming and bond breaking occur simultaneously, and thus the rate of reaction becomes dependent on the concentration of the incoming ligand.

Similarly, the slopes of the above plot gave the second order rate constants, k_2 and k_2' , to be $13 \pm 3 \text{ M}^{-1} \text{ s}^{-1}$ and $177 \pm 51 \text{ M}^{-1} \text{ s}^{-1}$. In the reaction of *N*-methylimidazole with $\text{NAC-Co}^{\text{III}}\text{MP8}$, we observe that the reaction is independent of ligand concentration and therefore must proceed through a dissociative (D) mechanism. The results show that the rate of reaction for methylamine being faster than *N*-methylimidazole with $\text{NAC-Co}^{\text{III}}\text{MP8}$. These differences in rate constants may be attributed to the size of the incoming ligands. *N*-methylimidazole is a secondary amine and is relatively more bulky than methylamine which is a primary amine; therefore it is easier for methylamine to attach to the metal centre compared to *N*-methylimidazole.

3.2.6 Kinetic Studies of Selected N-Donor Ligands by B_{12a}

The kinetics involving the ligand substitution of imidazole¹⁴⁴ and methylamine¹⁴⁶ with aquacobalamin has been reported by Marques and coworkers. However, we have decided to repeat these studies under the different conditions so as to have comparative data that are relevant to our project. The pseudo-first order rate constants were determined for the reaction between B_{12a} with *N*-methylimidazole and methylamine at pH 7.063 and 7.951, respectively. Similarly, as a result of the reactions being performed at different pH, k_{obs} values had to be converted into pH-independent rate constants using Equation 3.2. The usually inert Co^{III} -centre is exceptionally labile and the axial water ligand easily undergoes substitution with a variety of ligands as shown below in Figure 3.33.

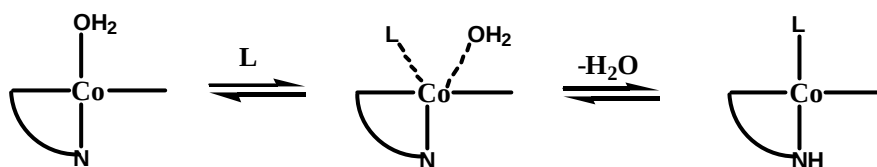


Figure 3.33: Shows schematic of ligand substitution in B_{12a}.¹⁵⁵

Table 3.9 below shows the calculated rate constants obtained when the data for the reaction between B_{12a} with *N*-methylimidazole were fitted to Equation 2.9.

Table 3.9: Kinetics of B_{12a} with NMI.

[NMI] / M	$k_{\text{obs}} / \text{s}^{-1}$	k_1 / s^{-1}
0.00010055	2.23E-03	5.36E-03
0.0001508	2.54E-03	6.11E-03
2.01E-04	2.96E-03	7.11E-03
2.51E-04	3.34E-03	8.02E-03
3.02E-04	3.70E-03	8.90E-03
3.52E-04	2.93E-03	7.03E-03
4.02E-04	4.72E-03	1.13E-02
0.0005208	5.53E-03	1.33E-02
6.03E-04	6.04E-03	1.45E-02
7.04E-04	7.01E-03	1.69E-02
0.0008044	7.48E-03	1.80E-02
0.000905	9.01E-03	2.16E-02
0.0010055	1.04E-02	2.50E-02
0.001207	1.14E-02	2.73E-02
1.41E-03	1.28E-02	3.09E-02
1.61E-03	1.62E-02	3.90E-02
0.00181	1.68E-02	4.04E-02
0.002011	1.98E-02	4.76E-02

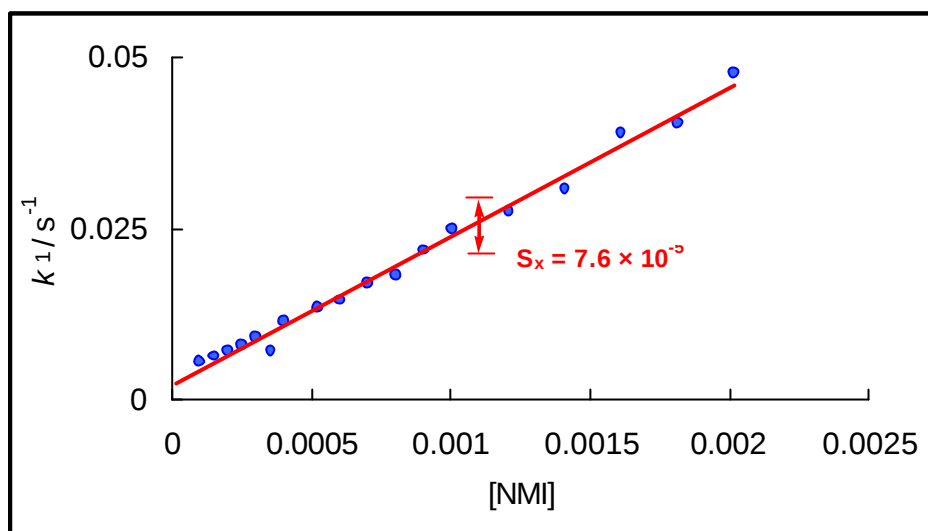


Figure 3.34: Plot of [NMI] against the observed rate constants for the reaction with B_{12a} .

The slope of the plot of k_1 versus [NMI] in Figure 3.34 shows that the rate at which ligand substitution occurs is dependant on the concentration of the incoming ligand. We propose that it involves the displacement of water and that a dissociative interchange (I_d) mechanism may be operating.¹⁵⁵ The second order rate constant, k_2 , for the reaction between *N*-methylimidazole and B_{12a} is $21.82 \pm 0.53 \text{ M}^{-1} \text{ s}^{-1}$. However, Marques *et al.*¹⁴⁴ reported a second order rate constant, k_2 , for the same reaction to be $16.6 \pm 0.3 \text{ M}^{-1} \text{ s}^{-1}$. Marques *et al.*¹⁴⁴ used KCl as an electrolyte and this would have formed a more inert chloro- B_{12a} complex which would account for the lower value obtained.

Table 3.10 below shows the calculated rate constants obtained when the data for the reaction between B_{12a} with methylamine were fitted to Equation 2.9.

Table 3.10: Kinetics of B_{12a} with MeHH₂.

[MeNH ₂]/M	$k_{1\text{obs}} / \text{s}^{-1}$	k_1 / s^{-1}
0.2505	7.24E-01	7.08E+02
0.5010	5.54E-01	5.42E+02
0.7515	1.03E+00	1.01E+03
1.2525	1.70E+00	1.67E+03
1.5030	1.84E+00	1.80E+03
1.7535	2.18E+00	2.13E+03
2.0040	2.21E+00	2.16E+03
2.2545	2.86E+00	2.79E+03
2.5050	2.93E+00	2.86E+03

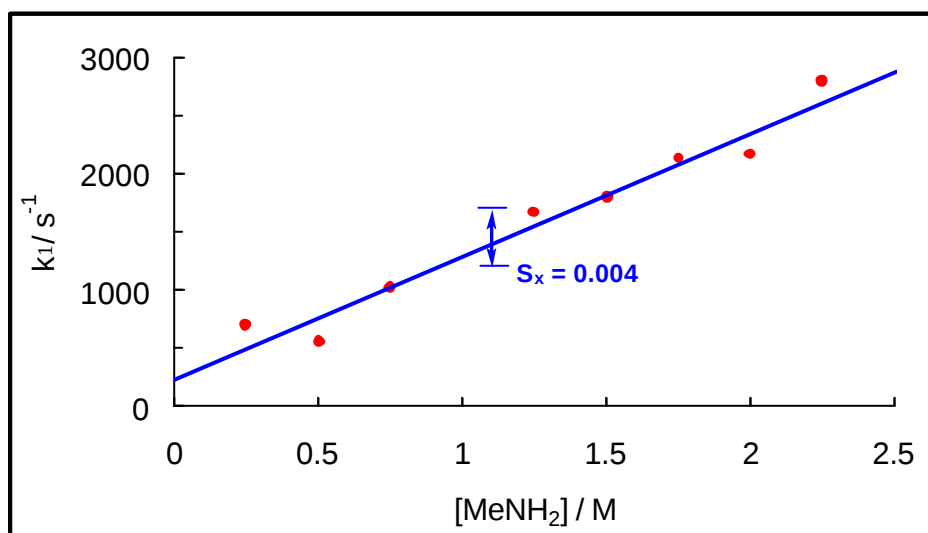


Figure 3.35: Plot of [MeNH₂] against the observed rate constants for the reaction with B_{12a}.

The slope of the plot of k_1 versus $[\text{MeNH}_2]$ in Figure 3.34 shows that the rate at which ligand substitution occurs is dependant on the concentration of the incoming ligand. Similarly, we propose that it involves the displacement of water and that a dissociative interchange (I_d) mechanism may be operating.¹⁵⁵ The second order rate constant, k_2 , for the reaction between methylamine and B_{12a} is $1.052 \pm 0.071 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. In a similar study Marques *et al.*¹⁴⁶ reported the second order rate constant for the same reaction to be 0.927 ± 0.0022 .

Table 3.11: Second order rate constants of $\text{NAC-Co}^{\text{III}}\text{MP8}$ and B_{12a} .

Ligand	Second order rate constant for $\text{NAC-Co}^{\text{III}}\text{MP8} / \text{M}^{-1} \text{ s}^{-1}$	Second order rate constant for $\text{B}_{12a} / \text{M}^{-1} \text{ s}^{-1}$
NMI	-	$k_2 = 16.6$ (a)
	-	$k_2 = 21.82 \pm 0.53$
MeNH ₂	$k_2 = 1.32 \pm 0.3 \times 10^4$	$k_2 = 9.27 \times 10^{-1}$ (b)
	$k_2' = 177 \pm 51$	$k_2 = 1.052 \pm 0.071 \times 10^3$

a – data obtained from reference 144

b – data obtained from reference 146

Table 3.11 shows that in the reaction of $\text{NAC-Co}^{\text{III}}\text{MP8}$ with *N*-methylimidazole and methylamine, two ligand substitutions are observed. In both reactions, we propose that the first rate constant is due to the substitution of the axial water molecule and the second due to the substitution of the proximal histidine residue giving rise to the formation of a bis-complex. Also when comparing the second order rate constants of both ligands with $\text{NAC-Co}^{\text{III}}\text{MP8}$, we observe that the rate of substitution of methylamine is significantly faster than *N*-methylimidazole. This may be attributed to the relative size of the ligands as mentioned earlier. In addition, the reaction of $\text{NAC-Fe}^{\text{III}}\text{MP8}$ with *N*-methylimidazole is independent of ligand concentration which suggests a dissociative mechanism. While

the reaction of $\text{NAC-Fe}^{\text{III}}\text{MP8}$ with methylamine the rate of ligand substitution is dependant on ligand concentration (suggests I_a mechanism).

Although the observed reaction rates of B_{12a} with *N*-methylimidazole compare very well with literature, the observed reaction rate of B_{12a} with methylamine do not. The results show that the rate of the reaction between $\text{NAC-Co}^{\text{III}}\text{MP8}$ and B_{12a} with *N*-methylimidazole and methylamine are significantly different. Overall it is clear that the solution chemistry of $\text{NAC-Co}^{\text{III}}\text{MP8}$ is complex and does not mimic the chemistry of $\text{NAC-Fe}^{\text{III}}\text{MP8}$ nor B_{12a} due to bisubstitution of the cobalt centre.

Chapter 4 Conclusions and Future Work

4.1 Conclusions

4.1.1 The synthesis and characterisation of products leading to the formation of *NAc-Co^{III}MP8*

In this project we synthesised *NAc-Co^{III}MP8* in an attempt to develop a model system to probe the chemistry of vitamin B_{12a}. All products formed during the synthesis of *NAc-Co^{III}MP8* were characterised using HPLC, UV-vis spectroscopy and FAB-MS with good results, where appropriate, that compared well with literature values.

We used the procedure developed by Munro and Marques with some modifications to synthesise *Fe^{III}MP8*.¹⁰⁹ The synthesis of *Fe^{III}MP8* was successfully performed in 55 % yield. The removal of the central *Fe^{III}* ion from *Fe^{III}MP8* was successfully accomplished by a process known as reductive demetalation.⁷⁹ *MP8* free base was successfully synthesised in 73 % yield. The *MP8* free base was further analysed using luminescence spectroscopy and characteristic triplet-triplet absorption band was observed on the emission spectrum. This provided further evidence that the reductive demetalation procedure was successful. *Co^{III}* was successfully inserted into the empty porphyrin cavity of the *MP8* free base using a procedure which was also adopted from the report by Primus and coworkers.⁷⁹ *Co^{III}MP8* was synthesised in 69 % yield. *Co^{III}MP8* was further analysed using luminescence spectroscopy, however, the characteristic triplet-triplet absorption band was not observed on the emission spectrum. The quenching of the triplet-triplet absorption band provided more evidence that remetalation was successful. Finally, acetylation of *Co^{III}MP8* was carried out according to methods developed by Munro and Marques.¹⁰⁹ *NAc-Co^{III}MP8* was synthesised in 58 % yield. Even if the acetylation step was done very early in the study, better yields would not have been obtained, since this would acetylate the nitrogen of the histidine residue instead of Cys-14 residue. The overall yield from *Fe^{III}MP8* to was calculated to be 29 %.

4.1.2 Solution Chemistry of NAc-Co^{III}MP8

We have used UV-vis spectroscopy to fully characterise the solution chemistry of monomeric NAc-Co^{III}MP8 over the pH range. Beer's law studies shows that NAc-Co^{III}MP8 remains monomeric in aqueous solution up to concentrations of at least 35 μ M. The extinction coefficient, ϵ , of NAcCo^{III}MP8 (pH 7.00, 25 °C) in distilled water and 1.0 M NaClO₄ was $1.80 \pm 0.01 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and $1.66 \pm 0.01 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, respectively.

The spectroscopic changes observed for NAc-Co^{III}MP8 during the course of spectrophotometric titration are very similar to those observed for NAc-Fe^{III}MP8,¹⁰⁹ with both being consistent with six successive ionisations. At pH <1.5, we propose that NAcCo^{III}MP8 exists as a six-coordinate diaqua complex. By analogy with NAc-Fe^{III}MP8, we attribute the first ($pK_1 = 2.0 \pm 0.3$) involves coordination of the *c*-terminal carboxylate group (Glu-21) of the appended polypeptide. The second acid range transition ($pK_2 = 2.8 \pm 0.1$) for NAcCo^{III}MP8 probably involves the deprotonation of the cationic His-18 and concomitant replacement of the *c*-terminal carboxylate by the neutral heterocyclic base. We have assigned pK_3 (3.9 ± 0.03) to the ionisation of one of the haem propanoic acid groups. The second haem propanoic acid group is assigned to the ionization with $pK_4 = 7.47 \pm 0.03$ and pK_5 (9.19 ± 0.04) was assigned to the ionisation of the cobalt-bound water molecule. Finally, we attribute pK_6 (12.07 ± 0.03) to the ionisation of the coordinated histidine *trans* to the OH⁻ to form the histidinate complex (His⁻-Co^{III}-OH⁻).

To further delineate the solution chemistry of NAc-Co^{III}MP8, its reaction with selected N-donor ligands was studied by monitoring the dependence of the absorbance at the Soret wavelength on the added ligand concentration. The formation constants between NAc-Co^{III}MP8 and *N*-methylimidazole and pyridine were determined successfully. When comparing the formation constants of NAc-Fe^{III}MP8, NAc-Co^{III}MP8 and B_{12a}, we found that the value of $\log K_1$ for NAc-Co^{III}MP8 is significantly higher than that reported for NAc-Fe^{III}MP8 and B_{12a}. In addition, we observed the formation of a bisubstituted complex in the reaction of NAc-Co^{III}MP8 with the selected N-donor ligands, and only

mono-substituted with $\text{NAC-Fe}^{\text{III}}\text{MP8}$ and $\text{B}_{12\text{a}}$. This strongly suggests that the Co(III)-His bond in $\text{NAC-Co}^{\text{III}}\text{MP8}$ is weaker than the same bond in $\text{B}_{12\text{a}}$ and the Fe(III)-His bond in $\text{NAC-Fe}^{\text{III}}\text{MP8}$.

In the reaction between $\text{NAC-Co}^{\text{III}}\text{MP8}$ with *N*-methylimidazole and methylamine we initially noticed that the data obtained did not follow convention pseudo-first order kinetics; instead there was some evidence for biphasic kinetics. In the reaction of *N*-methylimidazole with $\text{NAC-Co}^{\text{III}}\text{MP8}$, the results can be explained if the mechanism proceeds through a purely dissociative mechanism, i.e., if the rate of the reaction is controlled by the rate at which, firstly, the water molecule dissociates from the Co^{III} centre and, secondly, the histidine dissociates from the metal. The second order rate constant, k_2 , cannot be determined since the rate of reaction is independent of *N*-methylimidazole concentration. In the reaction of methylamine with $\text{NAC-Co}^{\text{III}}\text{MP8}$, the rate constants are dependent on the concentration and participation of the incoming ligand and we propose that it involves the displacement of water and histidine residue, respectively. The results suggest that a dissociative interchange (I_d) mechanism may be operating.¹⁵⁵ We propose that the displacement of water and histidine by methylamine involves an interchange mechanism (I_d), where the bond forming and bond breaking occur simultaneously, and thus the rate of reaction becomes dependant on the concentration of the incoming ligand.

Overall, the results show that the rate of reaction for methylamine with $\text{NAC-Co}^{\text{III}}\text{MP8}$ being faster than *N*-methylimidazole. As mentioned earlier, these differences in rate constants may be attributed to the size of the incoming ligands. The rate of reaction of $\text{B}_{12\text{a}}$ with *N*-methylimidazole and methylamine was also determined. Although the observed reaction rates of $\text{B}_{12\text{a}}$ with *N*-methylimidazole compare very well with literature, the observed reaction rate of $\text{B}_{12\text{a}}$ with methylamine do not. The results show that the rate of the reaction between $\text{NAC-Co}^{\text{III}}\text{MP8}$ and $\text{B}_{12\text{a}}$ with methylamine is significantly different.

The results obtained from the Beer's law studies and pK_a determinations of *N*Ac-Co^{III}MP8 are in excellent agreement with similar studies performed with *N*Ac-Fe^{III}MP8. However, it is clear that the ligand substitution and kinetic studies of *N*Ac-Co^{III}MP8 is complicated due to the formation of a bis-complex. The results presented in this work do give a general indication on how thermodynamically stable a Co^{III} ion is in a porphyrin ring and also to a very limited extent show that a porphyrin does not confer the same kinetic lability on the Co^{III} ion as the corrin ring.

4.2 Future Work

This work sets the foundation for future endeavours where much more in depth studies of thermodynamics of ligand coordination, kinetics of substitution processes, molecular modeling, reduction and self-exchange reactions, catalytic studies and various other studies on the solution chemistry of *N*-Ac-Co(III)MP8 can be unraveled.

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Appendices

Appendix A: HPLC Data

Table A1: HPLC analysis of MP8 in pH 7.00 buffer at $\lambda = 397\text{nm}$

Peak No.	Area %	RT	Area	BC
1	95	6.45	217600	01
2	5	6.15	11453	0.1
Total	100		229053	

Table A2: HPLC analysis of MP8 free base in pH 7.00 buffer at $\lambda = 403\text{nm}$

Peak No.	Area %	RT	Area	BC
1	1.689	4.63	1389	0.1
2	3.525	5.38	2899	02
3	9.235	5.64	7595	02
4	80.592	5.99	66279	02
5	4.959	6.49	4078	03
Total	100		82240	

Table A3: HPLC analysis of Co(III)MP8 in pH 7.00 buffer at $\lambda = 403\text{nm}$

Peak No.	Area %	RT	Area	BC
1	79.268	6.82	52448	02
2	9.484	7.43	6275	02
3	11.248	8.42	7442	03
Total	100		66165	

Table A4: HPLC analysis of *N*-Ac-Co(III)MP8 in pH 7.00 buffer at $\lambda = 397\text{nm}$

Peak No.	Area %	RT	Area	BC
1	1.844	5.21	1678	02
2	4.249	5.47	3867	02
3	66.103	5.99	60164	03
4	26.618	6.59	24226	01
5	1.187	7.05	1080	01
Total	100		91015	

Appendix B: FAB-MS Data

FAB-MS for MP8

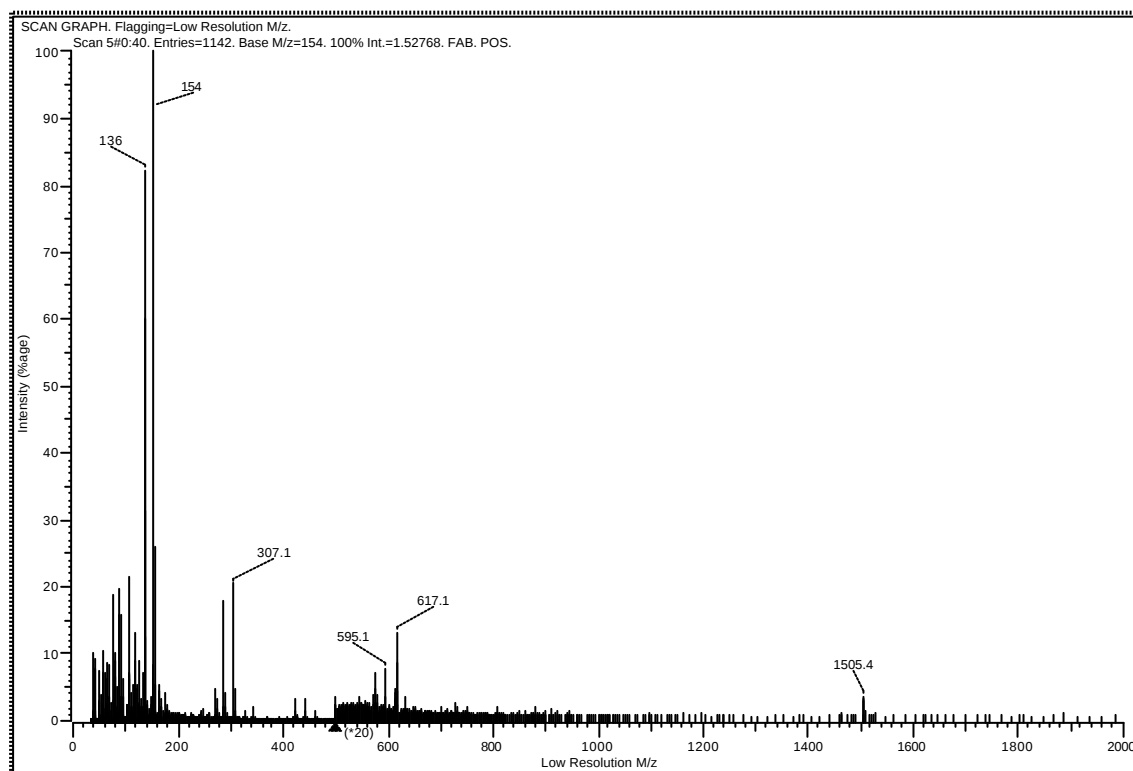


Figure B1: FAB-MS spectra of MP8 sample and matrix.

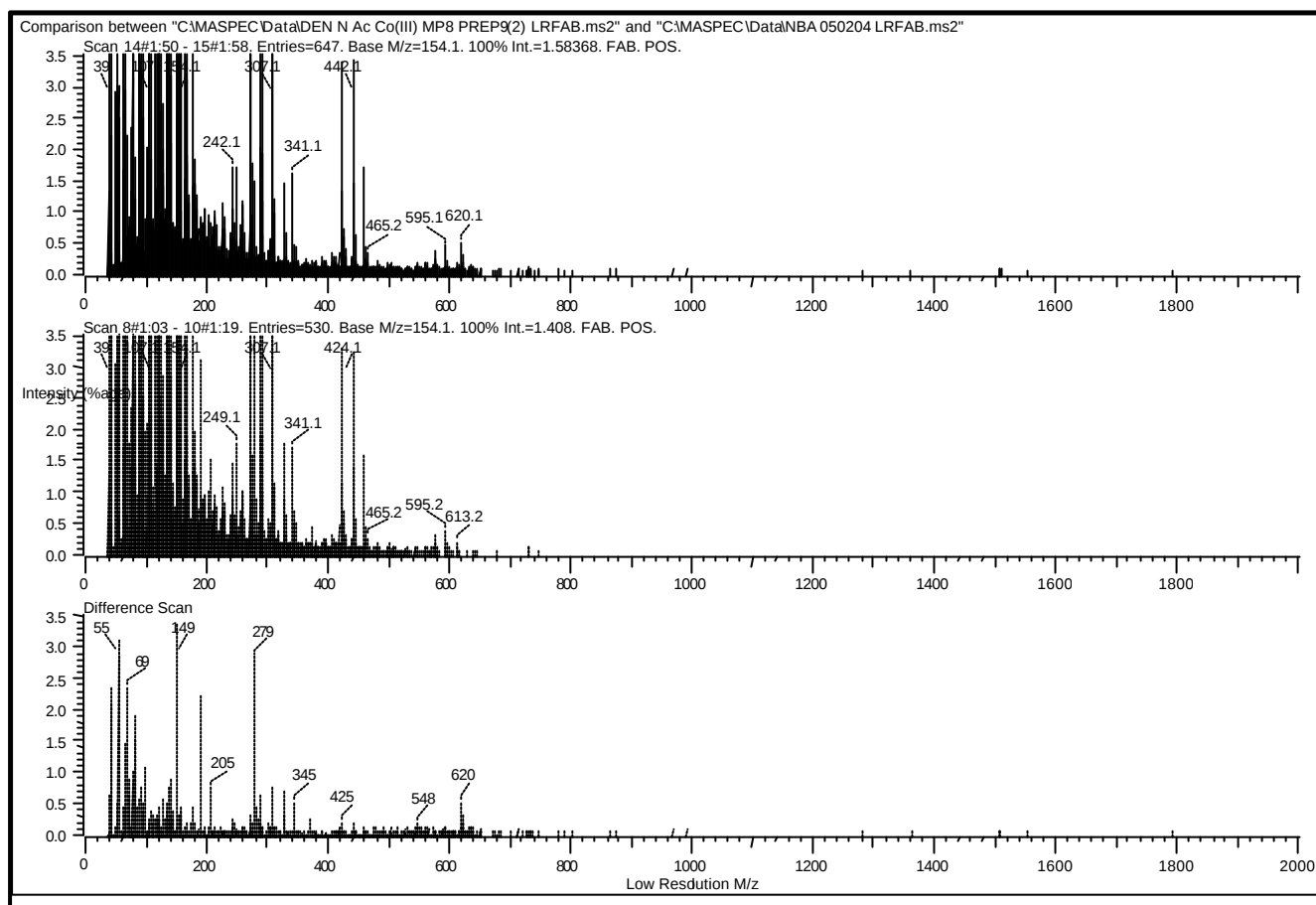


Figure B2: FAB-MS spectra of sample with matrix subtracted (bottom spectra).

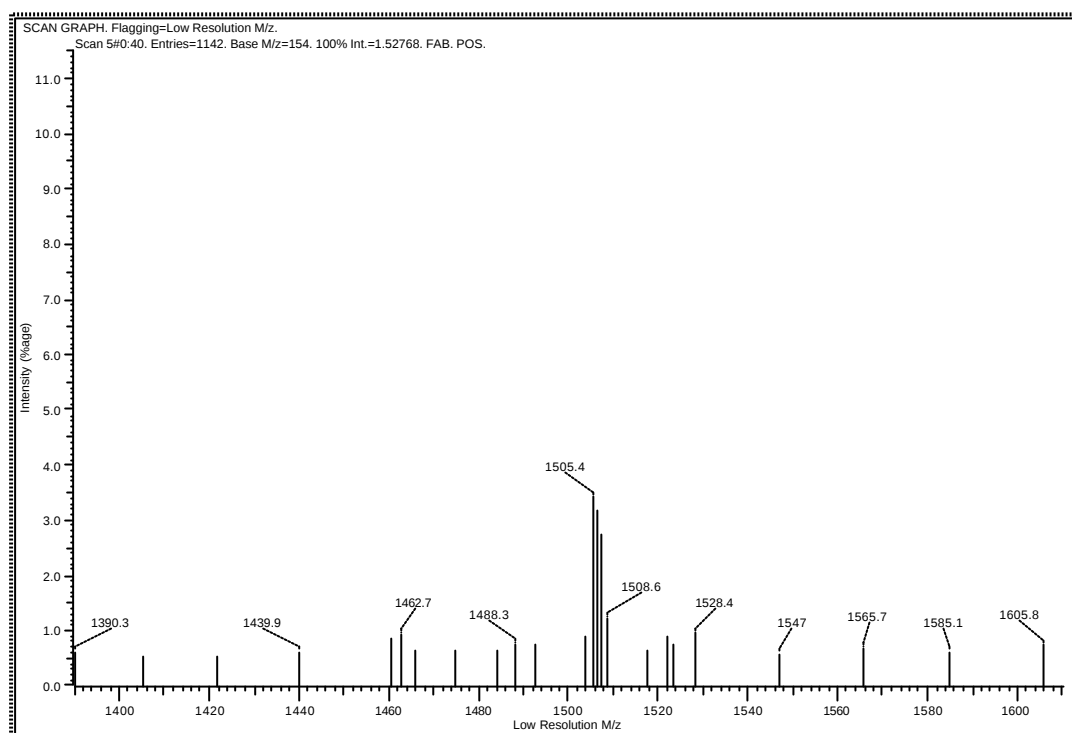


Figure B3: Isotopic distribution of sample.

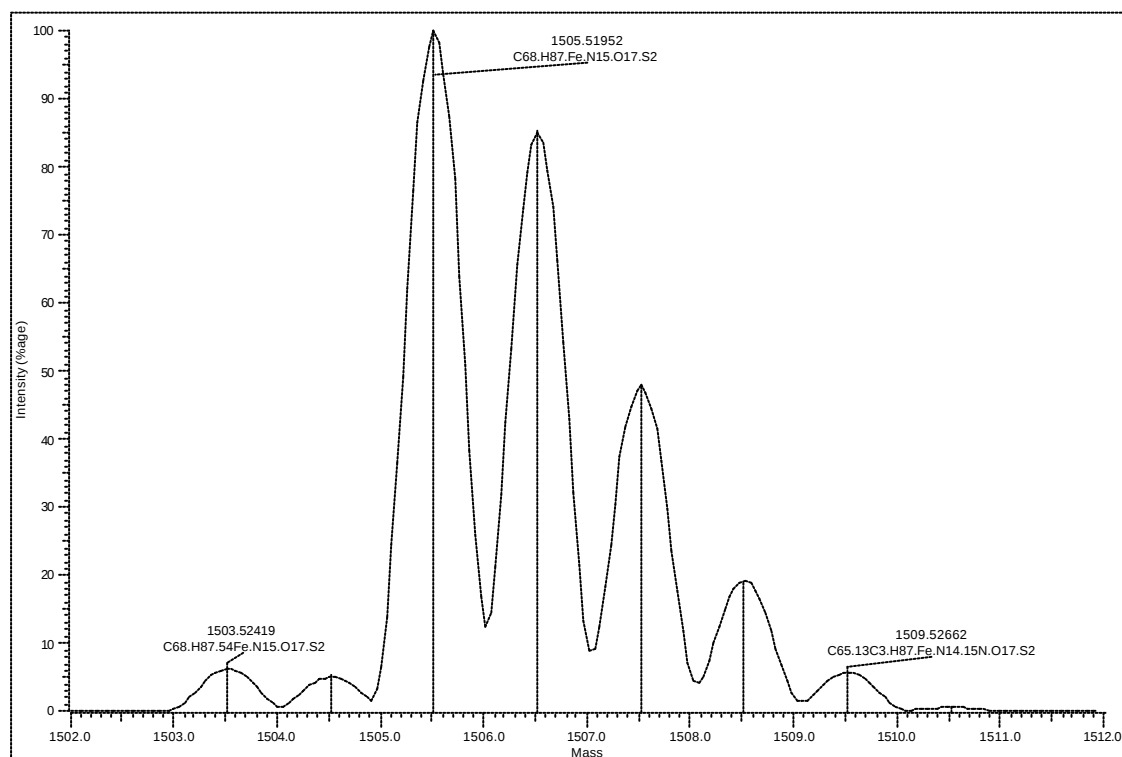


Figure B4: Theoretical isotopic distribution of sample.

FAB-MS for MP8 Free Base

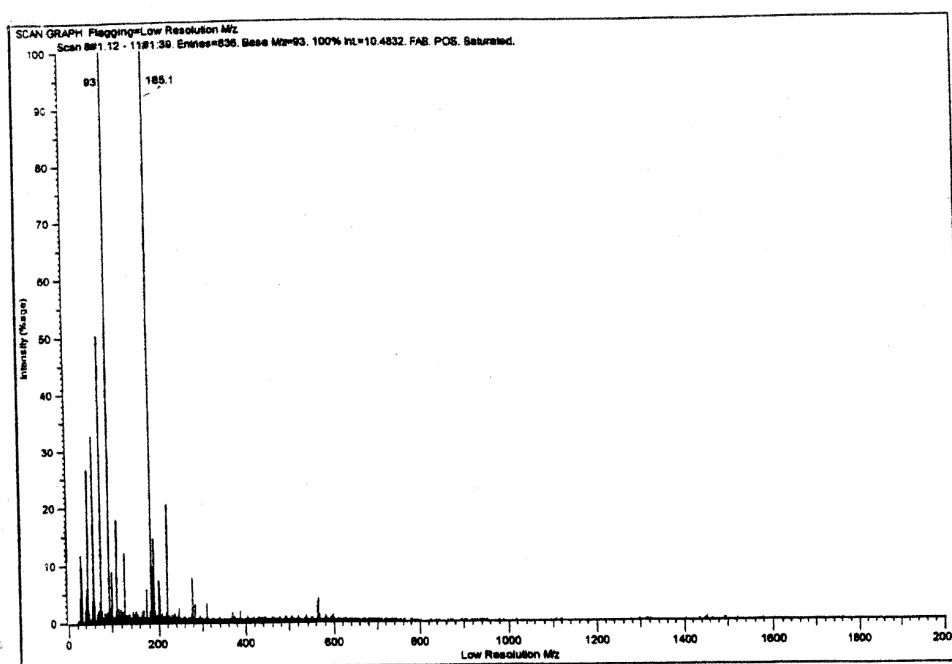


Figure B6: FAB-MS spectra of MP8 free base sample and matrix.

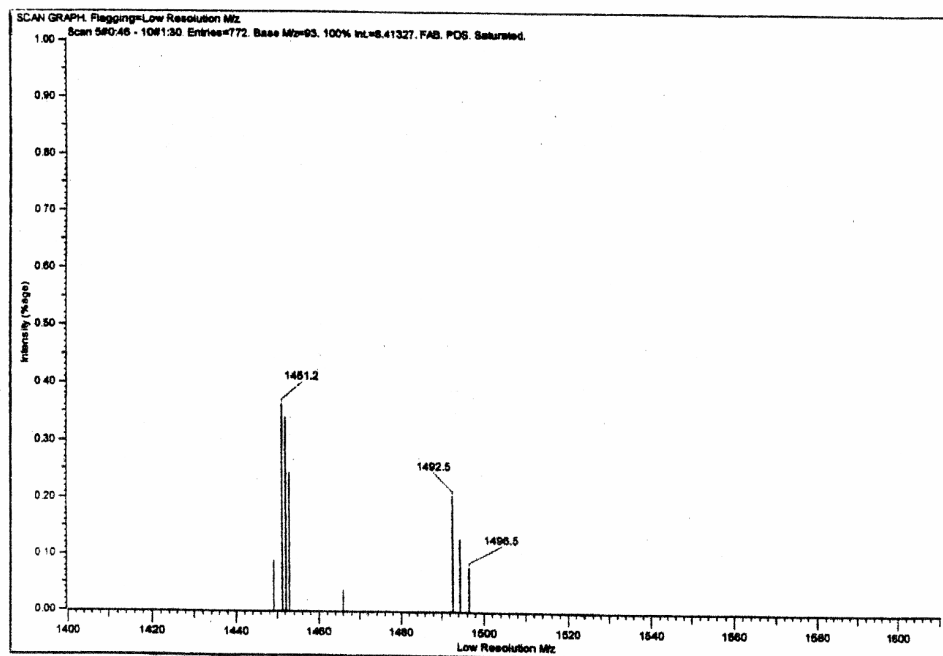


Figure B7: FAB-MS spectra of MP8 free base sample and matrix (enlarged view).

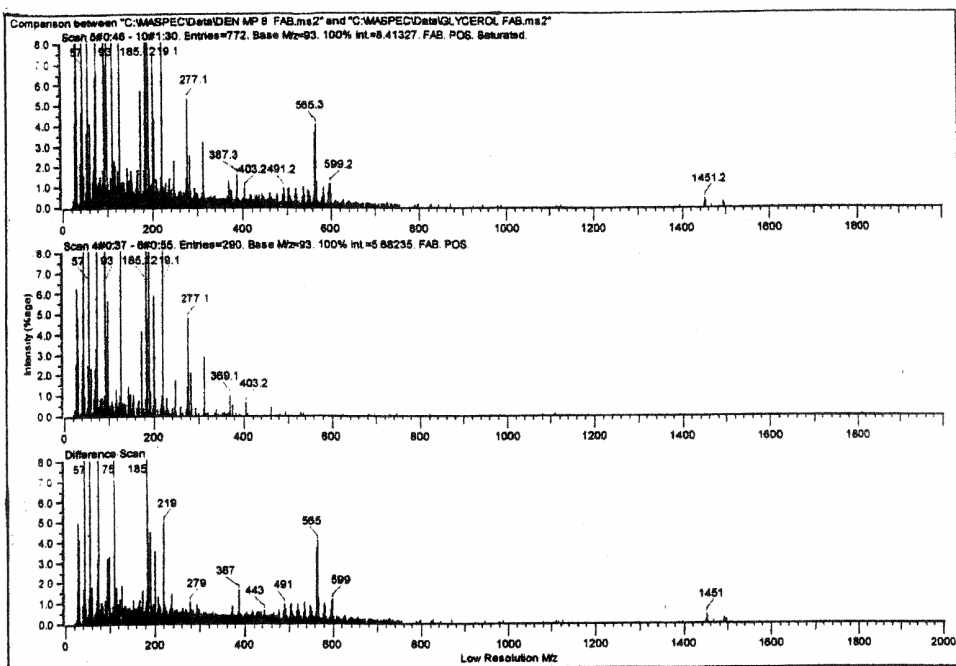


Figure B8: FAB-MS spectra of MP8 free base sample with matrix subtracted (bottom spectra).

FAB-MS for Co(III)MP8

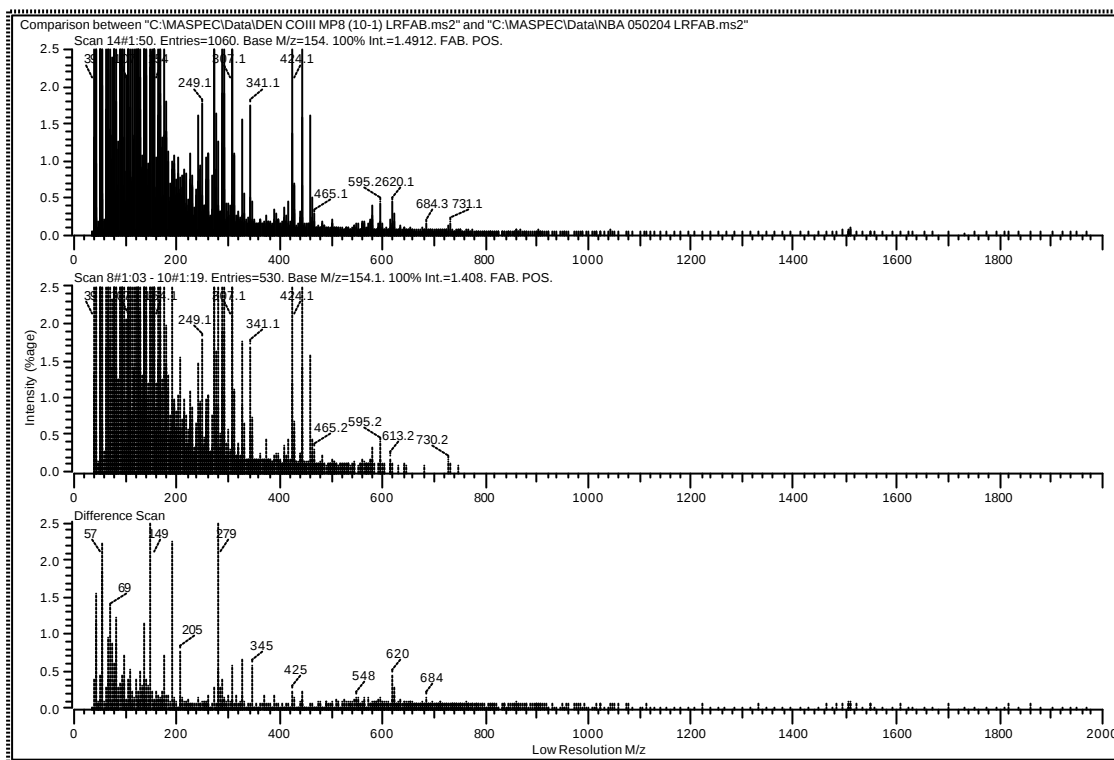


Figure B8: Fab spectra of sample and matrix.

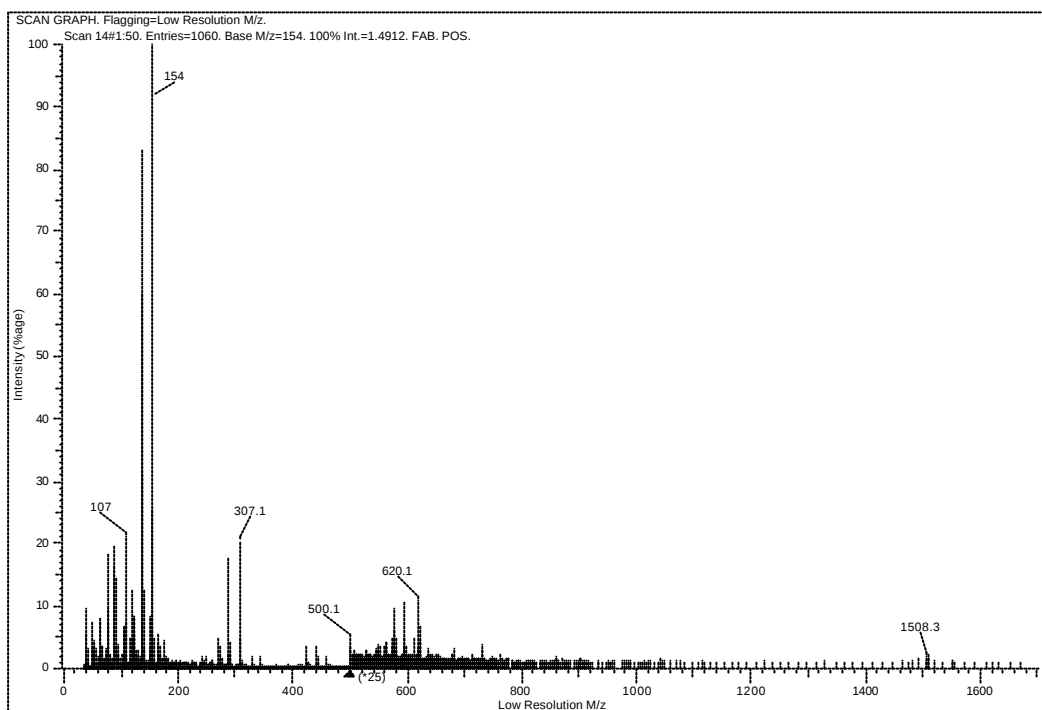


Figure B9: Fab spectra of sample with matrix subtracted(bottom spectra).

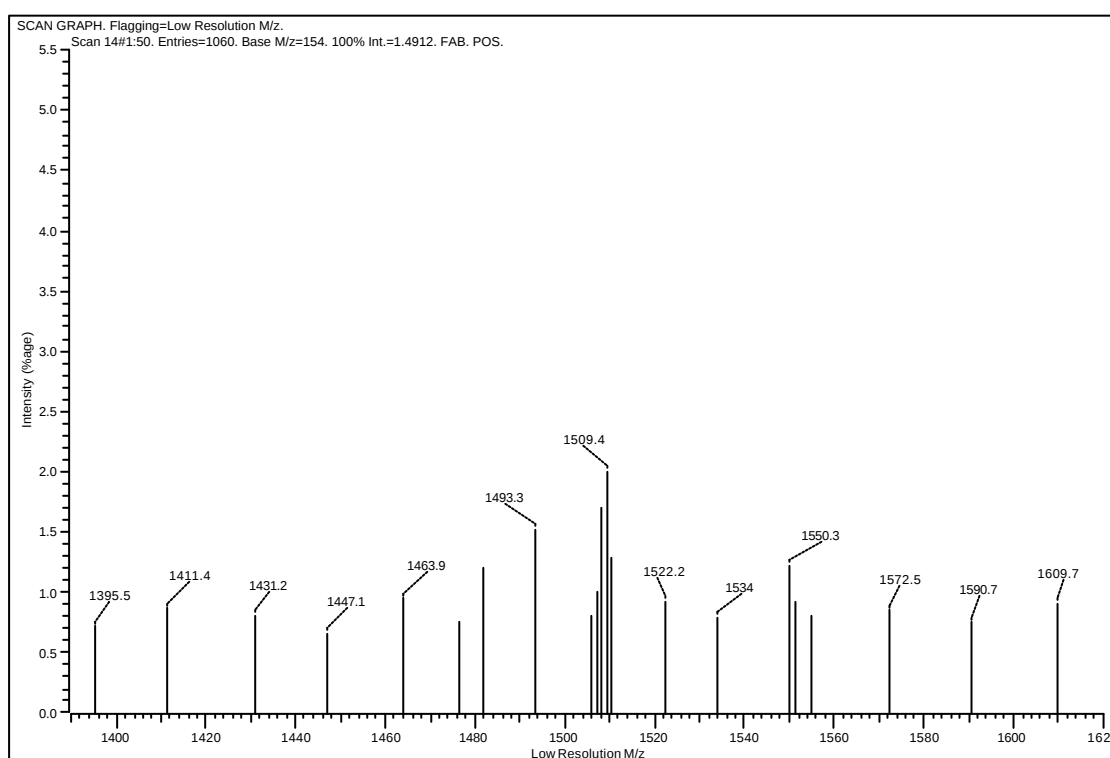


Figure B10: Isotopic distribution of sample.

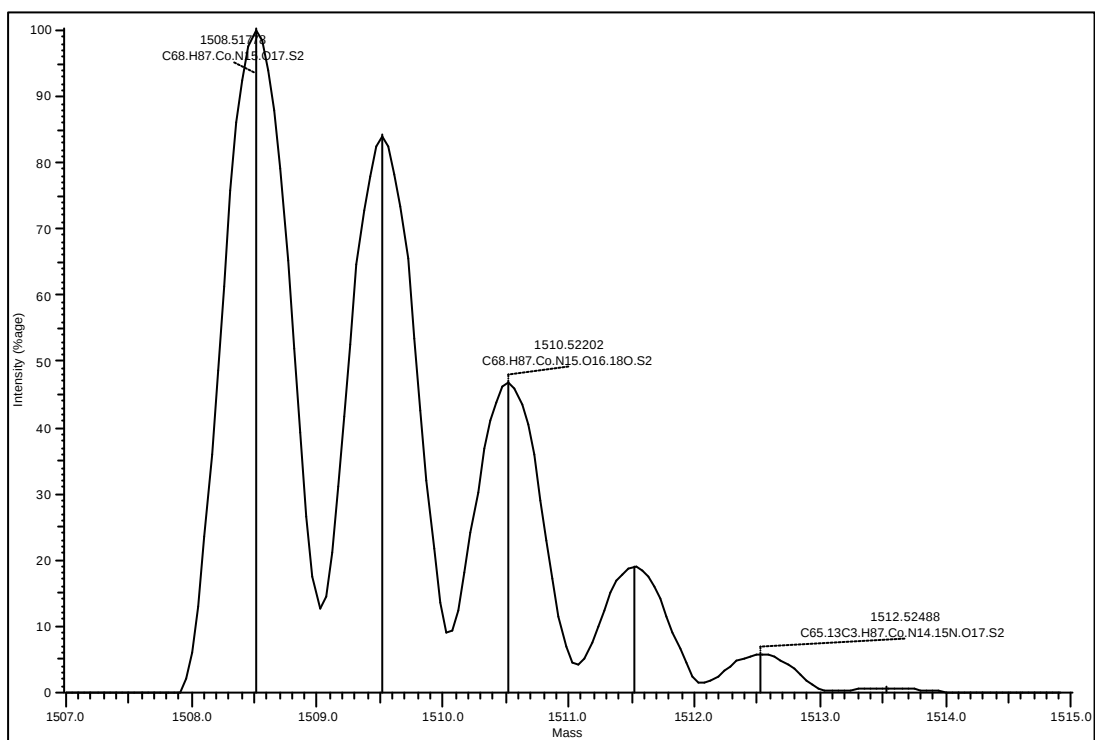


Figure B11: Theoretical isotopic distribution of sample.

FAB-MS for $\text{NAC-Co}^{\text{III}}$ -MP8

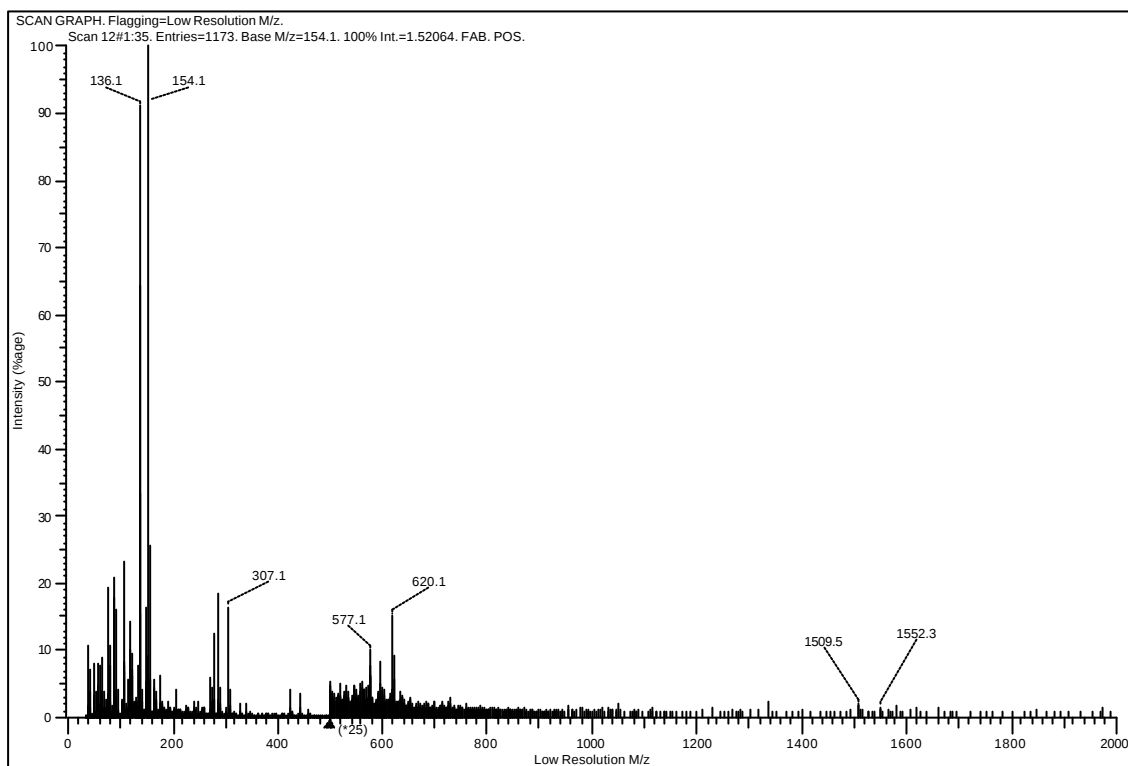


Figure B12: Fab spectra of sample and matrix.

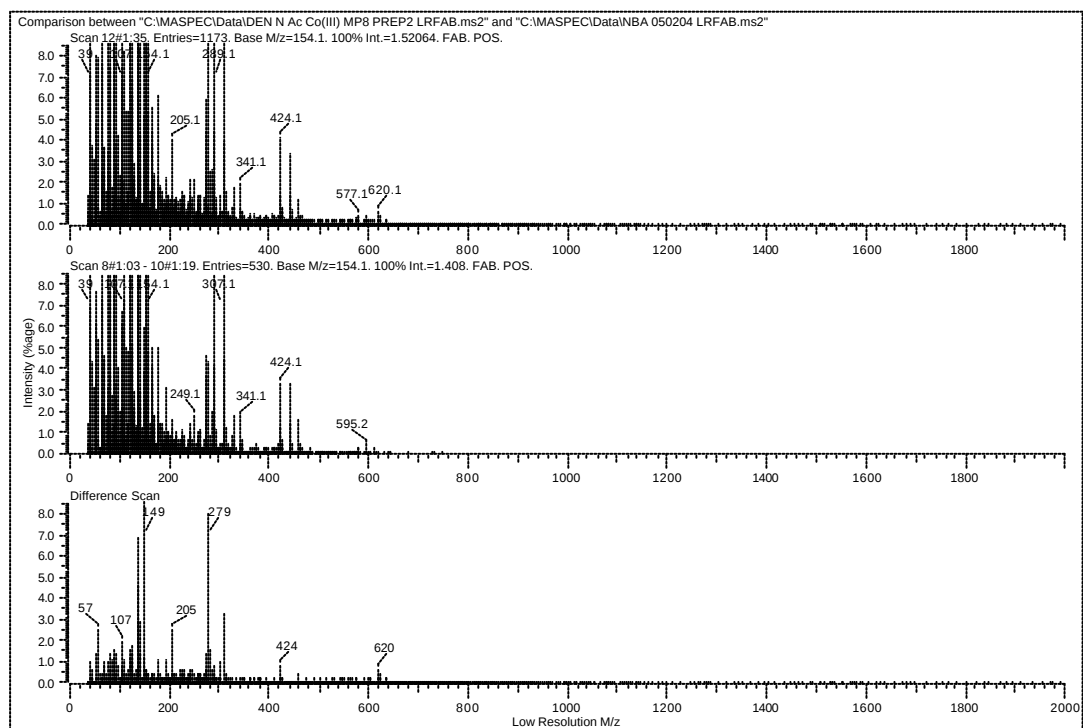


Figure B13: Fab spectra of sample with matrix sub tracked(bottom spectra).

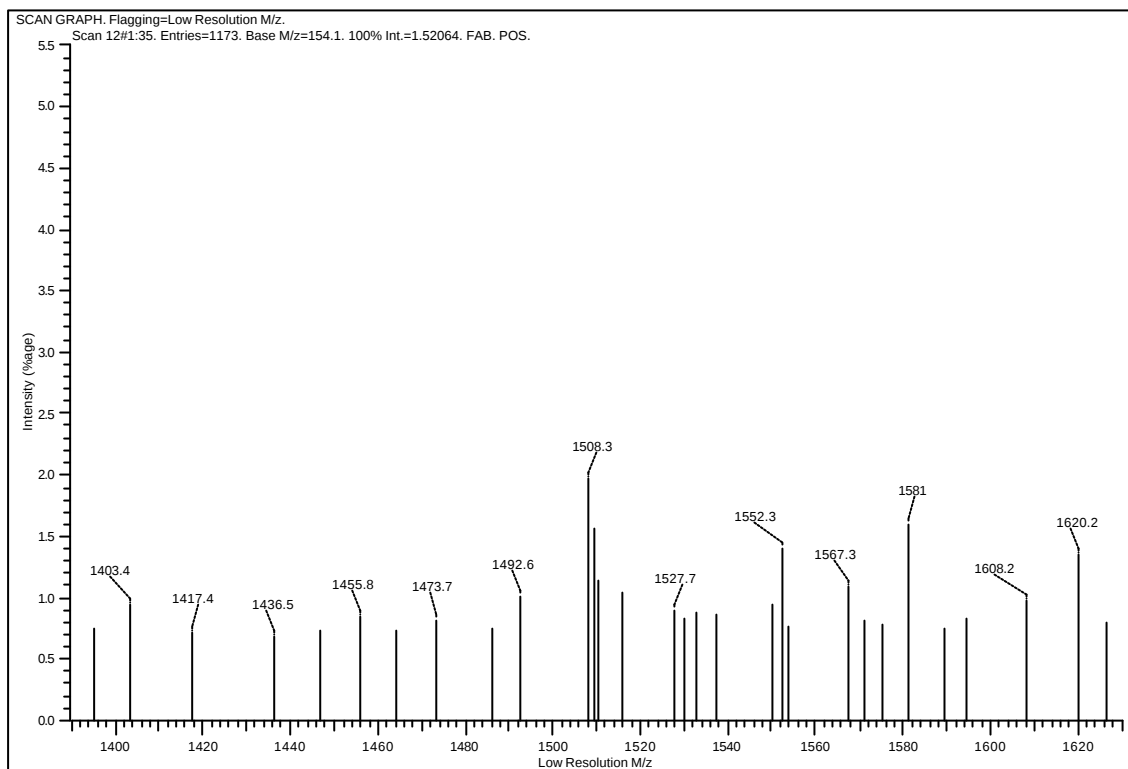


Figure B14: Isotopic distribution of sample.

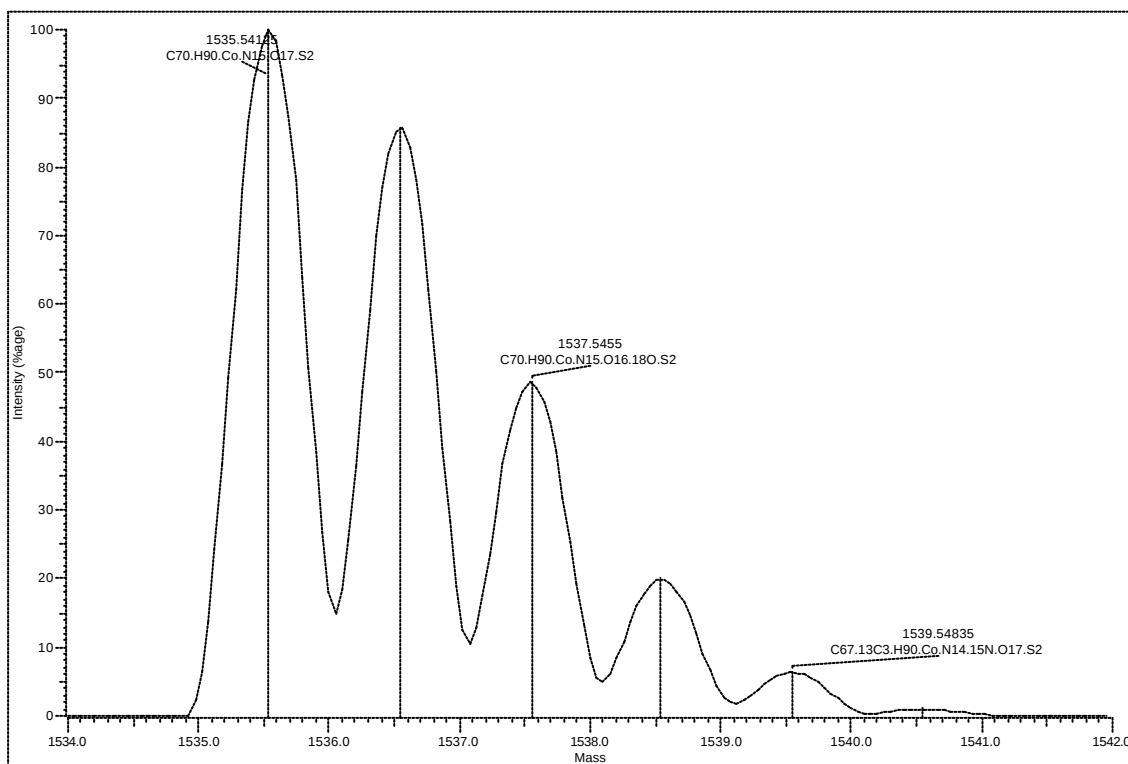


Figure B15: Theoretical isotopic distribution of sample.

Appendix C: Beer's Law Data

Table C1: Absorbance measurements for varying concentrations of *N*AcCoIII-MP8 in distilled water.

Absorbance at 420 nm	Concentration / M
0	0
0.0829	5.49E-07
0.1724	1.09E-06
0.2534	1.63E-06
0.3404	2.15E-06
0.4226	2.68E-06
0.5059	3.19E-06
0.589	3.7E-06
0.6703	4.21E-06
0.8379	5.2E-06
1.0036	6.16E-06
1.1656	7.11E-06
1.326	8.03E-06
1.5058	8.93E-06
1.6513	9.81E-06
1.7957	1.07E-05
1.9357	1.15E-05
1.733621	1.15E-05
2.057759	1.34E-05
2.37931	1.52E-05
2.707759	1.69E-05
3.041379	1.85E-05
3.389655	2.01E-05
3.681897	2.15E-05
4.127586	2.43E-05
4.739655	2.69E-05
5.156897	2.92E-05
5.500862	3.14E-05
5.983621	3.35E-05

Table C2: Absorbance measurements for varying concentrations of *N*AcCoIII-MP8 in 1.0 M NaClO₄.

Absorbance at 420 nm	Concentration / M
0	0
0.0828	5.49E-07
0.1684	1.09E-06
0.2468	1.63E-06
0.3336	2.15E-06
0.4152	2.68E-06
0.4889	3.19E-06
0.5671	3.7E-06
0.6413	4.21E-06
0.81	5.2E-06
0.9681	6.16E-06
1.123	7.11E-06
1.2739	8.03E-06
1.4251	8.93E-06
1.5367	9.81E-06
1.7113	1.07E-05
1.8456	1.15E-05
1.606034	1.15E-05
1.928448	1.34E-05
2.226724	1.52E-05
2.567241	1.69E-05
2.891379	1.85E-05
3.114655	2.01E-05
3.469828	2.15E-05
4.086207	2.43E-05
4.612931	2.69E-05
4.768966	2.92E-05
5.026724	3.14E-05
5.499138	3.35E-05

Appendix D: Spectroscopic pK_a Data

Table D1: Spectroscopic pK_a Data in Acidic Region

pH	Absorbance	pH	Absorbance
2.05	0.698	4.856	0.538
2.105	0.6998	4.982	0.5325
2.187	0.7024	5.111	0.5283
2.22	0.7035	5.243	0.5275
2.314	0.7045	5.332	0.5245
2.404	0.7031	5.466	0.5265
2.51	0.6981	5.614	0.528
2.576	0.6979	5.751	0.5298
2.652	0.6949	5.846	0.5355
2.706	0.6933		
2.805	0.6899		
2.9	0.6824		
3	0.6749		
3.14	0.665		
3.242	0.658		
3.364	0.6472		
3.45	0.6411		
3.532	0.635		
3.587	0.627		
3.644	0.615		
3.755	0.6101		
3.827	0.6046		
3.931	0.5965		
4.024	0.5865		
4.218	0.578		
4.411	0.5672		
4.529	0.5501		
4.646	0.548		
4.762	0.5448		

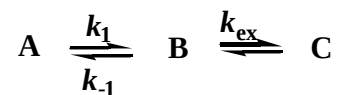
Table D2: Spectroscopic pK_a Data in Basic Region

pH	Absorbance	pH	Absorbance
5.49	0.937	8.434	1.0639
5.573	0.9377	8.504	1.0667
5.655	0.9384	8.574	1.0695
5.725	0.9408	8.645	1.0724
5.795	0.9431	8.742	1.0771
5.933	0.9444	8.838	1.0825
6.07	0.9456	8.935	1.0936
6.142	0.9445	9.03	1.1027
6.214	0.9433	9.142	1.1128
6.3	0.9422	9.254	1.1198
6.395	0.9451	9.326	1.1239
6.504	0.948	9.398	1.1279
6.591	0.9504		
6.678	0.9528		
6.766	0.9561		
6.901	0.9641		
7.036	0.973		
7.139	0.9775		
7.322	0.987		
7.402	0.992		
7.481	0.9971		
7.564	1.005		
7.647	1.0129		
7.739	1.0207		
7.914	1.0339		
8.003	1.0441		
8.117	1.0499		
8.231	1.0557		
8.333	1.0598		

Table D2: Spectroscopic pK_a Data in Basic Region with Pyridine

pH	Absorbance	pH	Absorbance
5.958	0.972	9.945	1.1989
6.101	0.9993	10.135	1.2021
6.2	0.9929	10.325	1.2052
6.304	0.9892	10.504	1.2056
6.41	0.9853	10.683	1.206
6.515	0.9874	10.855	1.199
6.61	0.9913	11.027	1.1919
6.717	0.9957	11.229	1.1805
6.826	1.0036	11.431	1.169
6.9	1.0099	11.587	1.1516
7.01	1.0162	11.743	1.1342
7.176	1.0226	11.875	1.1219
7.306	1.0336		
7.435	1.0473		
7.575	1.0548		
7.715	1.0623		
7.856	1.0715		
7.929	1.0827		
8.002	1.0926		
8.289	1.114		
8.576	1.1348		
8.722	1.1436		
8.868	1.1524		
9.014	1.1611		
9.123	1.1714		
9.239	1.1745		
9.356	1.1781		
9.548	1.185		
9.741	1.1919		

Appendix E: Derivation of Rate Constant Equation



$$\frac{dC}{dD} = k_{ex}[B]$$

Steady state on [B] :-

$$\begin{aligned} k_1[A] &= k_{-1}[B] + k_{ex}[B] \\ &= [B](k_{-1} + k_{ex}) \end{aligned}$$

$$\text{Therefore, } [B] = \frac{k_1[A]}{k_{-1} + k_{ex}}$$

$$\text{It follows that, } \frac{dC}{dD} = \frac{k_{ex}k_1[A]}{k_{-1} + k_{ex}}$$

Now usually $k_{ex} \ll k_{-1}$

$$\begin{aligned} \text{Therefore, } \frac{dC}{dD} &= \frac{k_{ex}k_1[A]}{k_{-1}} \\ &= k_{ex}K_1[A] \end{aligned}$$

$$\text{Therefore, } k_{obs} = k_{ex}K_1 \text{ (or } K_{OS1}k_{ex1})$$

