THE COORDINATION CHEMISTRY OF SOME COBALT CORRINOID AND IRON PORPHYRIN COMPLEXES

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ABSTRACT

The rôle of conformational change in the protein's control of the active metal site in haemoproteins and enzymes is examined with (i) cobalt corrinoid cofactors for the B_{12}-dependent isomerases, (ii) cytochrome c, and (iii) a model for cytochrome c, microperoxidase-8.

The half-lives ($t_{½}$) of homolytic fission of the cobalt carbon bond in the B_{12} coenzyme, adenosylcobalamin, and in methyl, ethyl and cyclobutylcobalamins have been determined in aqueous solution at 96 °C. The $t_{½}$ values increase (rates decrease) in the following ligand order: ethyl (ca. 9 min) < cyclobutyl (ca. 75 min) < adenosyl (ca. 180 min) < methyl (ca. 80 hrs). These values have been used to provide an estimation of $t_{½}$ ca. 10 years for the rate in the protein-free coenzyme at room temperature. Comparison with published data on the B_{12}-isomerases shows that the Co-C bond in the coenzyme is labilised by $\leqslant 10^{5}$ when bound by the apoenzyme and by a further $\geqslant 10^{5}$ in the presence of substrate to achieve the overall labilisation of $\geqslant 10^{10}$ (i.e. $t_{½} \sim 2.5$ ms) required for the enzymatic reaction.

The pK_a for the alkaline isomerisation of horse-heart cytochrome c (from Type III to Type IV, with cleavage of the iron-methionine (Fe-S) bond) has been confirmed to be 9.05 at 25°C, and extinction coefficients for Type III and Type IV at 695 nm are 867 and 83 M^{-1} cm^{-1}, respectively. Binding studies with cytochrome c in aqueous solution at 25 °C monitored the 695 nm charge-transfer absorbance band, present when the Fe(III)-S bond is intact. The specific binding of the perchlorate anion to cytochrome c occurs with K_{eq} ca. 5×10^{-3} M^{-1} (1:1 complex) and coulombic and chaotropic effects are implicated. Donor-acceptor complexes of caffeine and tryptophol with cytochrome c are formed where K_{eq} is ca. 6×10^{-4} M^{-1} (1:1 complex) and K_{eq} is ca. 9×10^{-2} M^{-1} (1:2 complex), respectively. Other drugs, including chlorpromazine, procaine and indole acetic acid, demonstrate these π - π interactions with cytochrome c and induce Fe-S bond cleavage.

An improved method for the preparation and purification of microperoxidase-8 (MP-8) and a TLC method as a rapid test of purity, have been developed. The binding of thioethers to MP-8 (possible models for the Fe-S bond in cytochrome c) and of drugs to MP-8 has been studied in 20% MeOH:H_2O (v/v) solution at 25 °C, where 2×10^{-6} M MP-8 is $\geqslant$ 97% monomeric at pH 7.4. N-acetyl-D,L-methionine binds to MP-8 with K_{app} ca. 1.9 M^{-1} at pH 7.4 and K_{app} ca. 2.1 M^{-1} at pH 6.7 (1:1 complexes): the 695 nm band characteristic of the cytochrome c Fe-S bond is exhibited. D,L-methionine binds to MP-8 with K_{app} ca. 155 M^{-1} at pH 7.4 (1:1 complex). Both caffeine and tryptophol form 1:1 donor-acceptor complexes with MP-8: K_{app} is ca. 151 M^{-1} at pH 7.4 and ca. 240 M^{-1} at pH 6.7, for caffeine, and K_{app} is ca. 60 M^{-1} at pH 7.4, for tryptophol. Other drug molecules, including chlorpromazine, demonstrate these π - π interactions with MP-8. Donor-acceptor complexes are formed with greater ease with these model complexes than with cytochrome c.

Declaration

I hereby declare that the work reported in this dissertation was carried out exclusively by me and that the dissertation has not been submitted for a degree at any other university.

Janine Aron

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To see a World in a Grain of Sand
And a Heaven in a Wild Flower,
Hold Infinity in the palm of your hand
And Eternity in an hour.

William Blake

ACKNOWLEDGEMENTS

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CHAPTER 1 - INTRODUCTION

1.1 Metallo-enzymes

Enzymes are biological catalysts found throughout the plant and animal kingdoms which catalyse a diversity of reactions essential to life. The metabolic rates of hundreds of pathways are controlled by enzymes; they usually display high specificity, both chemical and steric, for one or a small number of closely related substrates. Metallo-enzymes form a sub-group of the enzymes, and represent a partnership between a metal ion (e.g. copper(II)) or a metal complex with one or more ligands (e.g. iron porphyrins, cobalt corrinoids) providing the active catalytic site, and a protein which controls and enhances the activity of the metal. The protein is termed the 'apoenzyme' and, together with the metal ion or complex ('prosthetic group', 'co-factor' or 'coenzyme'), forms the enzyme. Metallo-enzymes are important in many areas of metabolism such as the enzymatic fixation of nitrogen (by nitrogenase enzymes), energy production or interconversion (e.g. by cytochromes) and detoxification mechanisms in the body (e.g. by peroxidases, catalases and cytochrome P-450). Some of the enzymatic reactions do not have analogues among protein-free complexes, such as carbon skeleton isomerisation by the isomerase enzymes (which incorporate cobalt corrinoid co-factors).

The enhanced catalytic activity of metallo-enzymes is due to a modification of the properties of the prosthetic group by the protein. The prosthetic group is tightly bound by the protein: the binding constant can be as high as 10^{31} (e.g. iron(III) in transferrin (Raymond et al., 1982)). The valency of the metal, the ligands and stereochemistry are fixed by the protein, sometimes inducing strain in the metal complex (e.g. copper(II) is forced to adopt a tetrahedral rather than its usual square planar configuration, in plastocyanin). Some pathways may be blocked by the protein, usually by steric means, while others may be introduced by the suitable positioning of groups such as thiols. The protein controls and modifies possible pathways to give the desired rate enhancement and specificity by varying one or more of:

(a) the kinetics of individual steps, (b) the thermodynamics of individual steps or (c) the thermodynamics of the overall reaction. A variation of thermodynamic properties coupled with changes in protein conformation, is the predominant mode for enhancing the catalytic activity of metallo-enzymes (Pratt, 1975). Thus the protein may

(i) alter the equilibrium constant for the coordination of a particular ligand by electronic or steric means (e.g. the coordination of oxygen by iron(II) in haemoglobin);
(ii) stabilise a ligand coordination number unusual in the protein-free species by steric means (e.g. the five-coordinate mono-imidazole iron(II) porphyrin in peroxidases, catalases and cytochromes);
(iii) vary the redox potential of the metal, for example by changing the hydrophobicity of its environment (e.g. in cytochrome c);
(iv) couple changes in the thermodynamic properties of the metal site with the binding of the substrate (e.g. induction of the normally unfavourable homolytic fission of the cobalt-carbon bond in the cobalt corrinoid cofactors of isomerase enzymes);
(v) couple changes involving sites distant from the metal with equilibria involving the metal (e.g. in haemoglobin).

For a more extensive review of the rôle of the protein in metallo-enzymes, see Pratt (1975).

The prosthetic group iron protoporphyrin IX (see Figure 1.1) provides an excellent example to show how the properties of the prosthetic group are altered in consequence of the attachment of different proteins. Different haemoproteins containing iron protoporphyrin IX display great versatility and diversity of function. Thus, haemoglobin and myoglobin are respiratory carriers which reversibly bind oxygen for transport or storage (Antonini and Brunori, 1971); the cytochromes have as principal function, electron transport in the mitochondrial respiratory chain (Lemberg and Barrett, 1973); mono-oxygenases, such as cytochrome P-450, hydroxylate hydrocarbons through the activation of oxygen (Hayaishi, 1962); catalases catalyse the decomposition of hydrogen

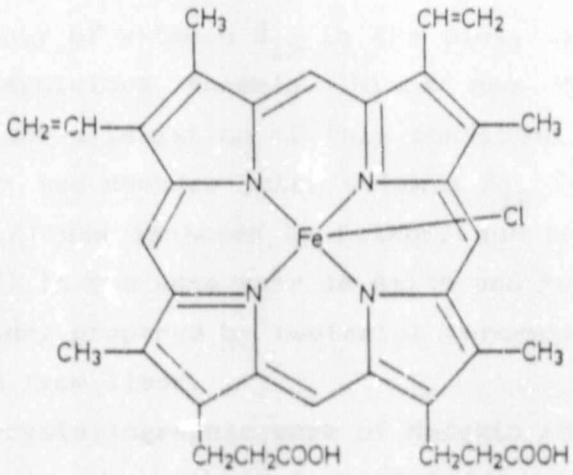

Fig. 1.1 Haemin chloride (Fe(III) protoporphyrin IX chloride).

peroxide, while peroxidases use hydrogen peroxide to oxidise a wide range of substances (catalases, which do not oxidise most of the substrates of peroxidases, may be regarded as peroxidases with a different specificity (Dixon and Webb, 1971)) (Frew and Jones, 1984); and terminal oxidases like cytochrome c oxidase, reduce oxygen to water (Caughey et al., 1976).

In this dissertation, the rôle of the protein in two types of metallo-enzymes has been studied: (i) the cobalt corrinoid-dependent isomerase enzymes using protein-free cobalt corrinoid models, and (ii) the haemoprotein cytochrome c, and a model for cytochrome c, the haem-octapeptide, microperoxidase-8.

1.2 Cobalt corrinoids used in this work

(a) History

Vitamin B_{12} is not synthesized by the human body. Yet a derivative of B_{12}, adenosylcobalamin, is the essential cobalt-cofactor for the in vivo interconversion of methyl-malonic and succinic acids by the enzyme methylmalonyl-coenzyme A mutase (the only known reaction in mammals requiring this B_{12} coenzyme) e.g.,

A deficiency of vitamin B_{12} in the diet, or its maladsorption, causes pernicious anaemia. Minot and Murphy (1926) first reported the alleviation of this condition by feeding patients raw liver; two decades later vitamin B_{12} (cyanocobalamin) (see Figure 1.2) was isolated by Folkers and co-workers (Rickes et al., 1948) in the same year as Smith and Parker (1948). Vitamin B_{12} is today prepared by bacterial fermentation rather than by isolation from liver.

The crystallographic work of Hodgkin (Hodgkin et al., 1956) aided by the chemical studies of Todd and Johnson (see Bonnet et al., 1957) established that B_{12} contains 5,6-dimethylbenzimidazole and cyanide anion as fifth and sixth ligands to a cobalt(III) ion. The cobalt ion resides in a corrin ring (which provides four planar nitrogen donors) akin to the porphyrin ring in haemoproteins (see the structure in Figure 1.2).

A biologically active form of vitamin B_{12} (vitamin B_{12} coenzyme) was discovered by Barker et al. (1958). The crystallographic studies of Lenhert and Hodgkin (1961) established that the macrocyclic structure and peripheral

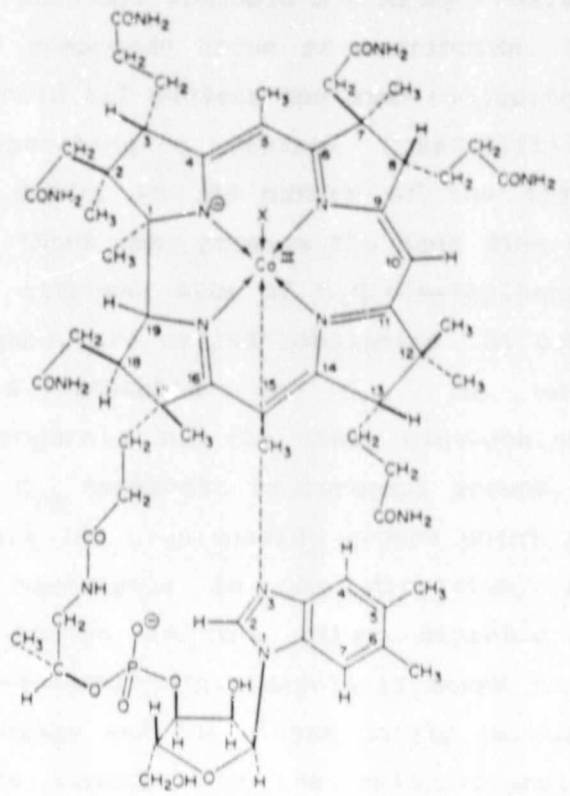

Fig. 1.2 The molecular structure of B_{12} (source: Pratt (1975)).

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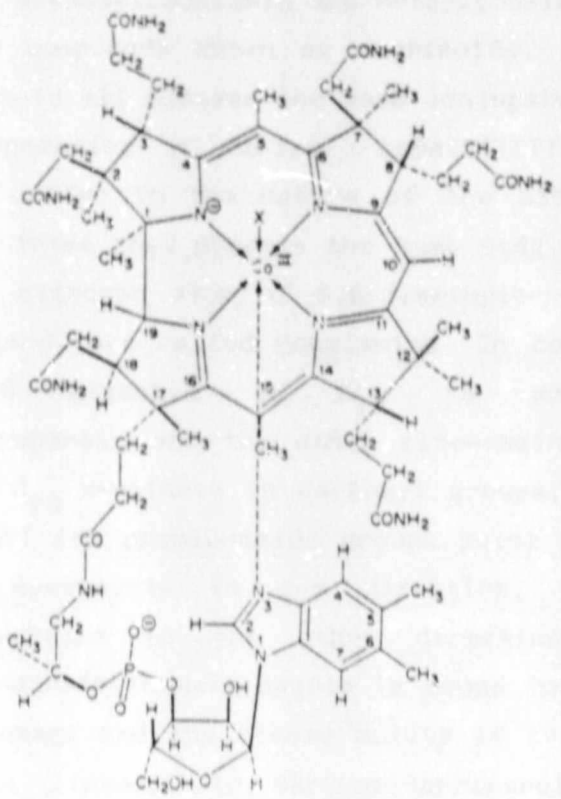


Fig. 1.2 The molecular structure of B_{12} (source: Pratt (1975)).

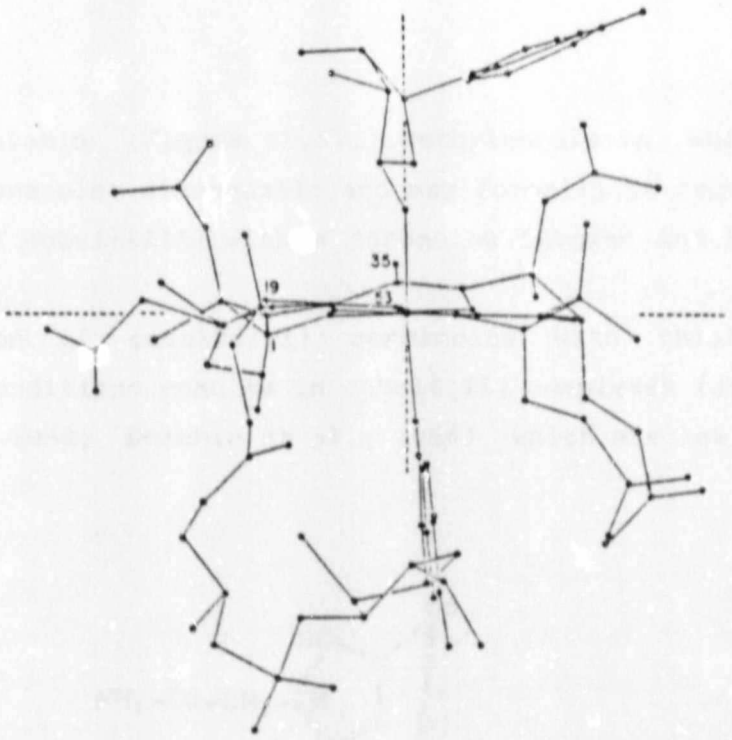
substituents of the coenzyme were the same as in cyanocobalamin, but that cyanide was replaced by the 5' carbon of an adenosyl group. The resulting Co-C sigma bond represents a unique feature of these corrinoids: sigma-bonded alkyl derivatives of metals of the first transition series were at the time of the discovery of the coenzyme considered to be rather unstable. Coenzyme B_{12} (adenosylcobalamin) (see Figure 1.3) was the first naturally occurring organometallic compound to be discovered, and adenosylcobalamin and related alkylcobalamins remain the only known organometallic complexes in nature. Methylcobalamin, known to be biologically active and essential for human metabolism (as cofactor for N^5-methyltetrahydrofolate homocysteine methyltransferase enzyme), was later discovered in vivo. The structure of this cofactor was recently determined by X-ray crystallography (Rossi et al., 1985).

In 1979, the total synthesis of vitamin B_{12} was reported by Woodward (Woodward, 1979).

(b) Structure

Cyanocobalamin, adenosylcobalamin and methylcobalamin form part of a family of compounds known as corrinoids. The naturally occurring corrinoid all possess the same conjugated corrin ring as B_{12}, incorporating a central cobalt(III) metal ion. Corrinoids may differ in the nature of the side chains and axial ligands; those that possess the same side chains as B_{12} itself and the nitrogen atom of 5,6-dimethylbenzimidazole as fifth axial ligand, are called cobalamins. In cobalamins, the propionic acid attached to C_{17} is amidated with (R)-1-amino-2-propanol, and the other side-chains at C_2, C_3, C_7, C_8, C_{13} and C_{18} terminate in carboxyl groups, converted to amide groups. All the propionamide groups point away from the almost planar macrocycle in one direction, and all the acetamide side-chains in the other direction. The other nitrogen of 5,6-dimethylbenzimidazole is bound to ribose in an α-glycosidic linkage and the ribose moiety is bound, in turn, via a phosphate linkage, to the aminopropanol side-chain. Cobinamides are corrinoids where the phosphate linkage to the

(i)



(ii)

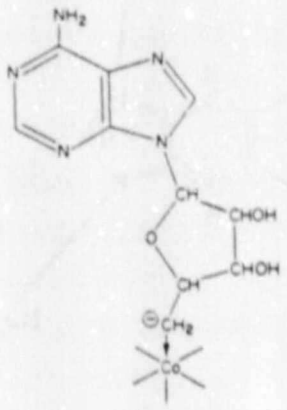


Fig. 1.3 (i) The structure of the cobalamin coenzyme (source: Lenhert and Hodgkin (1961)).

(ii) The 5'-deoxyadenosyl ligand present in coenzyme B_{12} (source: Pratt (1975)).

ribose moiety of the benzimidazole side-chain has been hydrolysed (see Figure 1.4). The coordinated benzimidazole is usually replaced by another ligand, often cyanide or water.

The sixth axial position in vitamin B_{12} is occupied by cyanide (CN^-). Cyanocobalamin is a diamagnetic, six-coordinate complex containing the d^6 cobalt(III) ion. The positive charges on the cobalt ion are offset by the single negative charges on the corrin ring, the cyanide and the phosphate.

Adenosylcobalamin (Figure 1.3), methylcobalamin and other cobalamins are also diamagnetic and may formally be regarded as complexes of cobalt(III) with a carbanion (Abeles and Dolphin, 1976).

Reduction of cobalt(III) corrinoids with thiol under anaerobic conditions results in cobalt(II) complexes (Jaselskis and Diehl, 1954; Dolphin et al., 1964) which are low spin d^7

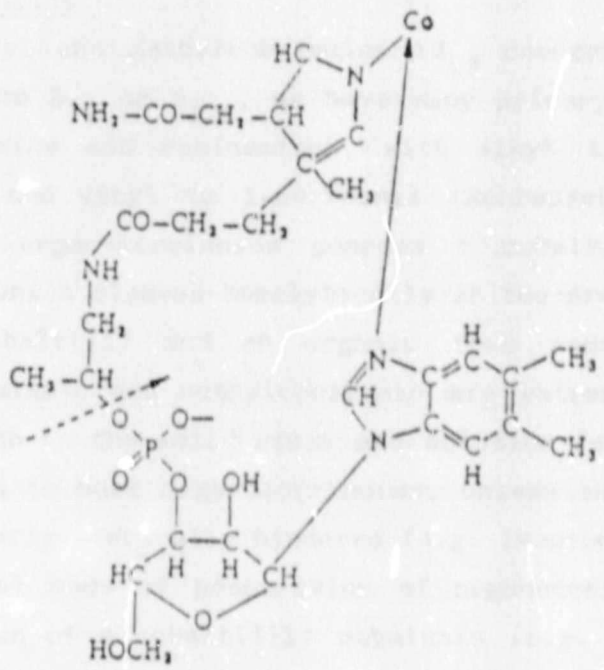


Fig. 1.4 The structure of cobinamides: hydrolysis at ⟶ of the C_{17} sidechain in cobalamins gives the corresponding cobinamide.

compounds with one unpaired electron. In neutral solution, the cobalt(II) B_{12} complex is probably five-coordinate with the benzimidazole base coordinated to the cobalt (Saveant, 1979). Further reduction of the cobalt(II) complex, or reduction of cobalt(III) with sodium borohydride, gives a green complex which has been established to be a monovalent cobalt complex (Hill et al., 1962). Co(I) corrinoids rapidly decompose in aqueous solution at low pH to give Co(II) and hydrogen,

probably via an intermediate cobalt hydride. Both Co(I) and Co(II) complexes are easily oxidised by air to give Co(III) complexes. Co(I) corrinoids are spin paired d^8 complexes. The benzimidazole base is not coordinated to the cobalt in the cobalt (I) B_{12} complex (Brodie and Poe, 1971).

The abbreviations B_{12} (cyanocobalamin), B_{12a} (aquocobalamin), and B_{12r} (cob(II)alamin) will be used in this dissertation.

(c) Organocorrinoids

Both adenosyl and methylcobalamins (B_{12} coenzymes) have been prepared from B_{12} or B_{12a}, as have many primary and secondary alkylcobalamins and cobinamides, with alkyl ligands ranging from ethyl and vinyl to 1-norbornyl (Schrauzer and Holland, 1971). All organocorrinoids possess a cobalt-carbon (Co-C) sigma bond which cleaves homolytically in the presence of light to give cobalt(II) and an organic free radical. However, adenosylcobalamin and methylcobalamin are extremely stable in the dark both in the solid state and solution (see Chapter 4); this applies to most organocorrinoids, unless the organoligand is particularly sterically hindered (e.g. isopropylcobalamin).

The usual mode of preparation of organocorrinoids is via the reduction of a cobalt(III) cobalamin (e.g. cyanocobalamin or hydroxocobalamin) or cobalt(III) cobinamide (e.g. aquocyanocobinamide or diaquocobinamide) to the corresponding cobalt(I) corrinoid (termed "supernucleophile" by Grate and Schrauzer (1979)), which is then reacted with compounds such as alkyl halides, expoxides, alkenes or alkynes, to give the desired product via S_N2 substitution (see Chapter 3) e.g.,

$$\text{Co(III)-OH}_2 \xrightarrow{\text{NaBH}_4} \text{Co(I)} \xrightarrow{\text{R-Hal}} \text{Co(III)-R} \quad .$$

Organocorrinoids engage in a diversity of reactions involving the cobalt coordination sphere; these are illustrated in Figure 1.5.

Co-R	$\rightleftharpoons$	Co(II)+R$^{\cdot}$	- HOMOLYTIC FISSION
Co-R	$\rightleftharpoons$	Co(I)+R^{+}	- REDUCTION
Co-R	$\rightleftharpoons$	Co-H+R-H	- β-ELIMINATION
Co-R	$\rightleftharpoons$	Co(III)+R^{-}	- HETEROLYTIC FISSION
Co-R	$\rightleftharpoons$	Co-R$^{:}$	- ORGANOLIGAND MODIFICATION

Fig. 1.5 Organocorrinoid reactions.

(d) Stable yellow corrinoids

Stable yellow corrinoids are sometimes formed as by-products of reactions with cobalamins that involve a change in the cobalt oxidation state. They are thought to arise from a free radical attack on the corrin ring: see for example, Grüning and Gossauer (1979). Stable yellow corrinoids (SYC) display a band at about 460nm in their absorption spectra; they are impervious to the effect of light, oxygen or cyanide, unlike B_{12r}.

(e) Enzyme reactions requiring corrinoids as cofactors

Three types of reactions in living organisms are catalysed by enzymes containing cobalt corrinoids as cofactors:

1. isomerisation reactions involving the 1,2-shift of a carbon, nitrogen or oxygen atom;
2. methyl transfer reactions, for example in the formation of methionine;
3. the conversion of ribonucleotides to 2'-deoxyribonucleotides, by the enzyme ribonucleotide reductase.

The enzyme reactions requiring cobalt corrinoid cofactors (i) adenosylcobalamin and (ii) methylcobalamin (in methyl transfer reactions), are classified and tabulated in Table 1.1.

The B_{12}-dependent isomerases together with the related ribonucleotide reductases are the first group of metalloenzymes (as opposed to haemoproteins) where the rôle of the protein is reasonably well understood. The mechanism whereby changes in protein conformation are coupled to cnanges on the metal ion is not known for certain, but several mechanistic proposals have been

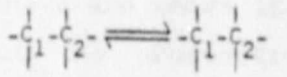
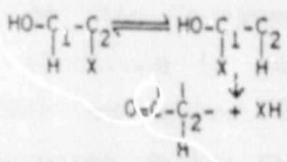
reported (e.g. Pratt, 1984; Finke and Hay, 1984).

In this work only the isomerase reactions are considered. In Chapter 4, model studies with protein-free cobalt corrinoids aim to clarify the rôle of the protein in isomerase enzymes. For a recent review of model studies concerning isomerase enzymes see Finke et al. (1984).

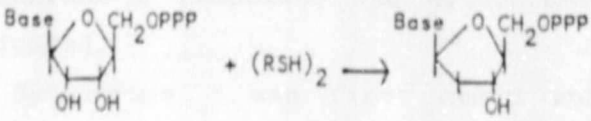
TABLE 1.1 Enzyme reactions requiring cobalt corrinoids (source: Chemaly (1980)).

Class 1 - Reactions requiring adenosylcobalamin

A. Rearrangement or Isomerization Reactions

Type of reaction		Enzyme
Carbon-skeleton rearrangement		Glutamate mutase Methylmalonyl-coenzyme A mutase α-Methyleneglutarate mutase
Migration of an amino group		L-β-lysine mutase D-α-lysine mutase D-ornithine mutase
Conversion of diols or amino alcohols to aldehydes		Dioldehydrase Glycerol dehydrase Ethanolamine ammonia-lyase

B. Conversion of ribonucleotides to 2'-deoxribonucleotides

Reaction	Enzyme
	Ribonucleotide reductase

$(RSH)_2$ = dithiol

Class 2 - Reactions involving methylcobalamin

Reaction	
$5\text{-}CH_3\text{-}THF + HS(CH_2)_2CH(NH_2)COOH \longrightarrow$	N^5-methyltetrahydrofolate:
$THF + CH_3S(CH_2)_2CH(NH_2)COOH$	homocysteine methyltransferase
$5\text{-}CH_3\text{-}THF + HS(CH_2)_2SO_3 \longrightarrow THF + CH_3S(CH_2)_2SO_3$	"Methane synthetase"
$5\text{-}CH_3\text{-}THF + CO_2 \longrightarrow THF + CH_3COO^-$	"Acetate synthetase"

THF=tetrahydrofolate

reported (e.g. Pratt, 1984; Finke and Hay, 1984).

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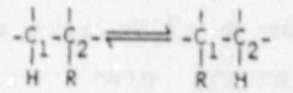
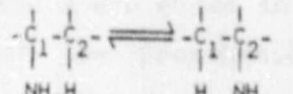
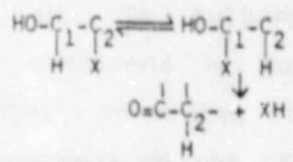
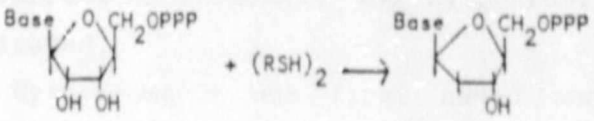
Carbon-skeleton rearrangement		Glutamate mutase Methylmalonyl-coenzyme A mutase α-Methyleneglutarate mutase
Migration of an amino group		L-β-lysine mutase D-α-lysine mutase D-ornithine mutase
Conversion of diols or amino alcohols to aldehydes		Dioldehydrase Glycerol dehydrase Ethanolamine ammonia-lyase

B. Conversion of ribonucleotides to 2'-deoxribonucleotides

Reaction	Enzyme
	Ribonucleotide reductase

$(RSH)_2$ = dithiol

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Reaction	
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$THF + CH_3S(CH_2)_2CH(NH_2)COOH$	homocysteine methyltransferase
$5\text{-}CH_3\text{-}THF + HS(CH_2)_2SO_3 \longrightarrow THF + CH_3S(CH_2)_2SO_3$	"Methane synthetase"
$5\text{-}CH_3\text{-}THF + CO_2 \longrightarrow THF + CH_3COO^-$	"Acetate synthetase"

THF=tetrahydrofolate

reported (e.g. Pratt, 1984; Finke and Hay, 1984).

In this work only the isomerase reactions are considered. In Chapter 4, model studies with protein-free cobalt corrinoids aim to clarify the rôle of the protein in isomerase enzymes. For a recent review of model studies concerning isomerase enzymes see Finke et al. (1984).

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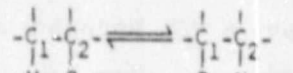
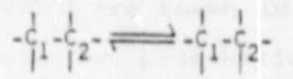
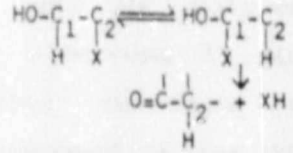
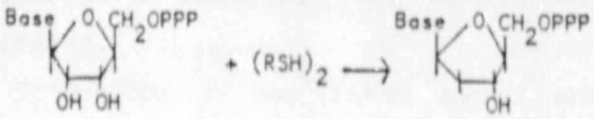
A. Rearrangement or Isomerization Reactions

Type of reaction		Enzyme
Carbon-skeleton rearrangement		Glutamate mutase Methylmalonyl-coenzyme A mutase α-Methyleneglutarate mutase
Migration of an amino group		L-β-lysine mutase D-α-lysine mutase D-ornithine mutase
Conversion of diols or amino alcohols to aldehydes		Dioldehydrase Glycerol dehydrase Ethanolamine ammonia-lyase

B. Conversion of ribonucleotides to 2'-deoxribonucleotides

Reaction	Enzyme
	Ribonucleotide reductase

$$+ (RS)_2 + H_2O$$

$(RSH)_2$ = dithiol

Class 2 - Reactions involving methylcobalamin

Reaction	
$5\text{-}CH_3\text{-}THF + HS(CH_2)_2CH(NH_2)COOH \longrightarrow THF + CH_3S(CH_2)_2CH(NH_2)COOH$	N^5-methyltetrahydrofolate: homocysteine methyltransferase
$5\text{-}CH_3\text{-}THF + HS(CH_2)_2SO_3 \longrightarrow THF + CH_3S(CH_2)_2SO_3$	"Methane synthetase"
$5\text{-}CH_3\text{-}THF + CO_2 \longrightarrow THF + CH_3COO^-$	"Acetate synthetase"

THF=tetrahydrofolate

1.3 Iron porphyrins used in this work

1.3.1 Cytochrome c

(a) History and description

The cytochromes are a group of haemoproteins whose principal biological function is electron and/or hydrogen transport to molecular oxygen in the respiratory chain, by virtue of a reversible valency change of their haem iron (International Union of Biochemistry, 1961). Cytochromes also play a rôle in photosynthesis (Hill, 1954) and in non-photosynthetic anaerobic and chemosynthetic bacterial reactions. Keilin first identified three different types of cytochrome (cytochromes a, b, and c) (Keilin, 1925; 1926) and showed that the respiratory function of cytochromes was related to a valency change on the haem iron. In fact, four main groups of cytochromes can be distinguished on the basis of their prosthetic groups: cytohaems a, b, c and d are shown in Figure 1.6. Cytochromes c are unique in that the prosthetic group, cytohaem c, is covalently bound to the peptide by thioether linkages to cysteine residues on the polypeptide chain. Cytochromes typically transfer electrons in multienzyme systems between themselves or other molecules such as the cytochrome reductases, dehydrogenases or the cytochrome peroxidases. This is illustrated in Figure 1.7 for the mitochondrial respiratory chain: the rôle of cytochrome c in electron transfer between cytochrome c reductase and cytochrome c oxidase is clearly indicated.

Cytochrome c was first named and described by Keilin (1925); its wide occurrence in cells from mammals to invertebrates and yeast has since been established. It is a small ($M_r \sim 12\,000$) monohaem protein, part of the terminal oxidation chain in the mitochondria, which transfers electrons from cytochrome c reductase to cytochrome c oxidase. The iron alternates between the ferric and ferrous states with a midpoint potential at pH 7 of 260 $\pm$ 20 mV (Lemberg and Barrett, 1973). The complete amino acid sequence is known for at least thirty-six species (Lemberg and Barrett, 1973, p. 136) ranging

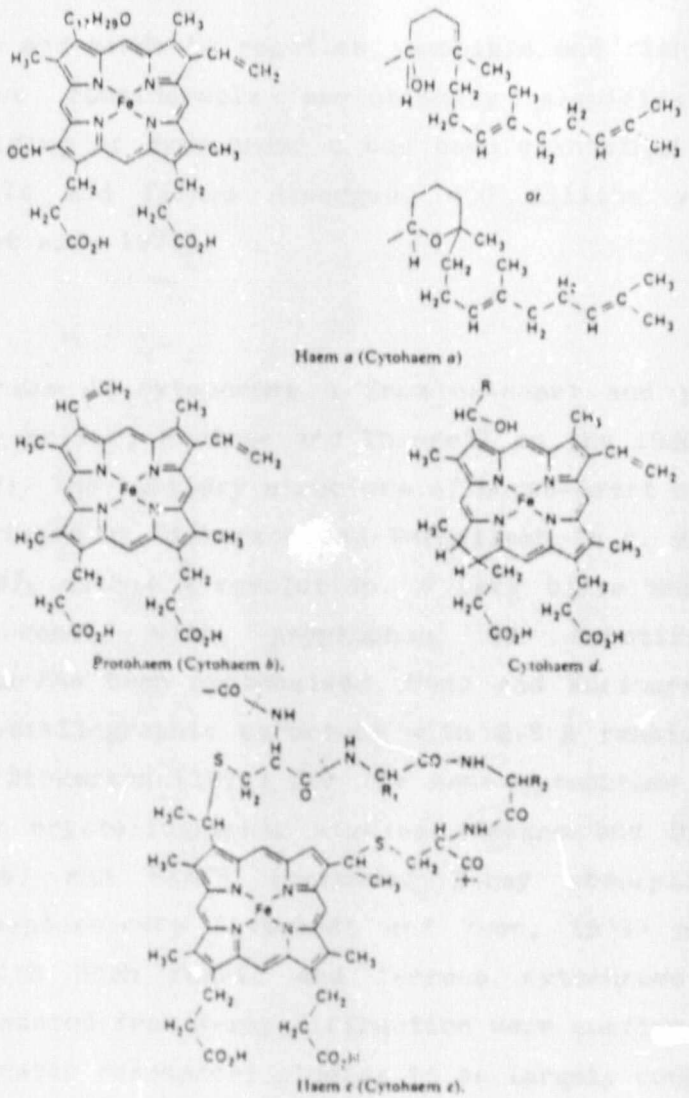
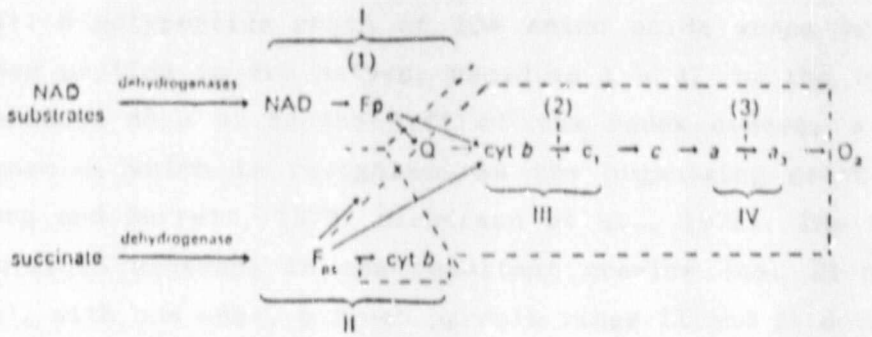


Fig. 1.6 Prosthetic groups of the cytochromes (source: Lemberg and Barrett (1973)).



The respiratory chain.

Cytochromes underlined and in space surrounded by interrupted line.
I, II, III, IV the four complexes that can be isolated (1), (2), (3) the three sites of phosphorylation.

Fig. 1.7 Cytochrome c in a multienzyme system (source: Lemberg and Barrett (1973)).

from mammals and birds to reptiles, amphibia and fishes. These studies have considerable evolutionary significance. The tertiary folding of cytochrome c has been maintained at least since mammals and fishes diverged, 400 million years ago (Dickerson et al., 1971).

(b) Structure

The preparation of cytochrome c from ox-heart and yeast was pioneered by Keilin, Hartree and Theorell in the 1930's (e.g. Keilin, 1930). The tertiary structure of horse-heart cytochrome c was established by Dickerson and Margoliash (e.g. Margoliash et al., 1968), with 4 Å resolution. A very close analogue of this cytochrome, with tryptophan 59 substituted by phenylalanine has been synthesized (Sano and Kurihara, 1969). An X-ray crystallographic structure with 2.8 Å resolution was reported by Dickerson (1971) for the same cytochrome species. Recent X-ray crystallographic studies (Takano and Dickerson, 1981a; 1981b) and EXAFS (extended X-ray absorption fine structure) measurements (Labhardt and Yuen, 1979) have been conducted with both ferric and ferrous cytochrome c. The structures deduced from X-ray diffraction were confirmed by NMR (nuclear magnetic resonance) studies to be largely conserved in solution (Moore and Williams, 1980). The structure of cytochrome c is shown in Figure 1.8. The X-ray studies confirmed the inclusion of the haem prosthetic group in a crevice of the protein molecule, as postulated by Ehrenberg and Theorell (1955a). A polypeptide chain of 104 amino acids wraps around the haem peptide in two halves, residues 1 - 47 to the right and residues 48 - 91 to the left of the redox centre, a low spin haem c, which is recognized as the organising principle (Lemberg and Barrett, 1973; Dickerson et al., 1971). The haem is contained sideways in the resultant crevice (ca. 21 Å in length), with one edge, between pyrrole rings II and II(methine bridge β), exposed to the solvent. Residues 94 - 104 return over the top of the molecule to the right and are the only genuinely α-helical residues. Four of the ligands to the haem iron are from the porphyrin itself. Histidine 18 is the fifth ligand, and the sulphur of methionine 80, the sixth. The

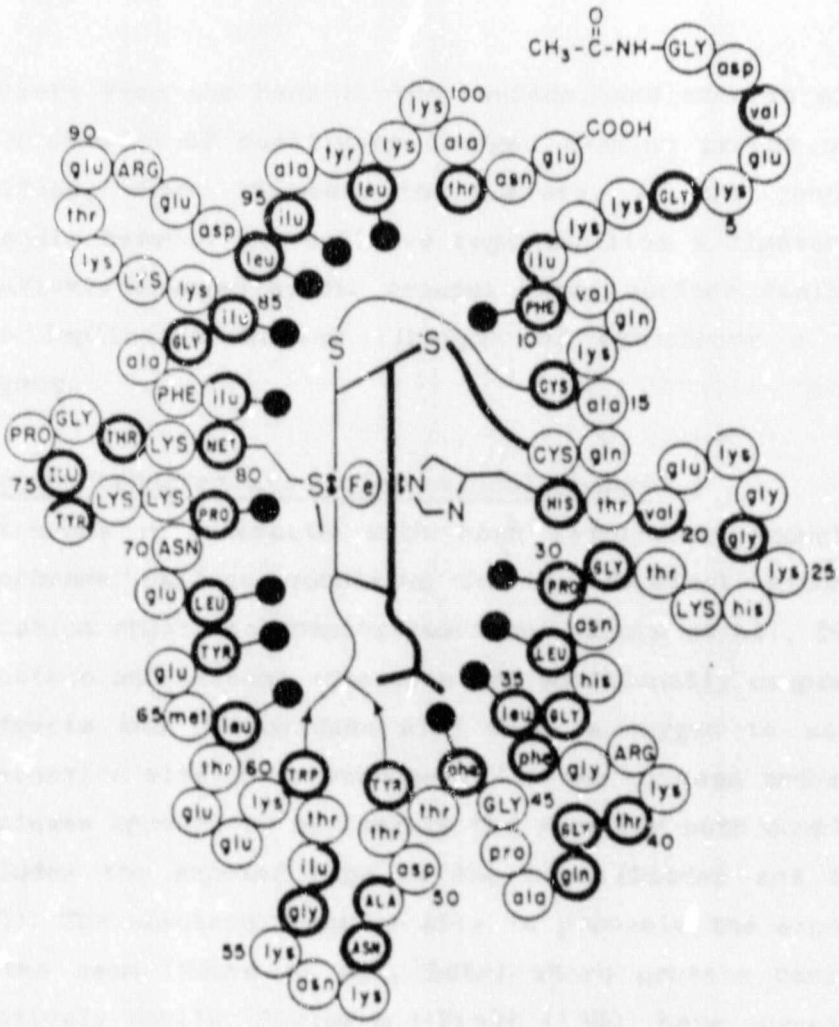


Fig. 1.8 The structure of cytochrome c (source: Dickerson et al. (1971)).

binding of methionine 80, postulated by Harbury et al. (1965) and supported by a model study (Shechter and Saludjian, 1967) and NMR studies (e.g. Wütrich (1969)), was confirmed by the X-ray study.

Histidine 18 and cysteines 14 and 17, which bind via thioether linkages to the porphyrin (pyrrole rings I (position 2) and II (position 4), respectively) extend from the right wall of the crevice. Methionine 80, which extends from the left wall, forms part of a largely invariant sequence of residues (67 - 82) which includes lysine residues 72, 73 and 79. Two channels filled with hydrophobic side-chains lead to the right

and left from the haem to the surface, and each is surrounded by a cluster of positively charged ε-amino groups of lysine residues, where it meets the surface. At the rear of the molecule between the positive regions, lies a cluster of nine negatively charged acidic groups. These surface features have been implicated in the linkages of cytochrome c to other enzymes.

(c) Electron transfer and conformational change

Cytochrome c interacts with both cytochrome reductase and cytochrome oxidase complexes in the terminal mitochondrial oxidation chain, in complex reactions (Moore et al., 1982). The reductase and oxidase molecules are additionally coupled to ATP synthesis and the oxidase also reduces oxygen to water. The interaction site on cytochrome c for the oxidase and reductase complexes appears to be largely the same for both complexes and includes the exposed edge of the haem (Rieder and Bosshard, 1980). The electron transfer site is probably the exposed edge of the haem (Moore et al., 1982) where protein residues are relatively mobile. Poulos and Kraut (1980) have suggested that phenylalanine 82 forms part of the electron transfer pathway. The electron transfer function has been modelled with iron, cobalt and ruthenium hexacyanides (Ragg and Moore, 1984) where the interaction site is the exposed haem edge and direct electron transfer probably occurs. An oxidation linked conformational change of the cytochrome c protein on the basis of differences in the ferric and ferrous species was suggested as early as 1933 (Zeile and Peuter, 1933). Reduced cytochrome c appears to be more compact and rigid than the oxidised species. It is less susceptible to digestion by trypsin (Okunuki et al., 1965) and shows a greater thermal stability than the oxidised molecule (Butt and Keilin, 1962). The methionine bond to haem iron, which in ferric cytochrome c is relatively easily broken by pH or increasing temperature (Ångstrom et al., 1982), is particularly stable in the ferrous species, even at extremes of temperature and pH (Moore et al., 1982). No denatured protein where the methionine-iron bond has been broken is found in lyophilised ferrous cytochrome c, as it is for the ferric

species (Avirам and Schejter, 1972). Refined X-ray structures of bonito cytochrome c have confirmed that differences exist between the two redox states (Takano and Dickerson, 1981a; 1981b).

Thus, cytochrome c is far more stable in the reduced than the oxidised state; the hydrophobic nature of the haem environment also serves to stabilise the ferrous relative to the ferric state. In consequence, the redox potential is relatively high (260 ± 20 mV) (Lemberg and Barrett, 1973). The nature of the conformational change is not known for certain, but minor changes in structure extending to the surface of the protein are likely (Moore et al., 1982). A detailed model based on X-ray studies has been proposed for the conformational change (Takano and Dickerson, 1981a; 1981b): it includes the movement of the haem group and axial ligands, some groups moving by up to 0.07 nm (asparagine 52 and tyrosine 67). X-ray and NMR data (Moore et al., 1980) agree that the major change in structure is at the back of the molecule, close to isoleucine 57. This is supported by the chemical modification studies of Bosshard and Zürrer (1980).

The rôle of the methionine-iron bond in the oxidation-linked conformational change remains controversial. NMR studies (Moore et al., 1980) of cobalticytochrome c and native cytochrome c reported a small increase in metal-sulphur interaction, on reduction, interpreted as a bond length increase. But X-ray crystallographic studies (Takano and Dickerson, 1981a; 1981b) and EXAFS measurements on cytochrome c (Labhardt and Yuen, 1979) and model complexes (Mashiko et al., 1981), contradict the assertion. However, "anomalous dynamic properties" (Moore et al., 1982) displayed by the bond in ferricytochrome merit its further investigation.

(d) The haem iron-methionine bond

The methionine ligand of cytochrome c is displaced by cyanide, azide and imidazole at pH 7 (Stellwagen, 1966; 1968; Gupta and Redfield, 1970; Sutin and Yandell, 1972) producing low-spin complexes, and by a residue at ca. pH 9.0, the identity of which has not definitely been established (Bosshard, 1981). The

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