4. Plastoquinone : Occurs in chloroplast and functions in photosynthesis, and closely related to coenzyme Q. It contains two CH_3 groups which replace the methoxy ($-OCH_3$) groups of coenzyme Q. In some bacterial respiratoy systems menaquinone takes place of coenzyme Q.

5. The Cytochromes : These respiratory carriers were discovered in animal cells in 1886 by Mc Munn. He called the compounds myo or histo-hematins.

Keilin's classical experiments in England in 1926–1927 demonstrated that these cell pigments (cytochromes) have been found in almost all living tissues and implied an essential role for these substances in cellular respiration.

Keilin's research showed that in every tissue usually there are three types of cytochromes to which he assigned the letters *a*, *b*, and *c*. The amount seemed to be proportional to the respiratory activity of the tissues, heart and other active muscles containing the largest amount of these pigments. The research on the cytochromes was facilitated by the fact that they absorb light of different wavelengths (Table 5.3).

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Table 5.3

Cytochromes	Alfa band	Beta band	Gamma band
Cytochrome *a*	605 mμ	517 mμ	414 mμ
Cytochrome *b*	563 mμ	530 mμ	430 mμ
Cytochrome *c*	550 mμ	521 mμ	416 mμ

The absorption spectra of oxidized and reduced cytochrome *c* are shown in (Fig. 5.15) note the positions of the α, β and γ bands of the reduced carrier. The three types of cytochromes (*a*, *b* and *c*) were distinguished by the positions of the absorption maxima of the α, β & γ bands of the reduced carrier. The three types of cytochromes (*a*, *b*, and *c*) were distinguished by the positions of the absorption maxima of the α, β and γ bands. As additional cytochromes were discovered and described, they were named $c_1, b_1, b_2, b_3, b_4, c_2, c_3, c_4, a_1, a_2, a_3$. The *b* type cytochromes include cyt aa_3 (cytochrome oxidase).

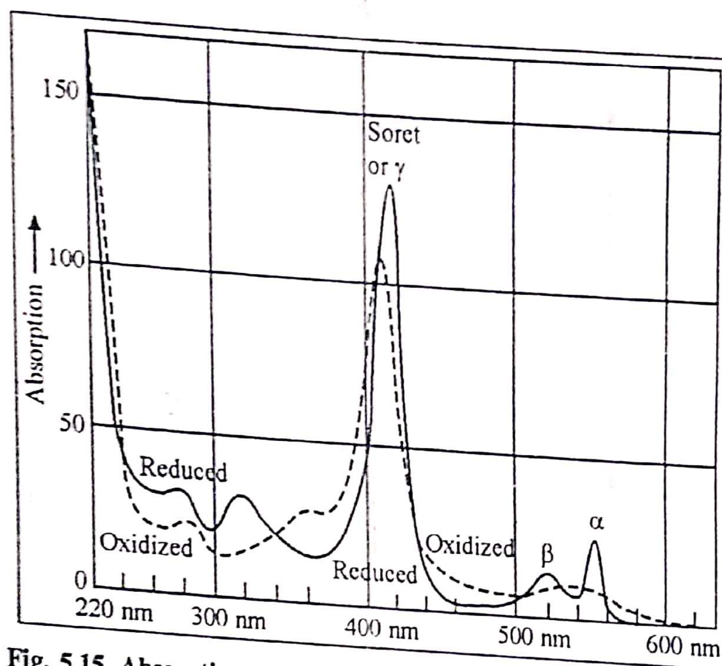

Fig. 5.15. Absorption spectra of oxidized and reduced cytochrome c.

The absorption spectra together with other properties of the cytochromes indicate that these compounds are conjugated proteins having an iron porphyrin as a prosthetic group for cytochrome *c* as shown in (Fig. 5.16).

It is protoporphyrin IX linked to cysteine residues in the protein through thioether linkage with the vinyl groups of rings (III) and (IV).

The prosthetic group of cytochrome *b* is protoporphyrin IX itself. The porphyrin of cytochrome *a* is porphyrin A, characterised chiefly by a long hydrophobic chain of hydrogenated isoprenoid unit. In this regard, porphyrin *A* resembles the porphyrin of chlorophyll (Fig. 5.17).

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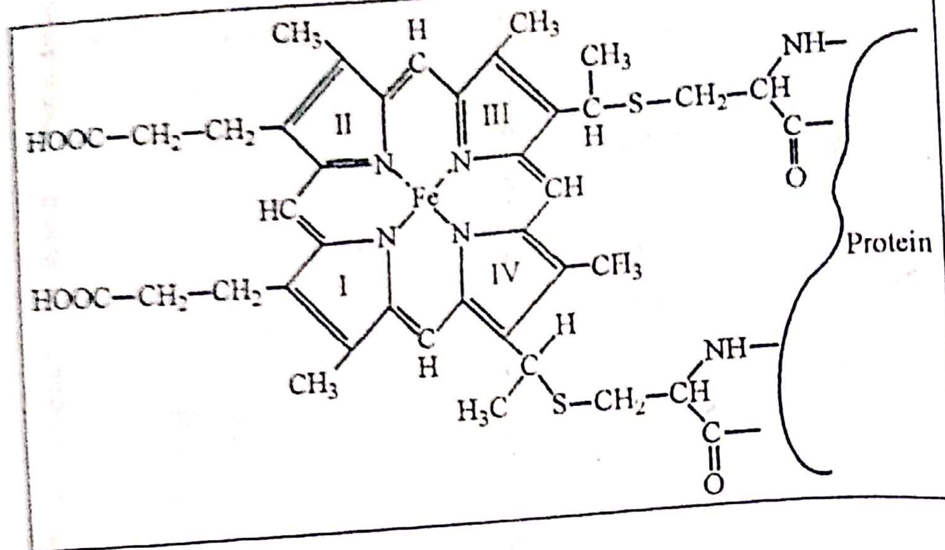

Fig. 5.16. Cytochrome *c*

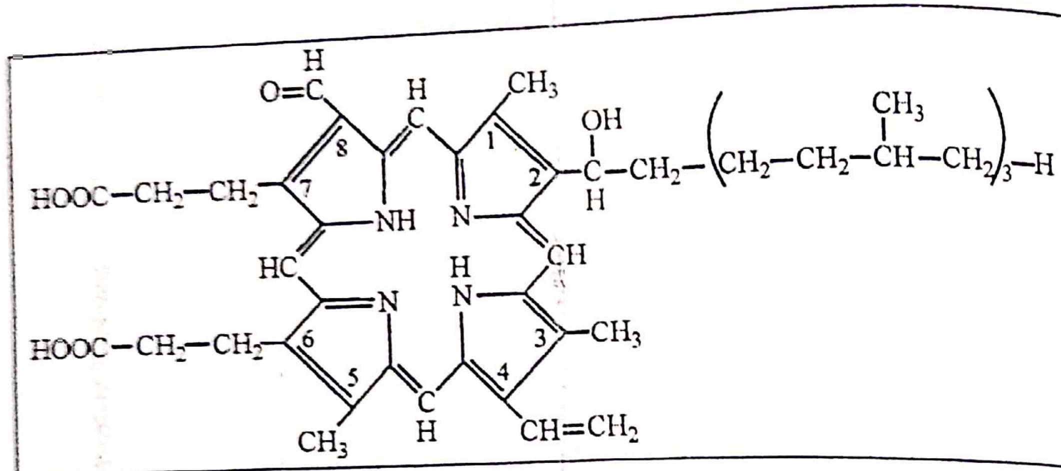

Fig. 5.17. Porphyrin A

The cytochromes tend to form complex with HCN, CO and H_2S, and such complexes are readily detected by their characteristic absorption spectra.

These reagents react by virtue of their ability to occupy one or both the two coordination positions of the Fe atoms that are not occupied by the nitrogen atoms of pyrrole rings of the porphyrin. In cytochrome c, where those two positions are occupied by other structures, complexes with HCN, CO and H_2S are not formed at neutral pH.

The cytochromes are capable of being alternately reduced and oxidized. The iron of the oxidized cytochromes is ferric iron; it is reduced to ferrous iron by the incorporation of one electron into the valence shell of the iron atom. This property allows the cytochromes to function as carrier in the electron transport process.

(a) Cytochromes *b* : Two-*b*-type cytochromes are cytochromes b_K (cyt_{560}) and cytochrome b_T (cyt b_{565}). The subscripts K and T stand for Keilin type cytochromes *b* and transducing cytochromes b_1 respectively. The two cytochromes are located in the middle of the membrane.

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Cyt b_T plays a direct role in energy conservation. The *b* cytochrome contains protoheme, which is also found in bacterial cytochrome *Q*.

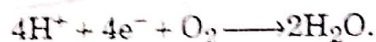
(b) Cytochrome b_5 It is a small molecule (m.w. 16,000) and occurs tightly bound to membranes of the endoplasmic reticulum. It involves in biosynthesis of fatty acids in the step converting beta-ketoacyl CoA to beta-hydroxyacyl CoA and in the conversion of α, β-unsaturated acyl CoA to acyl CoA. It is a component of NADH dependent stearyl CoA desaturase system (which converts stearyl CoA to oleil CoA).

(c) Cytochrome bc_1 Complex : This is made up of eight subunits–cytochrome c_1 (m-w. 29,000), Cytochrome *b* (m-w- 28,000), iron sulphur center (m- w- 24,000) and five other subunits. The total molecular weight will be in the range of 200,000. Except cytochrome *b* which is encoded by mitochondrial DNA, the other subunits are all encoded by nuclear DNA.

(d) Cytochrome c_1 : is an intrinsic protein with (MW 30,6000). It appears to be firmly bound to the membrane. The heme containing part of cytochrome c_1 is located on the *c*-side of the membrane from where it can easily transfer electrons to cytochrome *c*.

(e) Cytochrome aa_3 or cytochrome oxidase : Cytochromes *a* and a_3 are considered to be separate cytochromes. They have never been physically separated, and apparently cannot function independently. The functional unit of mammalian cytochrome oxidase consists of two heme molecules and two copper atoms ($Cu_α$ and $Cu_β$). Cytochrome *a* does not react with carbon-monoxide and therefore, is not inhibited by it. Cytochrome a_3, on the other hand, reacts with CO and is inhibited by it.

Cytochrome *c* oxidase contains four units (Cyt *a*; cyt a_3 , $Cu_α$ and $Cu_β$) which carry one electron each. Thus four electrons are stored in the enzyme prior to discharge. The four electrons ($4e^-$) combine with four protons ($4H^+$) and one molecule of oxygen to form water.



$$4H^+ + 4e^- + O_2 \longrightarrow 2H_2O.$$

(f) Cytochrome *c* : This is probably the most important class of electron transfer proteins,. involved in photosynthesis embedded in the thylakoid membranes of chloroplasts. They are also involved in the opposite process of cellular respiration in plants and animals which occurs in the membranes of mitochondria.

The heme group in cytochrome *c* is superficially the same as that in hemoglobin. However, the molecule will reveal subtle but highly important differences, *e.g.*, the vinyl groups have been converted to thioethers by covalent linkage to cysteine amino acid residues of the cytochrome protein chain. Also fifth and sixth coordination sites of iron are occupied by the imidazole nitrogen of a histidine amino acid residue and the sulphur of a methionine amino acid residue so the heme from cytochrome *c* is called heme *c*.

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Heme *c* in cytochrome *c*, is thus firmly covalently anchored to the protein amino acid sequence, which compares with the looser non-bonding interactions that bind heme to globin in Hb.

Heme *c* in cytochrome *c* has a redox potential of $E^0 = 0.25$ V. In fact cytochrome *c* is just one a family of cytochromes that forms an electron transporting chain, sequentially oxidising each other and so passing electrons from one to the next (*e.g.* *b* to c_1 to *c* to $a + a_3$ the suffixes were originally used by their discoverer, David Keilin; note the fact that these cytochromes are distinguished from each other by changes in their light absorption spectra) terminating with oxygen.

The polypeptide chain attached to the heme group in cytochrome *c* contains a variable number of amino acids; from 103–104 (Fig. 5.18).

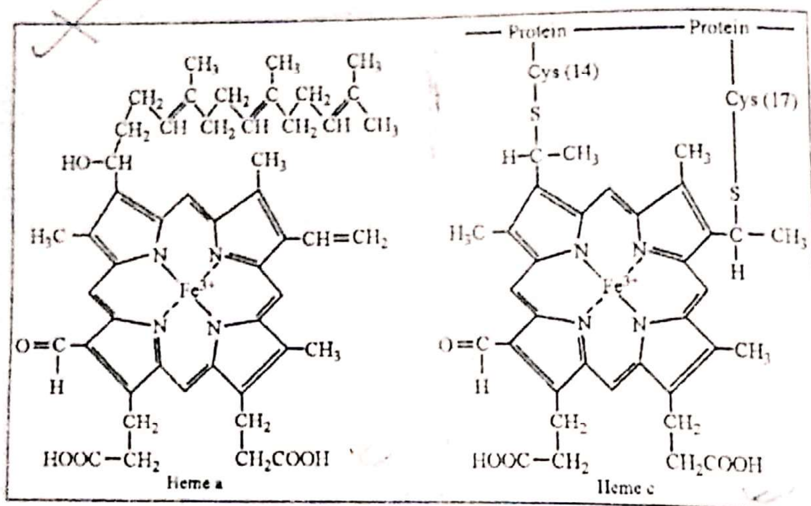


Fig. 5.18. Comparison of heme *a* (cytochrome *a*) and heme *c* (cytochrome *c*).

Mechanism of action of cytochrome *c* :

X–ray studies show that cytochrome *c* is a roughly spherical molecule with a groove down one side. Into this groove fits heme *c* with one edge (a mesoposition) (Fig. 5.17). The heme would, therefore, appear to be ideally placed to undergo fast electron transfer via a peripheral mechanism involving the porphyrin π system. Cytochrome *c* is probably electron carrier hemoprotein that links the complex of site (II) to cytochrome oxidase in site (III), but it is still not clear exactly what mechanism it uses for the oxidation and reduction of the heme moiety. However, in the reduced state, the Fe(II) porphyrin undergoes oxidation to form a π cation radical, which then undergoes rapid internal rearrangement of its electron so that the Fe(II) gets oxidised to Fe(III). This would be possible because in the low spin state the t_{2g} set of iron d orbitals are full [Fe (II) has a d^6 electron arrangement] and are strongly coupled to the porphyrin π system. Consequently, any perturbation of the porphyrin π system such as oxidation, would immediately be transmitted to the iron atom. This does not preclude the possibility that the heme iron in cytochrome *c* might be oxidised and reduced via a fast "outer sphere" axial process. This can be explained

using a peripheral or an axial mechanism. It is possible for both mechanisms to occur simultaneously, in so far as the oxidation could occur at the peripheral position of the porphyrin, while reduction of Fe [III] back to Fe [II] might involve electron transfer through one of the axial ligands (probably the axial sulphur). This, in turn, would require electrons to be

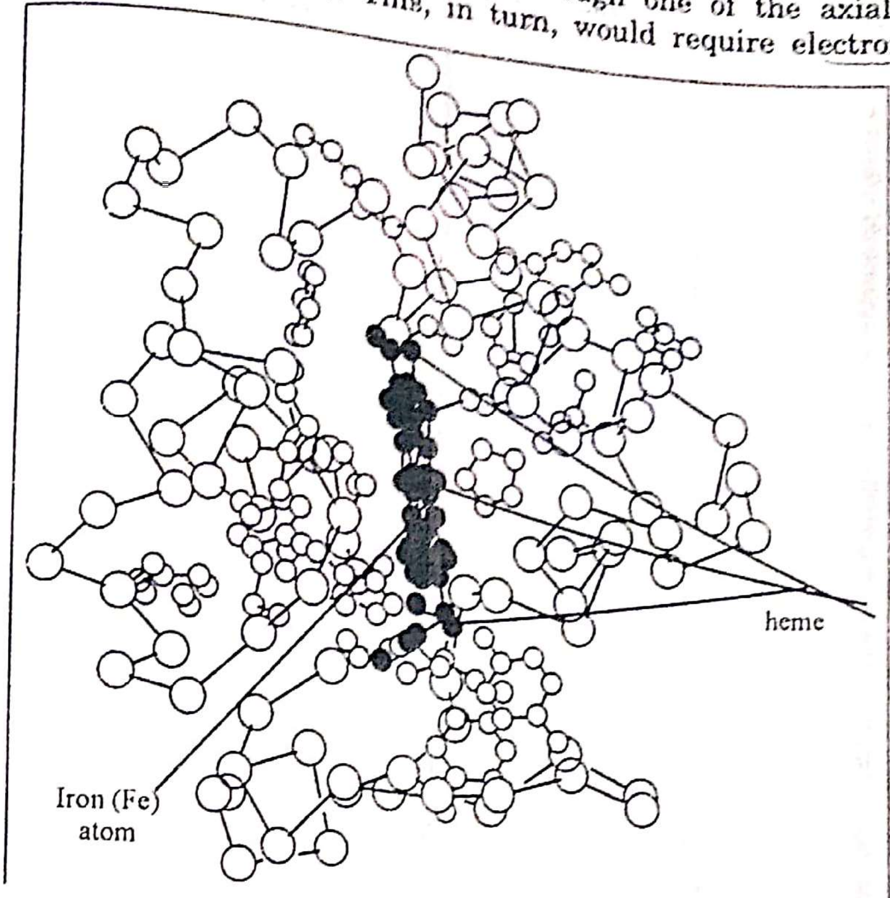


Fig. 5.19 (a). Cytochrome *c* and its heme group (a). The general disposition of the polypeptide chain showing the pocket in which heme resides.

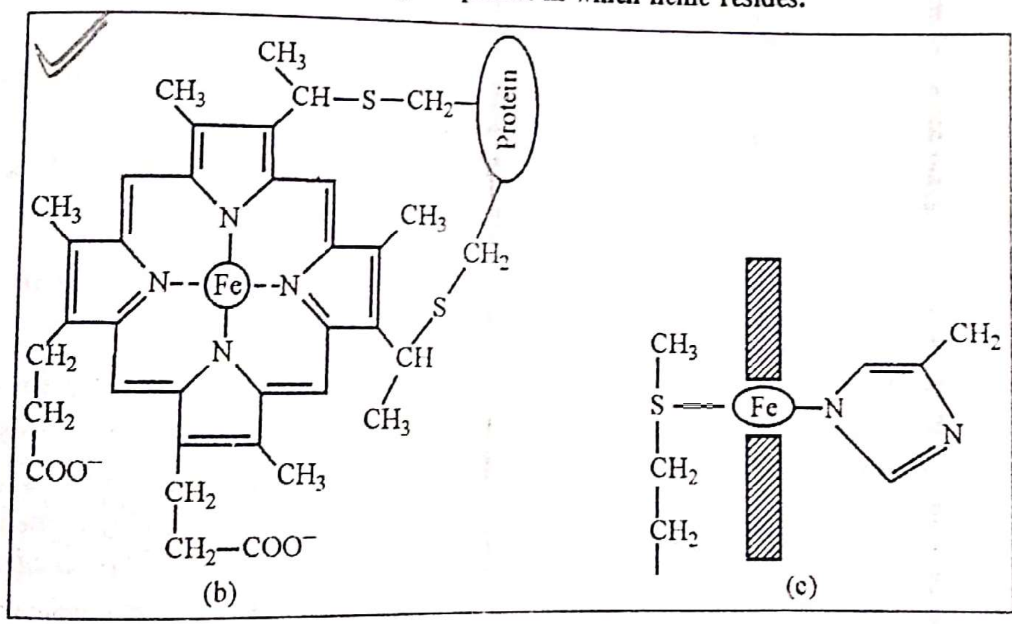


Fig. 5.19 (b) Structure of the cytochrome *c* heme group.
(c) A view perpendicular to the plane of the heme group showing bonding of heme iron to histidine and methionine residues.

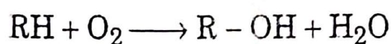
transferred through the protein chain to the heme, so that the sites of oxidation and reduction of cytochrome *c* could be separate from each other. It is known that the conformation of the protein around the heme is different in the oxidised and reduced forms of cytochrome *c*. In addition, measurements of the change of E° with pH for cytochrome *c* shows that acidic and basic amino acid residues near the heme are involved in the electron binding process. (Fig. 5.19a, b, c).

(g) Cytochrome P_{450} : The major enzyme in hydroxylation in site (I) is mono-oxygenase or cytochrome P_{450}. The reaction catalyzed by a mono-oxygenase is :

$$RH + O_2 + NADPH + H^+ \longrightarrow R\text{–}OH + H_2O + NADP^+$$

RH represents a very wide variety of drugs, carcinogens, pollutants and certain endogenous compounds such as steroids. The reaction catalyzed by cytochrome P_{450} can also be given as:

$$\text{Reduced cyt. } P_{450} \longrightarrow \text{oxidized cyt. } P_{450}$$



$$RH + O_2 \longrightarrow R - OH + H_2O$$

The major monooxygenases in the ER (endoplasmic reticulum) are cytochrome P_{450}. The cytochrome P_{450} are notable because 50% of the drugs ingested by patients are metabolized by species of cytochrome P_{450}. The same enzyme can also act as various carcinogens and pollutants.

Cytochrome P_{450} species :

1. These are hemoproteins.

2. They are present in high concentration in the membrane of the (ER) of liver. They are also present in the mitochondria as well as in the (ER) of the adrenal.

3. Six closely related species of cytochrome P_{450} present in liver (ER) act on a wide variety of drugs, carcinogens and xenobiotics.

4. The enzyme that uses NADPH in the reaction mechanism of cytochrome P_{450} is called NADPH–cytochrome P_{450} reductase.

5. The suitable lipid phosphatidyl choline, the major lipid found in the membranes of ER, is the component of the cytochrome P_{450} system.

6. The one other species named as cytochrome P_{-448} is specific for the metabolism of polycyclic aromatic hydrocarbons (PAH_S) and related molecules; hence it is said to be aromatic hydrocarbon hydroxylase (AHH). This enzyme is very important in the metabolism of (PAHs) and in carcinogenesis produced by this agent. Smokers have higher levels of this enzyme in some of their cells and tissues than nonsmokers.

Function of cytochrome in cellular electron transport :

Electron transport, and its corollary oxidative phosphorylation (the generation of ATP from ADP), are the final and most important stages of

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cellular respiration. All the enzymatic steps in which high energy molecules, such as carbohydrates, fats and amino acids are oxidized in aerobic cells lead to these last steps : electrons flowing towards oxygen from organic substrates, and reducing the oxygen to water. Energetically, this flow is "downhill" so

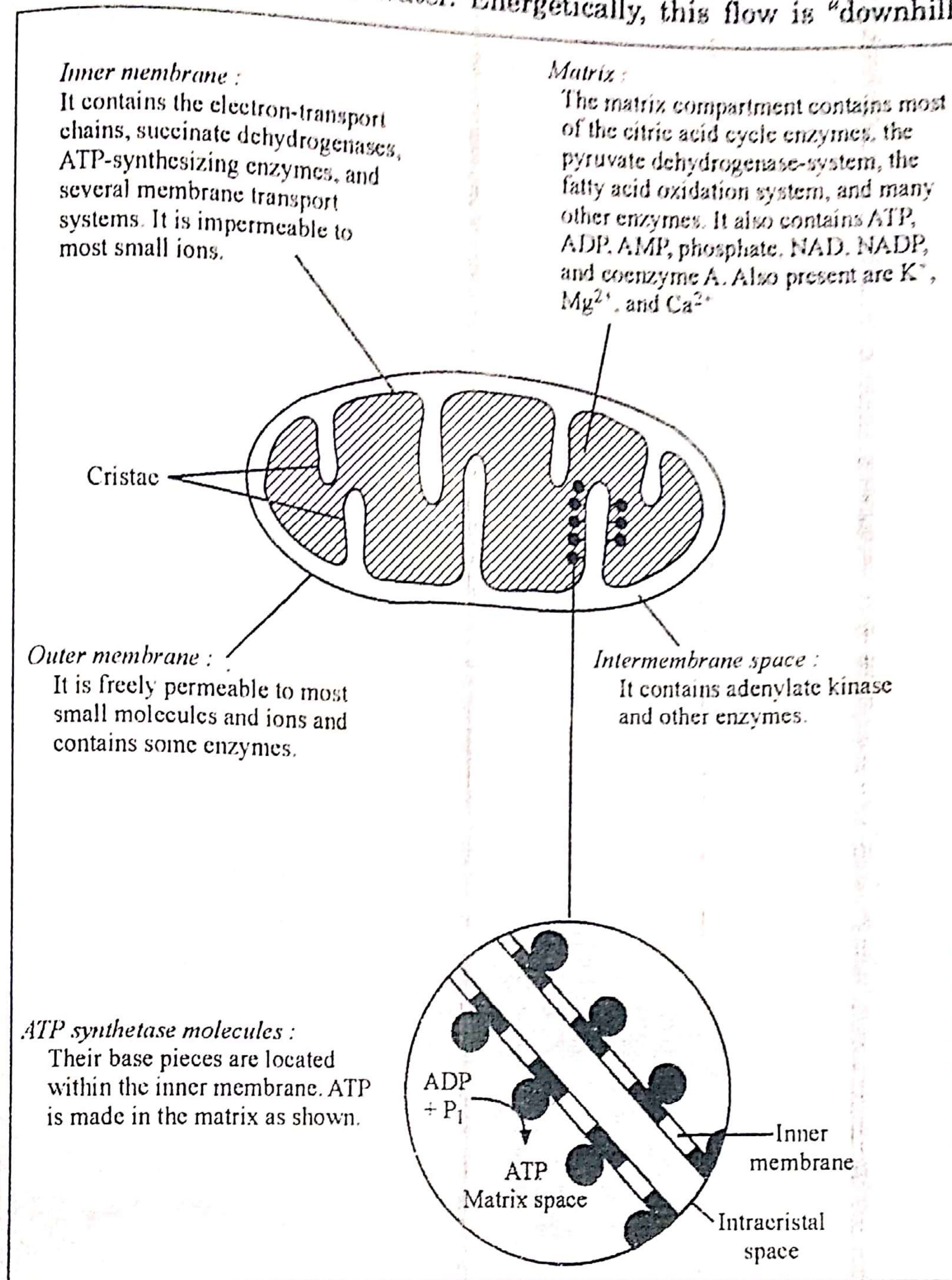

Fig. 5.20. The biochemical anatomy of mitochondria, showing the location of the enzymes of the citric acid cycle, the electron-transport chains, the enzymes catalysing oxidative phosphorylation, and the internal pool of coenzymes. The inner membrane of a single liver mitochondrion may have over 10,000 sets of electron transport chains and ATP synthetase molecules. The number of sets is proportional to the area of the inner membrane. Heart mitochondria, which have very profuse cristae and hence a much larger area of inner membrane, contain over three times as many sets of electron-transport systems as liver mitochondria. The internal pool of coenzymes and intermediates is functionally separate from the cytosolic pool.

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that electrons are losing energy. This energy is used in oxidative phosphorylation.

It can easily be over-looked, just how important ATP synthesis is, for it is the hydrolysis of ATP back to ADP that generates the energy we need to

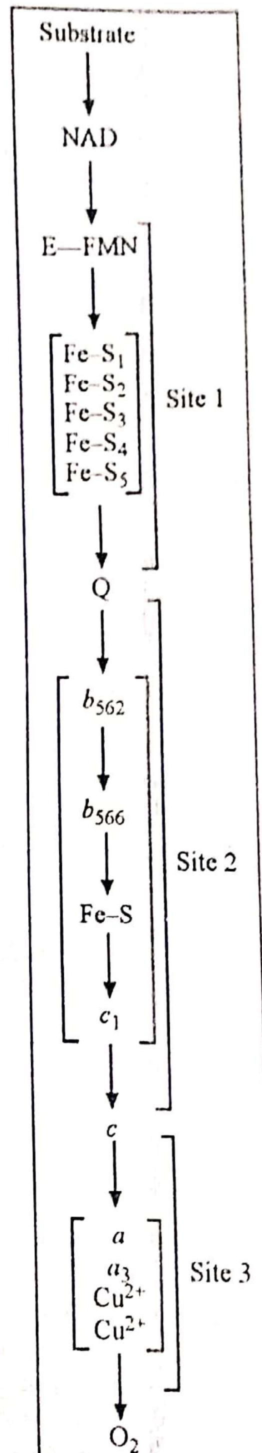

Fig. 5.21. The complete set of electron carriers of the respiratory chain. In site 1, there are at least five different iron-sulphur. In site-2, there are two different forms of cytochrome *b*, with different light-absorption peaks, as well as an iron-sulphur center distinct from those in site 1. In site 3, there are two copper ions in addition to cytochromes *a* and a_3. The precise sequence and function of all the redox centers is not known with certainty.

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live. Cytochrome c is just one of the links in the chain of electron carrier proteins that ultimately produce ATP.

The site of this electron-transporting chain, and the enzymes that produce ATP, are cristae within the cells mini power station, the mitochondrion. Each mitochondrion consists of a space, the matrix, which contains all the enzymes necessary for the oxidation of molecules necessary for the citric acid cycle. The cristae are finger like projections of the mitochondrial inner membrane on which the various electron–transporting and ATP–synthesising molecules are lined up.

The complete electron transporting chain consists of around 15 or more chemical groupings in three main sites. These sites are really collections of electron transporting proteins grouped together as functional complexes (Fig. 5.20).

The fourth complex connects site one to site two. Thus, the first complex in the chain consists of the enzyme NADH dehydrogenase and a group of around five closely linked proteins that contain electron rich iron sulphur clusters (Fig. 5.22).

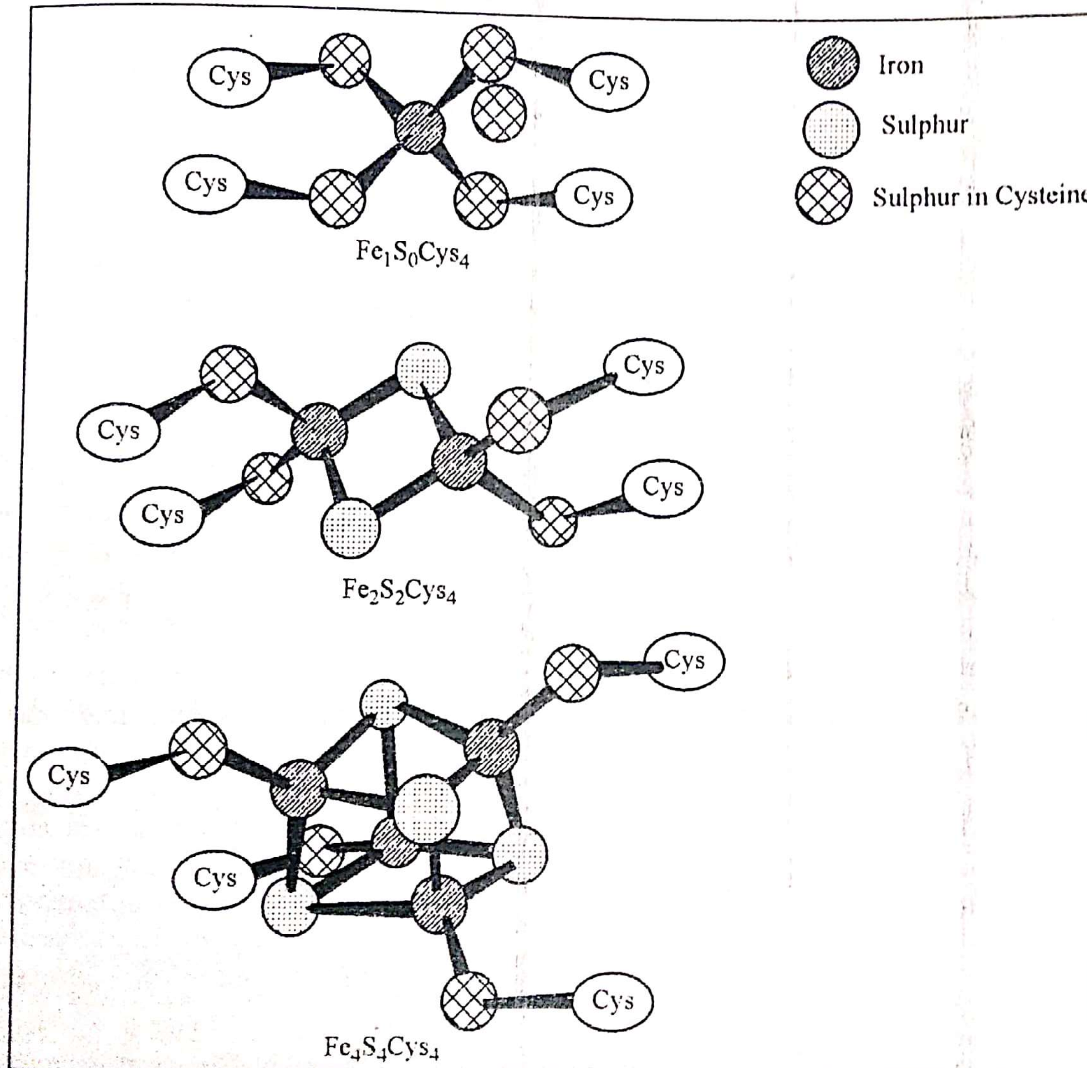

Fig. 5.22. The structure of iron-sulphur centers.

These are non-heme proteins vital to the flow of electrons through the chain. The second complex consists of the enzyme succinate dehydrogenase and its collection of iron sulphur proteins. The third complex contains the hemoproteins cytochromes b and c_1, and one iron sulphur protein. The final complex, the one that actually reduces oxygen to water, is called cytochrome oxidase and contains two heme groups (heme a and heme a_3) and two copper centers. Ubiquinone, also known as coenzyme Q, is a lipid soluble quinone which connects the first, second and third complex (Fig. 5.21).

The reason why these sites as complexes has to do with the way they are separated from the mitochondrial membrane. But how do they know that these electron carrier proteins function in the sequence that they do ?

The major components are arranged sequentially in order of their standard redox potential (E°), which are successively more positive as the chain proceeds towards oxygen (electrons tend to flow from electronegative to electropositive systems, causing a decrease in the available free energy). Second, each member of the electron transporting chain is specific for a given electron donor and acceptor. So NADH can transfer electrons on to NADH dehydrogenase but cannot short-circuit the chain by transferring them directly to cytochrome b or c. Thus the following processes are —

(1) Hydrogen atoms are enzymatically removed from the compound to be oxidized. The removal of H atoms is received by suitable coenzymes like NAD^+ or $NADP^+$. The enzyme NAD^+ or $NADP^+$ is thus reduced and becomes NADH or NADPH.

(2) Secondly, the hydrogen atoms are transferred again enzymatically to other coenzymes FAD and NAD^+ (or $NADP^+$) becomes regenerated so that it is ready for catching H atom again and FAD is reduced and becomes FADH.

(3) From FADH, H atom is transferred to ubiquinone or coenzyme Q. The H atom ionizes here, so that one electron is lost from this H atom. The removed electron is transferred to the cytochrome b containing Ferric ion (Fe^{+++}), which on receiving the extra electron becomes ferrous (Fe^{++}) ion *i.e.* it is reduced.

After losing the electron H atom becomes H^+ *i.e.* a proton and stays with coenzyme Q.

(4) From cytochrome b the electron is transferred to cytochrome c and then from cytochrome c to cytochrome a.

(5) By the action of the enzyme cytochrome oxidase (aa_3) (high affinity for oxygen) the extra electron from cytochrome a is transferred to molecular oxygen. As a result the molecular oxygen becomes negatively charged whereas cytochrome a is regenerated.

(6) Thus negatively charged oxygen combines with the proton and a molecule of H_2O is formed.

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Therefore ultimately oxygen is the final acceptor of hydrogen in biological oxidations (Fig. 5.23).

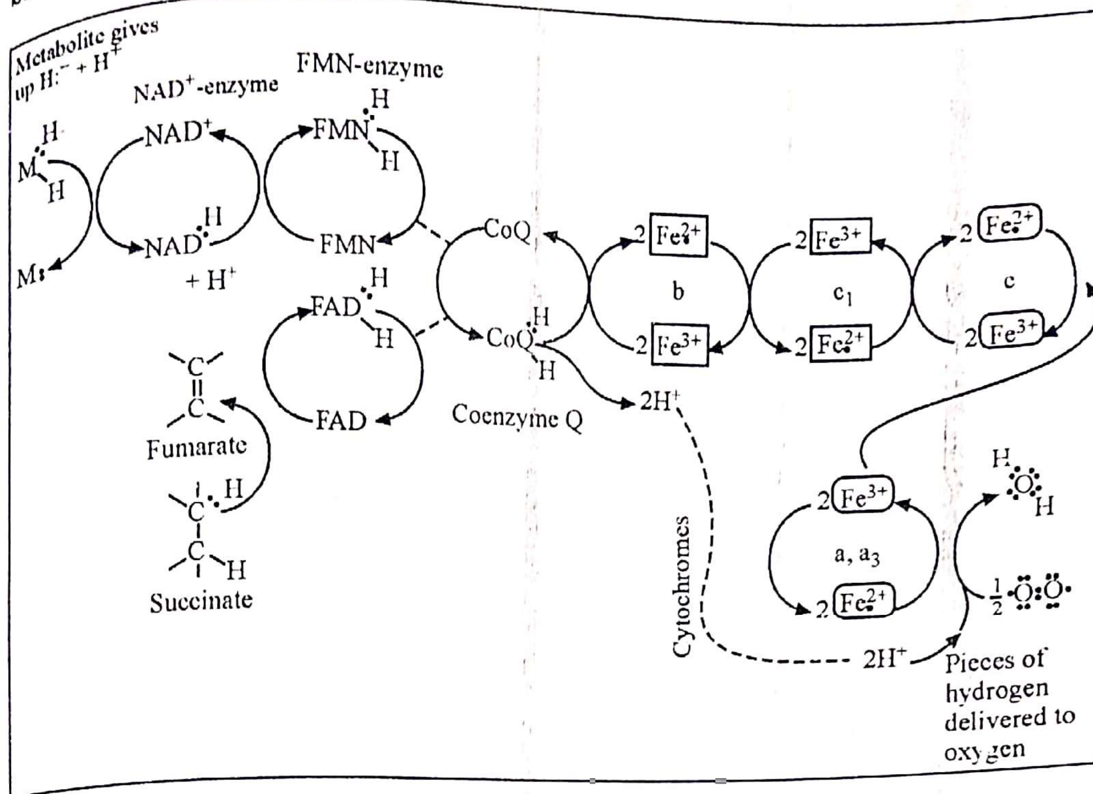

Fig. 5.23. The respiratory chain.

Iron–Sulphur Proteins : In 1957 Massey discovered that H_2S was formed upon precipitation of succinate dehydrogenase by acid. This was the first observation pointing to the presence of special form of sulphur in iron proteins that do not contain heme. Later acid labile sulphur attracted attention in connection with the investigation of a new class of electron carrier proteins. These were discovered in 1962 and were given the name ferredoxins. Ferredoxins participate in the transfer of electrons in diverse processes as photosynthesis, fixation of molecular nitrogen, assimilation of CO_2, reduction of nitrates and sulphite, hydroxylation of steriods, respiration of mitochondria etc. However, the name ferredoxin is given only to proteins that display exclusively electron carrier properties and no classical enzymatic function. All ferredoxins contain equivalent quantities of non-heme iron and acid labile or "inorganic" sulphur. Later it was established that the atoms of iron and acid labile sulphur form "clusters" the active sites of ferredoxins and a number of other non-heme iron sulphur protein. The Fe–S clusters are bonded to the protein iron SH groups of cysteine residues.

Four types of clusters are known : [1Fe–0S], [2Fe–2S], [3Fe–4S] and [4Fe–4S]. Electron paramagnetic resonance [EPR] at temperature below 100K is widely used in the study of proteins containing these clusters. Characteristic EPR signals are given by reduced forms of Fe–S proteins.

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The Fe–S clusters also give rise to characteristic absorption, circular dichroism and Mossbauer spectra (Fig. 5.24).

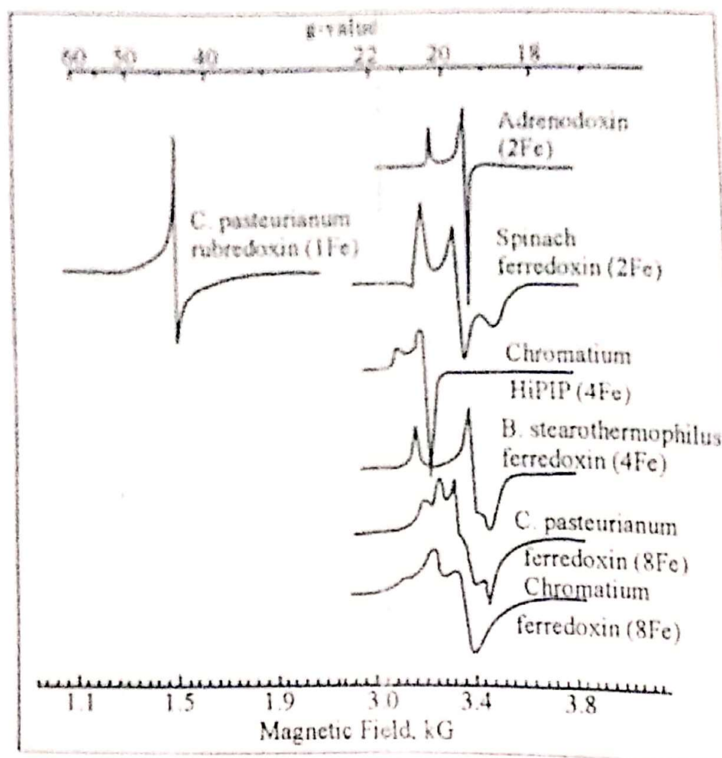

Fig. 5.24. EPR spectra of iron-sulphur proteins. Spectra of rubredoxin and HiPIP were measured in the oxidized state, spectra of the other proteins in the reduced state. The temperature of measurements was 77°K for the rubredoxin and adrenodoxin, and about 20°K for other proteins.

Iron sulphur clusters are observed not only in ferredoxins but also in bacterial rubredoxin, and in a number of enzymes, including hydrogenase, nitrogenase, nitrate and sulphite reductase, dihydroorotate dehydrogenase, glutamate synthetase, Xanthine and aldehyde oxidases, succinate and NADH dehydrogenases. The majority of these enzymes contain additional prosthetic groups — flavin, heme or molybdenum-besides the non-heme iron and labile sulphur. Some clusters e.g., in Xanthine oxidase of milk are of the [2Fe–2S] type while in others e.g., in bacterial hydrogenase are of the [4Fe–4S] type.

It is not yet clear whether clusters in enzymes participate only in electron transfer or are involved in substrate binding and activation. In case of Xanthine oxidase, clusters serve only as electron reservoirs that function to maintain the molybdenum as Mo(VI), for efficient reduction by xanthine and the flavin as $FADH_2$, for efficient oxidation by O_2.

1. Rubredoxin : These are non-heme iron proteins that participate in electron transfer processes. They contain Fe bonded to sulphur. Rubredoxin was first isolated from the bacterium *clostridium pasteurianum*, but it also occurs in other anaerobic bacteria. They are relatively low molecular proteins (~ 6000) and contain one iron atom which is bonded to four S atoms of cysteine ($SHCH_2CH(NH)_2CO_2H$) as part of a protein

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of about 54 amino acid residues. When Fe (III) is reduced to Fe (II) there is slight (0.05 Å) increase in the Fe–S distance, but the tetrahedral coordination is maintained. Iron is in the high–spin condition in both oxidation states (Fig. 5.25).

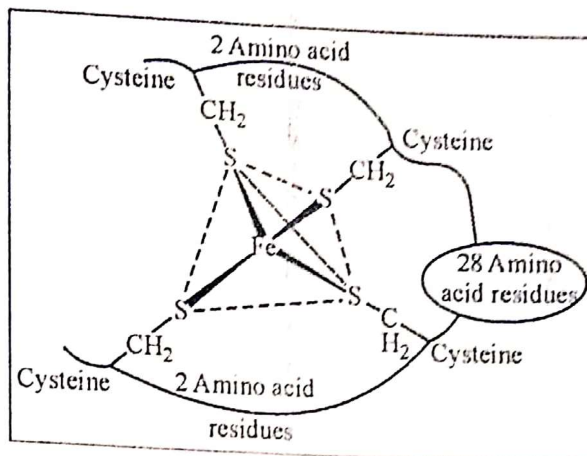

Fig. 5.25. The environment of the iron atom in the rubredoxin molecule.

2. Ferredoxin : is a non-heme iron containing proteins, iron and sulphur. There are several types of ferredoxin. The four known classes of iron sulphur proteins are:

(a) [1Fe – 0S] : This is the simplest rubredoxin (cys – S)$_4$ Fe. It is a bacterial protein of uncertain function with molecular weight of about 6000. The single iron atom is at the center of tetrahedron of four cysteine ligands. Fe–S distance is 2·267Å. (Fig. 5.26).

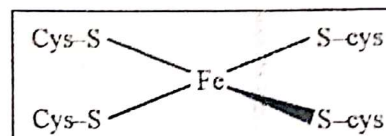

Fig. 5.26. Fe, S in bacterial rebredoxin.

(b) [2Fe – 2S] : Clusters of the [2Fe–2S] type have been found in the ferredoxin of plants, bacteria and mammals. The majority of these proteins have molecular weight between 10,000 and 12,500 and contain at least four residues of cysteine. Their oxidation reduction potentials E_0, vary from –0.42 to –0.24V. The structure for the Fe–S clusters has been postulated on the basis of a number of physico- chemical investigations (Fig. 5.27).

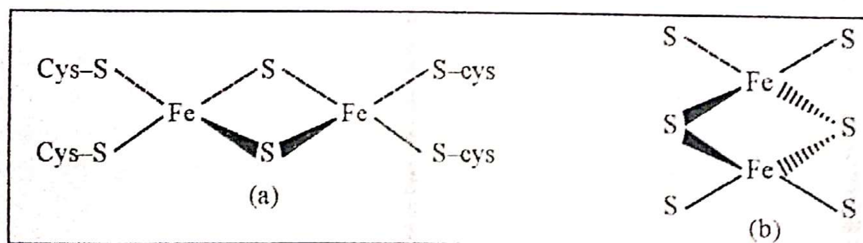

Fig. 5.27. (a) Fe_2S_2 in photosynthetic ferredoxin.
(b) Fe_2S_2 in S. *platensis* ferredoxin.

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The proposed structure of the [2Fe–2S] cluster agrees with the properties of a synthetic analog the anion bis (O-xylyl-α, α′-dithiolato-μ_2-sulfidoferrate (III)) (Fig. 5.28).

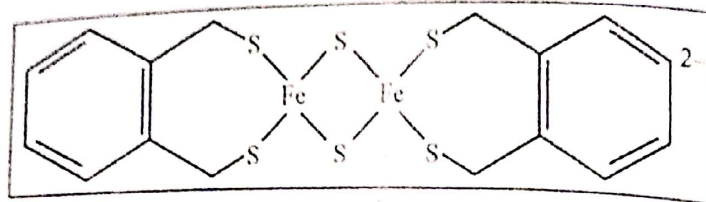

Fig. 5.28

The electronic, Mossbauer and NMR spectra, and magnetic susceptibility of this compound are very much similar to those of the oxidized [2Fe–2S] ferredoxins. Thus, this synthetic cluster compound can be regarded as an adequate model for the active site of ferredoxins.

(c) [3Fe – 4S] : These contain Fe_3S_4 (S – cys)$_3$ unit. This species is most easily visualized as an Fe_4S_4 cube missing one corner. The oxidized form of the three iron centers contains three Fe (III) ions. The reduced form of this contains three Fe (III) ions and one Fe (II) ion (Fig. 5.29).

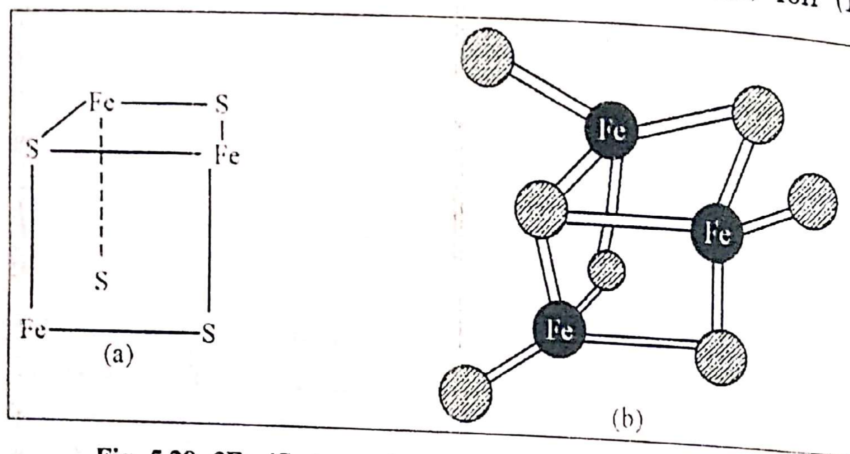

Fig. 5.29. 3Fe-4S cluster in *A. Vinelandii* ferredoxin (a, b).

(d) The structure of [4Fe–4S] clusters : Cluster of the [4Fe–4S] type are found in a large number of bacterial ferredoxins which contain four or eight atoms of iron, and also in "high potential iron sulphur protein, HiPIP". The latter, isolated from the photosynthetic bacterium *chromatium*, which contains four atoms each of Fe and labile sulphur and four residues of cysteine. It has molecular weight of 9,254 and high value of E_0 of +0.35 V. In contrast to most ferredoxins, which give EPR signals in reduced state, HiPIP shows an EPR signal in oxidized state (g_{11} = 2.12; $g_{\perp}$ = 2.04).

The structure of the HiPIP cluster was established by X-ray crystallography at a resolution of 2Å, shown in Fig. (5.30).

The cluster forms a distorted cube in four opposite corners of which are located atoms of Fe. The labile sulphur atoms are located in the remaining four corners of the cube. Each atom of iron is bound to four atoms of sulphur : three of acid labile sulphur which form sulphide bridges,

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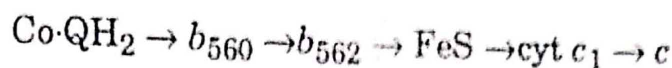
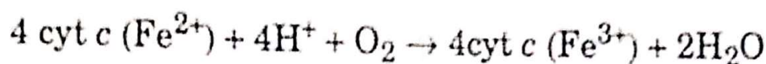
It contains two b-type cytochromes, b_{560} and b_{562}; a c-type cytochrome c_1; and an iron-sulphur center.

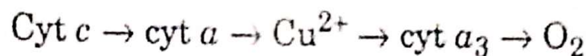


$$Co{\cdot}QH_2 \rightarrow b_{560} \rightarrow b_{562} \rightarrow FeS \rightarrow \text{cyt } c_1 \rightarrow c$$

4. Complex (IV) : The complex contains cytochrome a, two Cu^{+2} ions and cytochrome a_3; the flow of electron is as follows : (Fig. 5.32)



$$4 \text{ cyt c } (Fe^{2+}) + 4H^+ + O_2 \rightarrow 4\text{cyt } c\ (Fe^{3+}) + 2H_2O$$



$$\text{Cyt } c \rightarrow \text{cyt } a \rightarrow Cu^{2+} \rightarrow \text{cyt } a_3 \rightarrow O_2$$

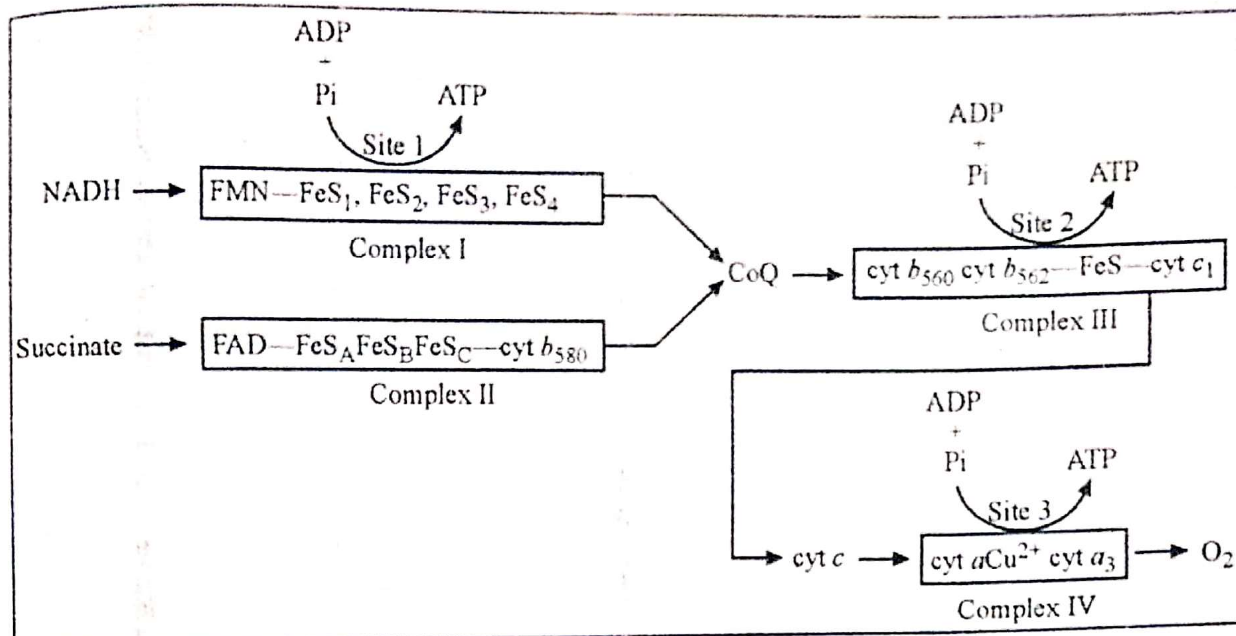


Fig. 5.32. The cytochrome electron transport chain (a) complexes I, II, III and IV are shown together with the specific carrier components for each complex.

Synthetic Models :

1. Models of cytochrome P_{450} : Cytochrome P_{450} are membrane-bound mono-oxygenase enzymes that homogeneously catalyse oxygen atom transfer to bound non-polar substrates. In fact, enzymes collectively classed as P_{450} are not really cytochromes : their function is oxygen atom transfer, not electron transport.

P_{450} use NADPH to reduce dioxygen, one oxygen atom is reduced to water while the second is transferred to a wide variety of endogenous (*e.g.* steroids, fatty acids. leukotrienes and prostaglandins) and exogenous (*e.g.* drugs, pesticides, anaesthetics, solvents and chemical carcinogens) substrates.

The structure of the camphor-hydroxylating enzyme, for example, from *Pseudomonas putida* shows that the camphor-binding site the heme, and the oxygen-binding site are all in close proximity within the protein. In addition, the camphor binding site is lined with hydrophobic amino acid residues and protected from the outside world by a reversible cap. The heme is attached to the protein only via axial sulphur atom of a cysteine residue, with a water molecule, near enough to the iron to be a second

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of (3–). For the *chromatium* HiPIP the charge of the reduced form would be (2+) and that of the oxidized form (3+). The (2+) state is diamagnetic whereas both (3+) and (1+) states are paramagnetic and are detected by EPR measurements.

In the ferredoxin of *Azotobacter vinelandii* both a high potential cluster ($E_0' = +0.34$ V) and a low potential cluster ($E_0' = -0.42$ V) have been observed.

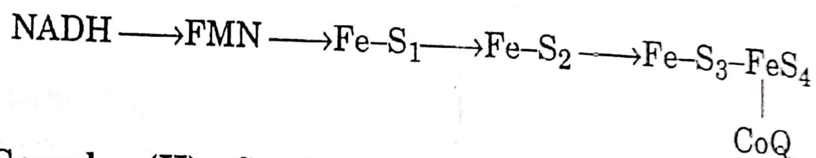
From EPR spectra of this ferredoxins it appears that despite their widely different potential both clusters function between the C and C^+ states, *i.e.*, between the oxidized and "super-oxidized" forms as in the HiPIP clusters. A HiPIP-cluster has also been observed in mammalian succinate dehydrogenase. It has a potential of +0.06 V, gives an EPR signal in the oxidized state, and together with two other iron-sulphur clusters found in this enzyme, seems to play an important role in the transfer of electrons in the respiratory chain.

Recently a binuclears [2Fe–2S] cluster exhibiting EPR signals of the HiPIP type has been found in pig heart aconitase.

Functions of Iron–sulphur proteins : Some enzymes are metallic ions linked to the apoenzyme for transfer of electrons in oxidation-reduction reactions. Such enzymes are called metalloproteins. The commonly involved ions are those of iron and copper. Proteins bound with sulphur atom are called iron-sulphur proteins (Fe – S).

They function as :

1. Complex (I) : Complex (I) contains four iron sulphur centers. There is a large difference in the E° values of centers ranging from –0·330 – 0·020 V; therefore the flow of electrons is from NADH to FMN to the iron sulphur proteins where they flow from the iron-sulphur center with the lowest E_0 to highest.



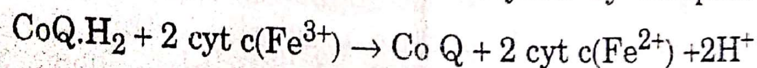
$$NADH \longrightarrow FMN \longrightarrow Fe\text{–}S_1 \longrightarrow Fe\text{–}S_2 \longrightarrow Fe\text{–}S_3\text{–}FeS_4 \;|\; CoQ$$

2. Complex (II) : Catalyzes the reduction of CoQ by electrons removed from succinate.

[Succinate + CoQ ⟶ Fumarate + $CoQ{\cdot}H_2$]. This complex, contains FAD, composed of four polypeptides with molecular weight of 70,000, 27,000, 15,000 and 13,000.

The two larger peptides contain the catalytic site for the oxidation of succinate; the smaller (27,000) subunit of two contains an Fe_4S_4 center. The two smallest peptides (15,000 and 13,000) contain an Fe_2S_2 center and *a*, *b* cytochrome.

3. Complex (III) : The reacton catalyzed by complex (III) is :



$$CoQ.H_2 + 2\ cyt\ c(Fe^{3+}) \rightarrow Co\ Q + 2\ cyt\ c(Fe^{2+}) + 2H^+$$

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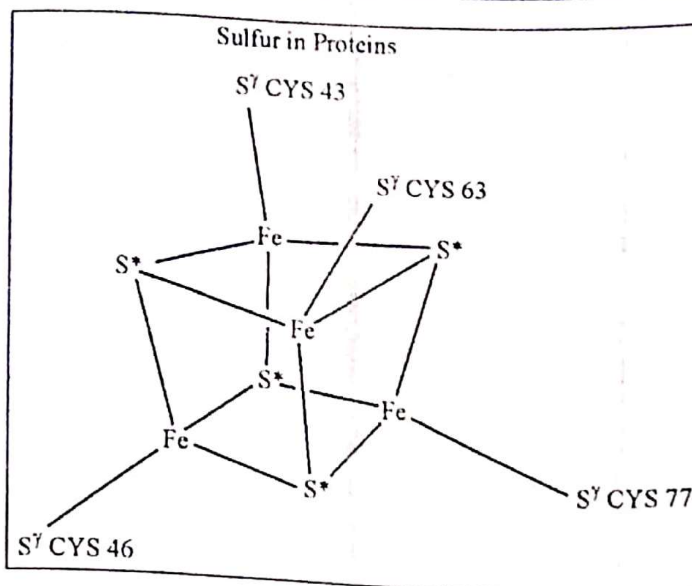

Fig. 5.30. Structure of the [4Fe–4S] cluster in the high potential iron-sulphur protein from *chromatium vinosum*. S* denotes an atom of acid-labile sulphur.

and one S^{γ} atom of a cysteine side chain. The size of the cluster is slightly diminished upon oxidation, the length of the Fe–S* and Fe–S^{γ} bonds being 2.26 and 2.20Å, respectively, in the oxidized form and 2.32 and 2.22Å in the reduced form.

Clusters with similar or nearly identical structures have been discovered in the ferredoxin of *Peptococcus aerogenes*. This protein, like the well studied ferredoxins of *clostridium*, contains eight atoms of iron and labile sulphur and eight residues of cysteine. Its molecular weight is about 6,000 and value of E_0' is about –0.4 V. The absorption spectrum of the oxidized form has a maximum at 390 nm which is diminished in the reduced form.

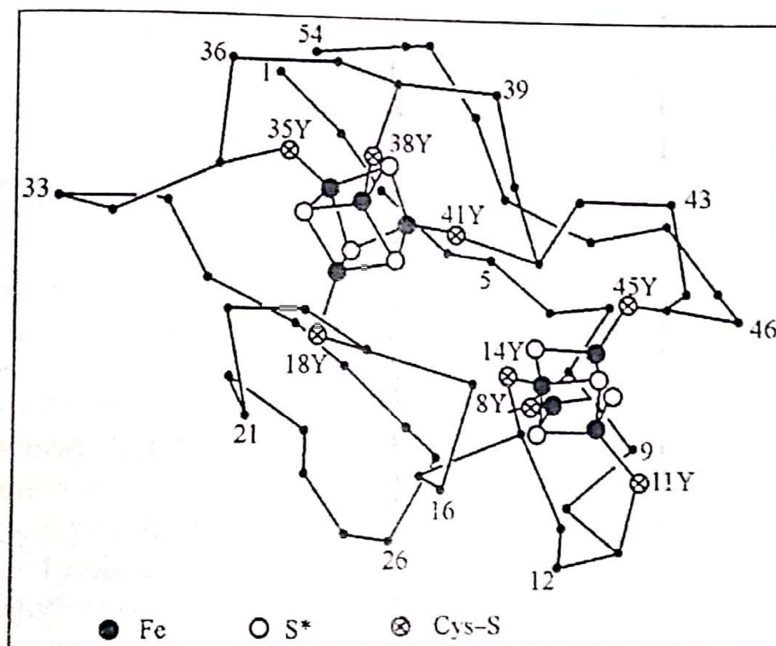

Fig. 5.31. Structure of the ferredoxin from *Peptococcus aerogenes*. The path of the peptide chain and the [4Fe–4S] clusters is shown.

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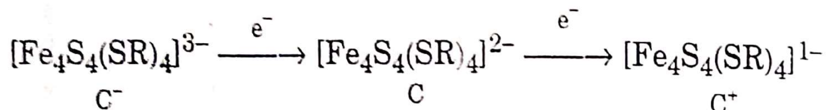
X-ray crystallographic analysis showed that this ferredoxin contains two identical [4Fe–4S] clusters, located in hydrophobic surroundings 12Å from each other. Each cluster appears to accept a single electron. Analysis of the EPR spectrum suggests interaction between the clusters. The clusters are attached to the protein by bonds between the iron atoms and SH groups of cysteine residues. Furthermore, there are 15–18 NH ...... S hydrogen bonds connecting the acid-labile and cysteine sulphur atoms to peptide groups of the protein (Fig. 5.31 on page 109).

The Fe–S clusters in the *chromatium* HiPIP and in the ferredoxin of *P. aerogenes* should have the same cube like structure and at the same time such great differences in physical properties. The difference in potential is 0.75V.

HiPIP is diamagnetic in the reduced state and paramagnetic in the oxidized form, while the reverse is true for the bacterial ferredoxins. The optical and magnetic properties of the oxidized ferredoxins of bacteria and reduced HiPIP are very much similar. These facts can be rationalized on the assumption that the Fe–S clusters can exist in one of three formal oxidation state denoted as C^+ $(1Fe^{2+} + 3Fe^{3+})$, C $(2Fe^{2+} + 2Fe^{3+})$ and C^- $(3Fe^{2+} + 1Fe^{3+})$.



$$\underset{C^-}{[Fe_4S_4(SR)_4]^{3-}} \xrightarrow{e^-} \underset{C}{[Fe_4S_4(SR)_4]^{2-}} \xrightarrow{e^-} \underset{C^+}{[Fe_4S_4(SR)_4]^{1-}}$$

C^+ is characteristic of oxidized HiPIP, C of reduced HiPIP or oxidized ferredoxin, and C^- of reduced ferredoxin. Under normal conditions, HiPIP is not reduced to the C^- state, nor is ferredoxin oxidized to the C^+ state. This behaviour apparently results from peculiarities of the protein surrounding the clusters. It has been established that the number of NH ...... S hydrogen bonds in the ferredoxin is significantly greater than in HiPIP.

This distinction may be one of the reasons that the clusters in the two proteins are stabilized in different oxidation states.

The term "high-potential iron sulphur protein" is used only for the protein from photosynthetic bacteria that was given this name initially. Otherwise, the use of the term is discouraged for two reasons : (1) The three dimensional structure of the active center of *chromatium* HiPIP is essentially the same as that of ferredoxins. The magnetic properties differ because the Fe–S clusters may assume three different oxidation levels. (2) It has become evident that other *iron-sulphur* proteins may have the unusual oxidation level of the *chromatium* protein without a high mid point oxidation-reduction potentials. Conversely, ferredoxins with the usual oxidation level and magnetic properties, may have high-mid point potentials.

It has also been suggested that the sulphur atoms of the bound cysteine residue not be included in the calculation of the formal charge of the clusters. Thus, the charge of the oxidized cluster of ferredoxins would be (2+) instead of (2–) and that of the reduced form (1+) instead

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axial ligand. Thus, according to the criteria, the heme bound iron cycles through a range of oxidation states relatively easily (Fig. 5.33).

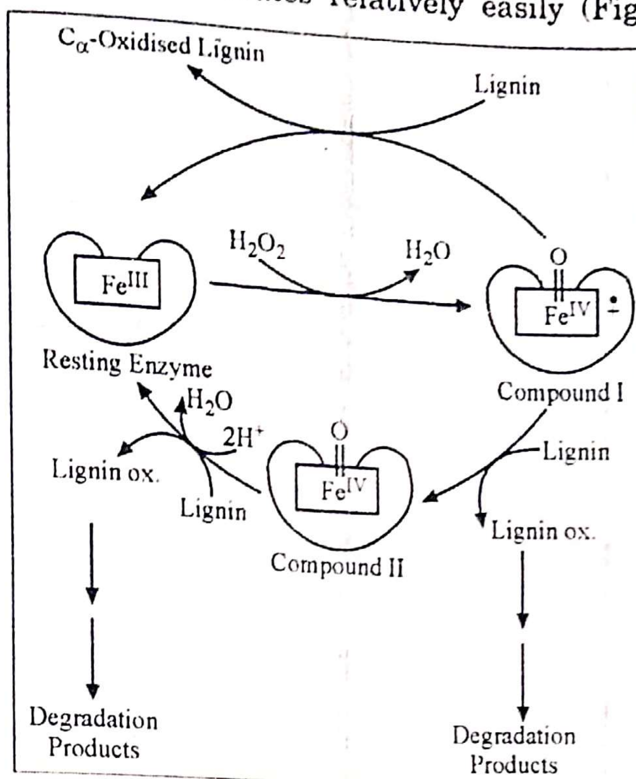

Fig. 5.33. Possible catalytic cycle.

In its resting state, the iron complex is in equilibrium between a five coordinate, high spin Fe (III) and a six coordinate, low spin Fe (III) complex. Binding of the substrate favours the five coordinate species as the water molecule in the sixth position is displaced. Uptake of an electron is favoured by the high spin Fe (III) which is reduced to Fe (II) and binds oxygen to form a stable, low spin, six-coordinate intermediate. On uptake of a second electron, the oxygen undergoes cleavage to yield water and an oxo-iron intermediate. It is this that oxygenates the substrate. The thiolate fifth ligand pushes electron density onto the coordinated oxygen, bringing about cleavage.

Molecules containing reduced oxygen species *e.g.*, alkyl hydroperoxides, peracids, periodate, hypochlorite, hydrogen peroxide, amine oxides and iodosoarenes are also capable of reducing dioxygen in the presence of P_{450}.

Most of the metalloporphyrin systems used as P_{450} models are variations on the theme of meso tetraarylporphyrins, with a variety of substituents in the meso-positions.

These models manage to combine effective stereospecific oxidation of the products, protection of the iron-porphyrin catalyst from oxidative degradation, high shape and regioselectivity and asymmetric induction. The central metal used in most of these systems is iron or manganese; the latter being more effective at alkane hydroxylation, in the presence

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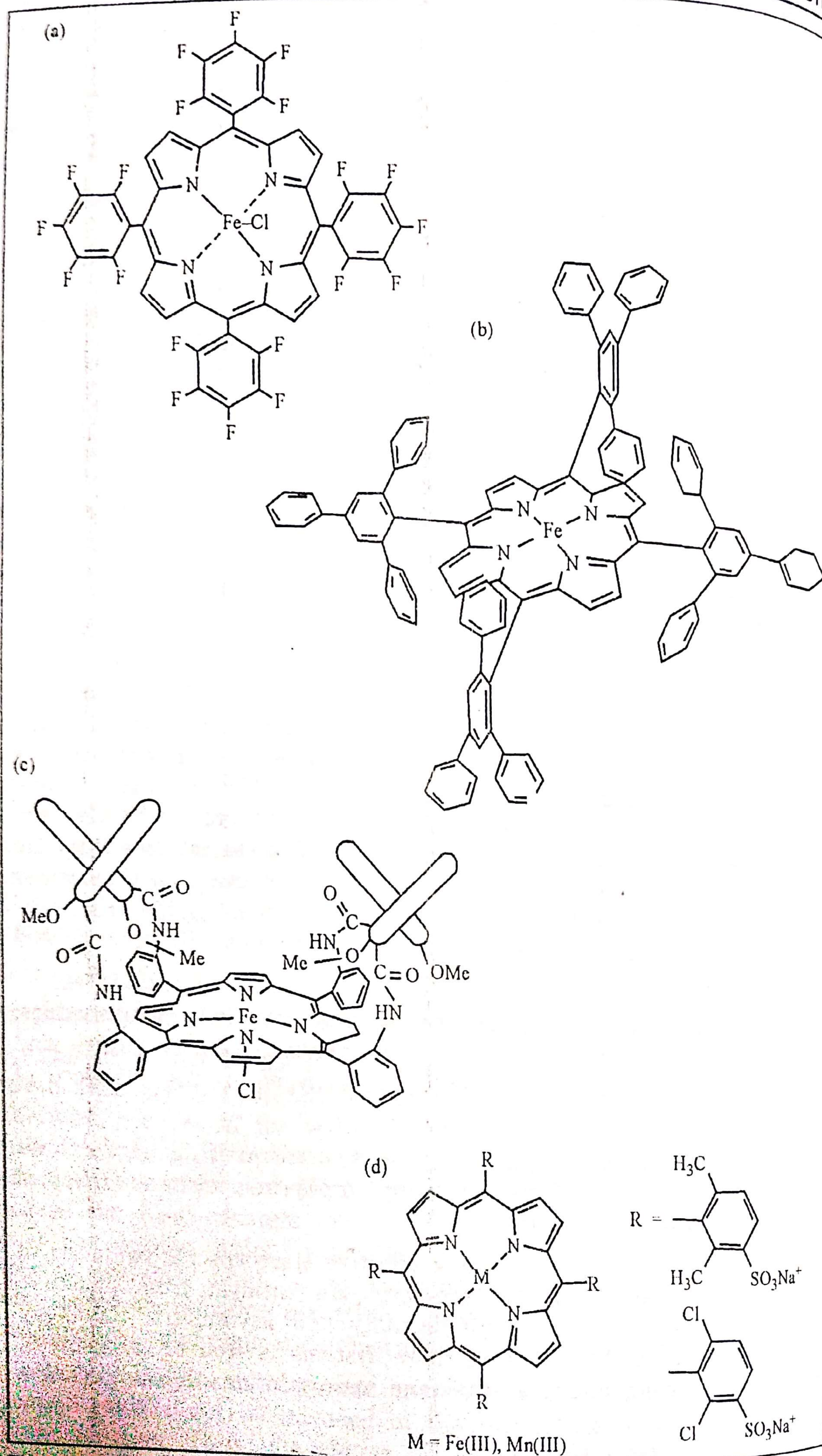

Fig. 5.34. Synthetic Porphyrin that model cytochrome P_{450}, (a) Protection of metal center from oxidation (b) Shape and regioselectivity and (c) asymmetric induction. In (a) the space filling

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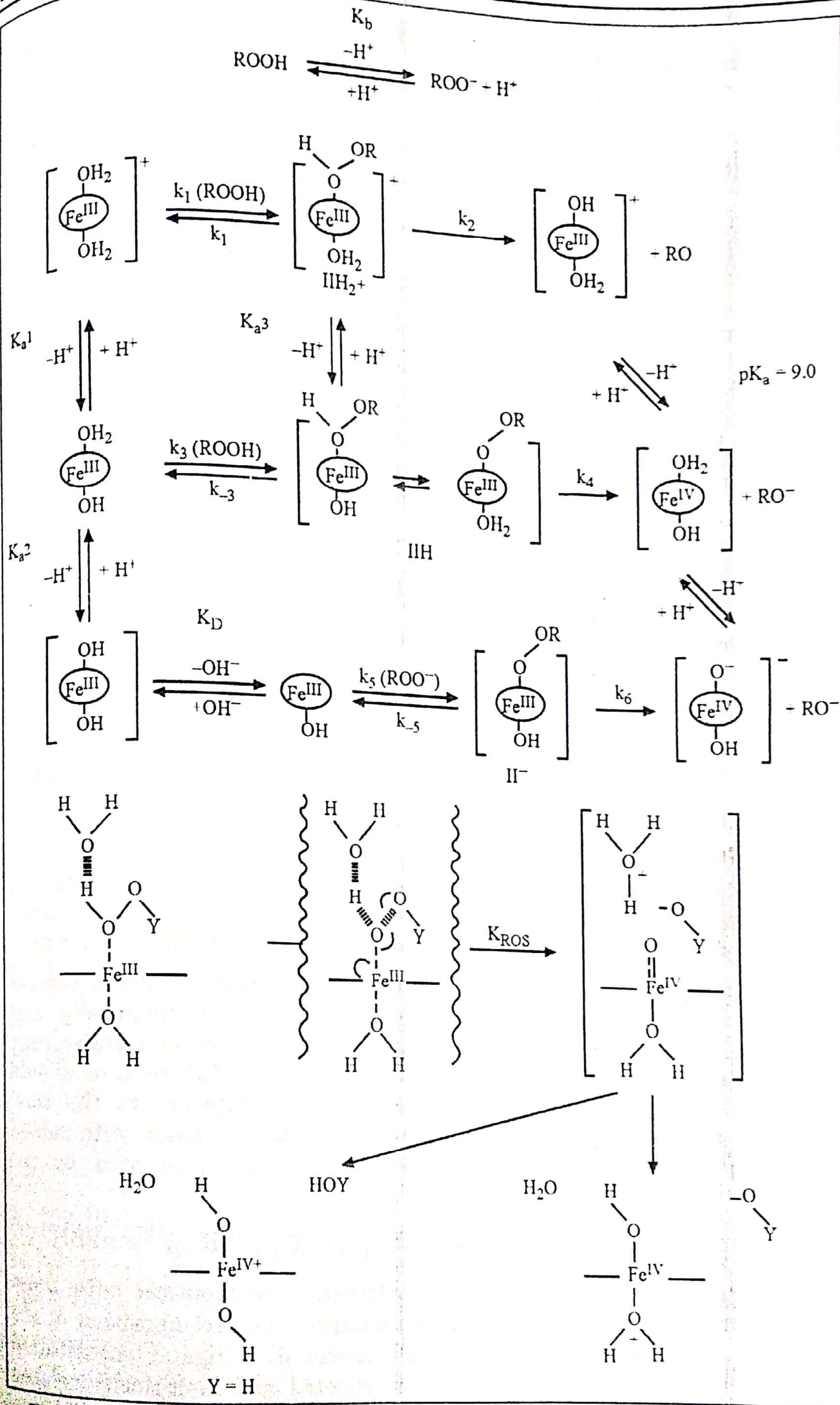

Fig. 5.35. Mechanism of reaction of water-soluble iron porphyrins with alkyl peroxides in aqueous media.

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of sodium hypochlorite. When the substrate was not present in excess, only the Mn porphyrins were resistant to self-destruction and the most effective ligand proved to be mesotetrakis (2, 6 dichlorophenyl) porphyrin (Fig. 5.34).

One of the problems with much of the work on P_{450} models is that reactions are performed in organic solvents in which it is not possible to obtain information about the reaction mechanism involved.

This is because the proton activity in organic solvents is not easily determined. It is only in an aqueous solution that the conditions necessary for oxygen transfer, such as ionic strength, acidity and ligand species concentration are best controlled and data (*e.g.* electrochemical and kinetic) are best interpreted.

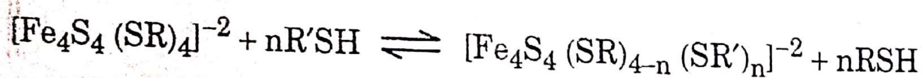
Owing to the steric hindrance of the eight ortho-substituents on the meso artyl rings (methyl or chloro), the metal complexes do not form μ-oxo dimers, nor do they aggregate in solution, as does meso-tetrakis (4–sulphonatophenyl) porphyrin and its complexes. The reactions of alkyl peroxides with the water soluble iron complexes are shown in (Fig. 5.35).

The reaction is first order in metal (III) porphyrin and ROOH. Bruice concludes that oxygen transfer reactions with alkyl peroxides occur from the complex. (Porph) M^{III} $(OH)_2$ (ROOH), and two others, one in which the ROOH is ionised and one in which a water ligand is ionised. Also, the rate of oxygen transfer increases upon stepwise proton ionisation. The model system involves a rate limiting homolytic fission of the hydroperoxide O-O bond prior to oxidation of any substrate.

Synthetic Iron-Sulphur Proteins

The general formula $(Fe_4S_4\,(S{-}R)_4]^{n-}$ of synthetic iron-sulphur complex in which R is an alkyl or aryl group. These complexes are very much similar to the clusters found in HiPIP and in the oxidized ferredoxins.

Besides their electron transfer capacity, the synthetic clusters possess a type of reactivity that allows their modification in chemically and biologically interesting ways. The presence in the clusters of tetrahedrally coordinated paramagnetic iron suggested that the metal centers should undergo facile substitution of the thiolate ligands external to the core. Indeed, the tetranuclear dianions have been shown to react with added monofunctional thiols in a substitution equilibrium represented by the equation :



$$[Fe_4S_4\,(SR)_4]^{-2} + nR'SH \rightleftharpoons [Fe_4S_4\,(SR)_{4-n}\,(SR')_n]^{-2} + nRSH$$

In all cases the fully substituted tetramers are identical with those produced by direct synthesis, and in no instance has decomposition of the Fe_4S_4 core been observed over the full course of a ligand substitution reaction. Using this kind of reaction, complicated and biologically related structures have been incorporated around Fe_4S_4 cores. Initially it was shown that acetyl-L-cysteine-N-methylamide and $[Fe_4S_4(S{-}tBu)_4]^{2-}$ reacted to form

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the "monopeptide" complex $[Fe_4S_4(S\text{-cys (Ac) NHMe})_4]^{2-}$. Subsequently, these reactions were extended to N-protected C-amide glycyl-L-cysteinyl glycyl oligopeptides.

Replacement of the original simple ligands with cysteine containing peptides that imitate the cluster environment in proteins is of great interest. The next step may be incorporation, via ligand substitution reactions, of natural cysteine-containing fragments of ferredoxins around the Fe_4S_4 core. Studies of such synthetic clusters may provide a better understanding of the effects of protein structure on the functions and properties of the active sites of ferredoxins and other iron-sulphur proteins.

The substitution reaction with benzenthiol in partially aqueous media has been also used for the quantitative removal (extrusion) and subsequent characterization of intact Fe_4S_4 and Fe_2S_2 cores from ferredoxins, nitrogenase, hydrogenase, xanthine oxidase and aconitase.

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UNIT 6

NITROGENASE

The third fundamental process in nature that is carried out by living cells in addition to photosynthesis and respiration is nitrogen fixation. This process, in turn, is part of the cycle of reactions known as the nitrogen cycle. Many constituents of the living cell contain nitrogen; they include proteins, amino acids, nucleic acids, purines, pyrimidines, porphyrins, alkaloids, and vitamins. The nitrogen atoms of these compounds eventually travel the nitrogen cycle, in which the nitrogen of the atmosphere serves as a reservoir. Nitrogen is removed from the reservoir by the process of fixation; it is then returned by the process of denitrification.

Components of Nitrogen Cycle :

Several inorganic nitrogen compounds as well as a myriad of organic nitrogen compounds, can be considered as components of nitrogen cycle. The former include N_2 (gas), NH_3, nitrate ions (NO_3^-), nitrite ions (NO_2^-) and hydroxyl-amine (NH_2OH). At a glance, it is apparent that the nitrogen atom can possess a variety of oxidation numbers. Some of these are as follows :

Nitrate ion	Nitrite ion	Hyponitrite ion	Nitrogen gas	Hydroxyl amine	Ammonia
NO_3^-	NO_2^-	$N_2O_2^{2-}$	N_2	NH_2OH	NH_3
Oxidation No. + 5	+ 3	+ 1	0	− 1	−3

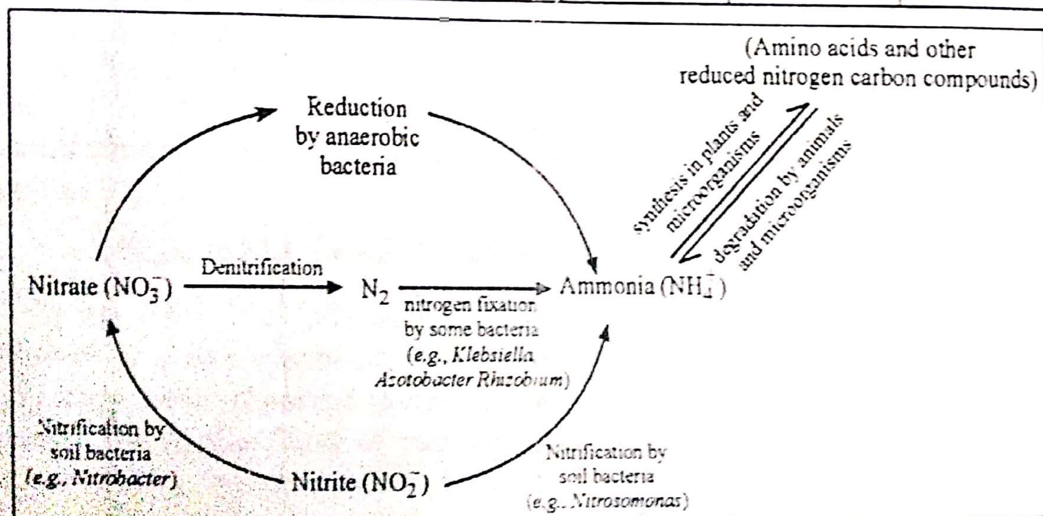

Fig. 6.1. The nitrogen cycle.

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Thus, in nature nitrogen may exist in highly oxidized form (NO_3) or in highly reduced state (NH_3).

For plant growth, nitrogen must be present in an available form, namely either as ammonia or the nitrate anion. Ammonia is provided from two principal sources, namely biological nitrogen fixation and nitrogenous fertilizers. (Fig. 6.1)

Basic requirements : The basic requirements of nitrogen fixation are :

(1) Presence of enzyme nitrogenase and hydrogenase.

(2) A protective mechanism for the enzyme nitrogenase against O_2.

(3) A non-heme iron protein-ferredoxin as electron carrier.

(4) Hydrogen donating system (viz. pyruvate, hydrogen, sucrose, glucose, etc.)

(5) A constant supply of ATP.

(6) Presence of thiamine pyrophosphate (TPP); Coenzyme-A, inorganic phosphates and Mg^{++} as cofactors,

(7) Presence of cobalt and molybdenum; and

(8) A carbon compound for trapping released ammonia.

The key enzyme–nitrogenase : The enzyme nitrogenase is present in bacteroids (in case of symbiotic N_2 fixation of legumes) and in other N_2 fixing organisms. Recent studies have shown that the enzyme nitrogenase consists of two protein components (or subunits). They are composed of two metalloproteins which are commonly referred as :

(i) non-heme iron protein commonly called Fe-protein or dinitrogen reductase and

(ii) iron molybdenum protein called MoFe protein (or dinitrogenase) or MFe (M = Mo, V, and Fe) cofactor. The Fe protein component is common to all nitrogenases is a dimer with a single Fe_4S_4 cluster bound between

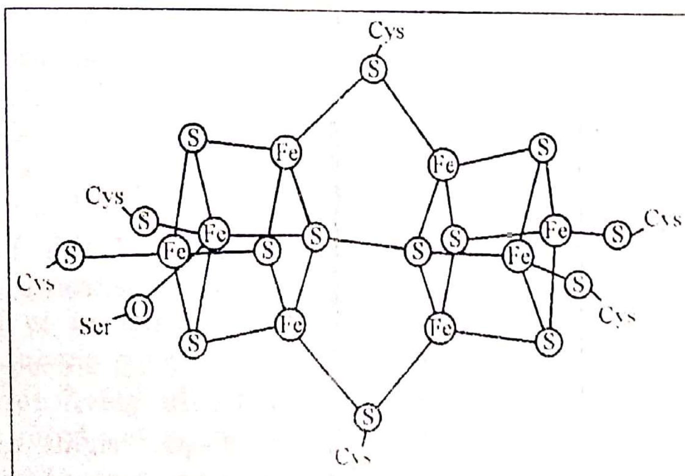

Fig. 6.2. Model for the structure of the P cluster in nitrogenase.

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two equivalent subunits and frequently known as "P cluster" as shown in (Fig. 6.2).

Recent studies indicate that the P-cluster probably is of the type 8 Fe–7S instead of 8Fe–8S type.

The function of Fe-protein components is to react with ATP and reduce MoFe protein which then reduces N_2 to ammonia.

Nitrogenase which catalyzes the reduction of N_2 to NH_3 consists of two proteins:

(i) The MoFe protein, a ~ 220–KD protein of subunit structure $\alpha_2 \beta_2$ that contains two proteins.

(ii) The Fe protein, a ~ 64 KD protein dimer of identical subunits that contain Fe.

Much of the Fe in these proteins contains [4Fe – 4S] clusters in each dimer and two ATP binding sites. The Fe protein in [4Fe – 4S] clusters is generally considered to undergo one electron redox cycle between the $[4Fe-4S]^{+2}$ state and $[4Fe-4S]^{+}$state and this clasps a ferredoxin unit (Fe_4S_4) between them by forming Fe–S bonds to two cysteine residues in each subuint (Fig. 6.3).

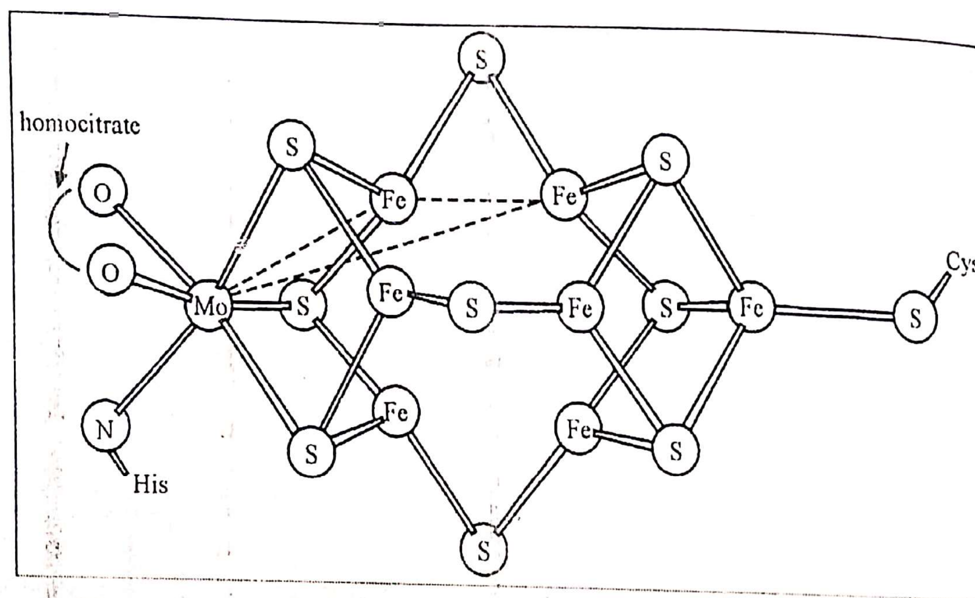

Fig. 6.3. Representation of the Core of FeMoCo.

The MoFe protein contains "P cluster" and the Fe-Mo cofactor (FeMoCo). The dimagnetic P cluster is involved in electron transfer between the Fe protein and the FeMoCo. Later it is believed to be the site of substrate reduction. The structure of FeMoCo cluster isolated from *A. Vinelandii* and *C. Pasteurianum* is shown in (Fig. 6.3).

The two entities, $MoFe_3S_3$ (left) and Fe_4S_3 (right) are bridged by three sulphur atoms. The Fe–Fe distances between bridged ion sites average 2·5 Å, close enough to be considered as metal-metal bonded. Only two protein ligands Cys and His coordinate the cofactor to the protein, resulting

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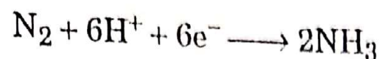
in the unusual situation in which six Fe atoms bridged by non-protein ligands are three–coordinate.

EXAFS studies indicate very little change in the coordination of the Mo-atom in Mo-nitrogenase during enzyme turnover; an indication that N_2 probably does not bind to molybdenum but instead to iron.

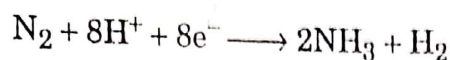
Mode of Action of Nitrogenase

The overall reaction is as follows :



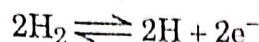
$$N_2 + 6H^+ + 6e^- \longrightarrow 2NH_3$$

As shown in the equation, the reduction of nitrogen to ammonia requires at least 6 protons and 6 electrons. Besides, it requires at least 12 molecules of ATP because at least 4 ATP molecules are needed for each pair of electron transferred to N_2. Very recently, in view of the fact that N_2 fixation is always accompanied by evolution of some H_2, the equation is modifed as follows :



$$N_2 + 8H^+ + 8e^- \longrightarrow 2NH_3 + H_2$$

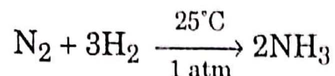
The enzyme hydrogenase is present in almost all the microbes involved in N_2 fixation. The enzyme catalyzes :



$$2H_2 \rightleftharpoons 2H + 2e^-$$

Biological Nitrogen Fixation :

In contrast to the chemical fixation of nitrogen is biologcal fixation, occurs at one atmosphere pressure and at the temperature of living cells in the presence of appropriate enzymes.



$$N_2 + 3H_2 \xrightarrow[1\ atm]{25^\circ C} 2NH_3$$

Biological fixaton of nitrogen is accomplished either by nonsymbiotic micro-organisms that can live independently or certain bacteria living in symbiosis with higher plants. The former group includes aerobic organisms of the soil (*e.g. Azotobacter*), soil anaerobes (*e.g. Clostridium* sp.), photosynthetic bacteria (*e.g. Rhizobium rubrum*), and cyanobacteria (*e.g. Anabaena* sp.). The symbiotic system consists of bacteria (*Rhizobia*) living in symbiosis with members of the Leguminoseae such as clover, alfalfa and soyabeans. Legumes are not the only higher plants that can fix nitrogen symbiotically. About 190 species of shrubs and trees, including the Sierra sweet Bay, ceanothus and alder, are nitrogen fixers.

An essential feature of symbiotic fixation is the development of nodular tissue that forms on the roots of legumes after infection by a strain of Rhizobia, specific for the given legume. The legume alone is unable to fix nitrogen, free living Rhizobia bacteria can fix N_2 only when grown with a limiting supply of organic nitrogen and oxygen.

Biological nitrogen fixation is carried out by a highly conserved complex of proteins called the nitrogenase complex (Fig. 6.4). The two key components

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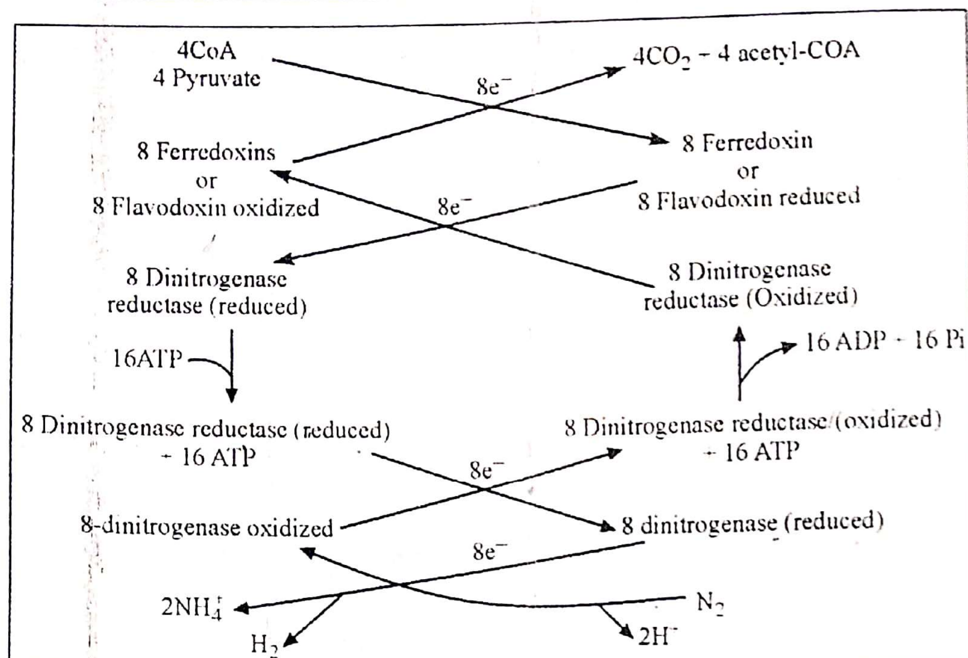

Fig. 6.4. Nitrogen Fixation by nitrogenase complex.

of this complex are dinitrogenase reductase and dinitrogenase. Dinitrogenase reductase (M_r 60,000) is a dimer of two identical subuints (Fig. 6.5).

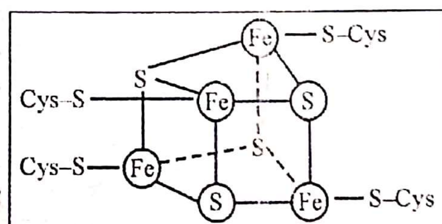

Fig. 6.5. Fe–4S centers (ferredoxins)

It contains a single Fe_4-S_4 redox center which can be oxidized and reduced by one electron. It also has two binding sites for ATP. Dinitrogenase is a tetramer with two copies of two different subunits (combined M_r 240,000) (M_r = relative molecular mass). Dinitrogenase contains both iron and molybdenum, and its redox center has a total of 2Mo, 32Fe, and 30S per tetramer. About half of the Fe and S is present as four Fe_4-S_4 centers. The remainder is present as part of a novel iron molybdenum cofactor of unknown structure. A form of nitrogenase that contains vanadium rather than molybdenum has been detected and both types of nitrogenase systems can be produced by some bacterial species. The vanadium enzyme may be the primary nitrogen fixation system under some environmental conditions, but it has not been well characterized.

Nitrogen fixation is carried out by a highly reduced form of dinitrogenase, and requires eight electrons : six for the reduction of N_2 and two to produce one molecule of H_2 as an obligate part of the reaction mechanism.

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Dinitrogenase is reduced by the transfer of electrons from dinitrogenase reductase (as in fig. 6.4). Dinitrogenase has two binding sites for the reductase, and the required eight electrons are transferred to dinitrogenase one at a time, with the reduced reductase binding and the oxidized reductase dissociating from dinitrogenase in a cycle. This cycle requires the hydrolysis of ATP by the reductase.

The immediate source of electrons to reduce dinitrogenase reductase varies, with reduced ferredoxin (Fig. 6.5), reduced flavodoxin, and other sources playing role in some systems. In atleast one instance, the ultimate source of electrons is pyruvate (as in Fig 6.4).

In the reaction carried out by dinitrogenase reductase, both ATP binding and ATP hydrolysis bring about protein conformational changes that evidently help to overcome the high activation energy of nitrogen fixation. ATP binding to the reductase shifts the reduction potential (E_0) of this protein from –250 to –400 mV, an enhancement of its reducing power that is required to transfer electrons to dinitrogenase.

Two ATP molecules are then hydrolyzed during the actual transfer of each electron from dinitrogenase reductase to dinitrogenase.

Another important characteristic of the nitrogenase complex is an extreme lability when oxygen is present. The reductase is inactivated in air, with a half life of 30 s.

The dinitrogenase has a half-life of 10 min. in air. Free living bacteria that fix nitrogen avoid or solve this problem in a variety of ways. Some exist only anaerobically or repress nitrogenase synthesis when oxygen is present. Some aerobic bacteria, such as *Azotobacter vinelandii*, partially uncouple electron transport from ATP synthesis so that oxygen is burned off as rapidly as it enters the cells. When fixing nitrogen, cultures of these bacteria actually warm up as a result of their effort to remove oxygen.

The nitrogen fixing *Cyanobacteria* use still another approach. One of every nine cells differentiates into a heterocyst, a cell specialized for nitrogen fixation, with thick walls to prevent oxygen from entering.

The symbiotic relationship between leguminous plants and the nitrogen fixing bacteria in their root nodules solve both the energetic requirements of the reaction and the oxygen lability of the enzymes. The energy required for nitrogen fixation was probably the evolutionary driving force for this association of plants with bacteria.

The bacteria in root nodules fix hundred times more nitrogen than their free living cousins under conditions generally encountered in soils.

To solve the oxygen toxicity problem, the bacteria in root nodules are bathed in a solution of an oxygen binding protein called leghemoglobin. Leghemoglobin efficiently delivers oxygen to the electron transfer system of the bacteria, and it binds all the oxygen so that it cannot interfere with nitrogen fixation, the efficiency of the symbiosis between plants and bacteria

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is evident in the enrichment of soil nitrogen brought about by leguminous plants. This enrichment is the basis of crop rotation methods used by many farmers, in which planting of non-leguminous plants (such as corn) that extract fixed nitrogen from the soil are alternated every few years with planting of legumes such as alfalfa, peas or clover.

Nitrogen fixation is the subject of intense study because of its immense practical importance. The expense of producing ammonia industrially for use in fertilizers increase with the cost of energy supplies, and this has led to efforts to develop recombinant or transgenic organisms that can fix nitrogen. Recombinant DNA techniques are being used to transfer the DNA that encodes nitrogenase and related enzymes into non-nitrogen fixing bacteria and plants. Success in these efforts will depend on overcoming the problem of oxygen toxicity in any cell producing nitrogenase.

Molybdenum Nitrogenase :

Nitrogenase is an enzyme involved in the fixation of nitrogen which occurs in bacteria. This enzyme is comprised of two protein chains. The lower molecular weight protein contains an Fe_4S_4 cluster. The larger protein, which, itself is tetrameric, involves two molybdenum atoms, and large number of iron atoms and sulphide ions. Both proteins are required for activity. Although the iron-sulphur clusters are thought to be the redox centers, molybdenum is vitally important. It has been shown that bacteria grown in the presence of tungsten (VI) oxide rather than molybdenum (VI) oxide, can incorporate tungsten but show no nitrogen fixing activity. It is possible, therefore, that the nitrogen actually coordinates with molybdenum during the fixation process (Fig. 6.6 a, b).

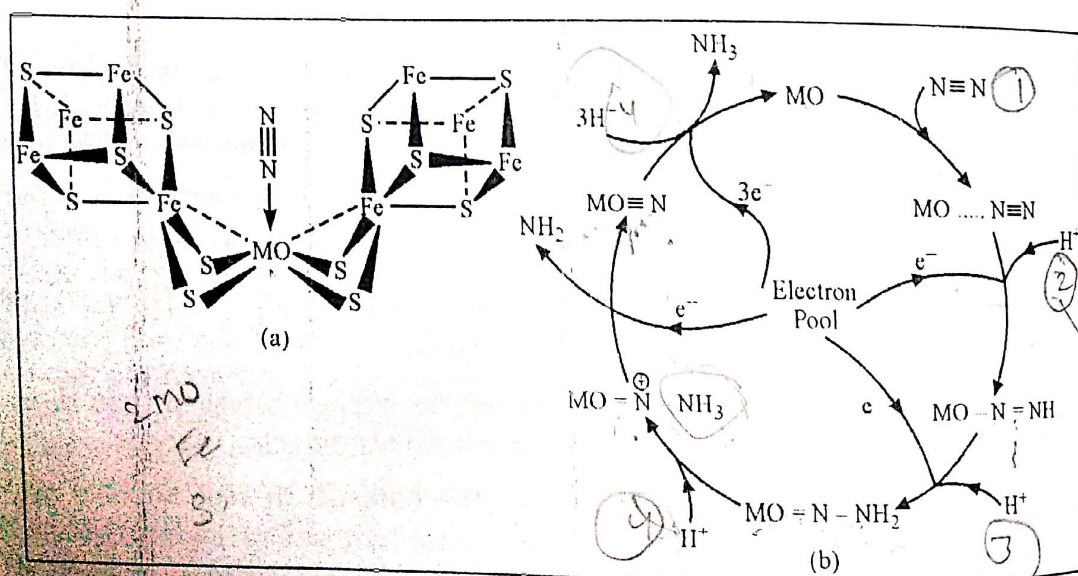

Fig. 6.6. (a) Proposed arrangement of Mo, S and Fe in the active site of nitrogenase. (b) Proposed catalytic cycle of nitrogen fixation and conversion.

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Spectroscopic and other evidences :

1. The Molybdenum iron protein :

A. Protein structure : The molybdenum iron protein component of nitrogenase has been purified extensively from *Azotobacter vinelandii, Klebsiella Pneumoniae, Bacillus polymyxa, Rhodospirillum rubrum* and *clostridium pasteurianum.*

From each of these three sources the protein is an $\alpha_2 \beta_2$ tetramer of subunits. The α chain molecular weight is reported to be approximately 60,000 while the β chain is approximately 50,000 from *Klebsiella* and *Clostridium* and 59,000 from *Azotobacter*. The total molecular weight of the molybdenum iron protein is thus 220,000 from *Klebsiella* and *Clostridium* and 240,000 from *Azotobacter.*

The proteins from *Azotobacter* and *Clostridium* have been crystallized and determination of the structure has begun.

More precise quantitation of iron and sulphur in this labile system is difficult by chemical analysis. The most accurate assignments of composition probably come from the rationalization of spectroscopic experiments. The localization of these iron, sulfur and molybdenum atoms in individual clusters and investigation of the structure of those clusters constitutes a fair share of the recent progress in the nitrogenase field.

B. Metal clusters : The molybdenum-iron protein isolated from every source examined displays a characteristic EPR spectrum with apparent g values near 4·32, 3·65 and 2·01 at temperature near 4K. As the sample temperature is raised above 15K, the signals rapidly broaden and disappear. These unusual g values may be described adequately as deriving from the ground state Kramer's doublet of an $S = 3/2$ system. Integration of low temperature EPR spectrum of the *Azotobacter vinelandii* protein gave a spin concentration of 0·91 spins per molybdenum atom, implying the presence of two spin systems per protein molecule. The presence of the $S = 3/2$ EPR signal depends on the redox state of the molybdenum iron protein.

Assignment of the iron and molybdenum atoms to distinct clusters was made using a combination of EPR and Mossbauer spectroscopy and reinforced by chemical experiments.

Mossbauer spectra : Mossbauer spectra obtanied at 1·5 K of the semi-reduced molybdenum-iron protein from *Azotobacter vinelandii* were decomposed into four components named "D", "Fe^{2+}", "S" and "M" Table (6.1).

Table 6.1 : Decomposition of the Mossbauer spectrum of the semi-reduced molybdenum-iron protein taken at 30 K^a.

Spectral component	δ (mm/S)	ΔE_Q (mm/S)	% of total Absorption
"D"	0·64 (3)	0·81 (3)	42 (3)
"M"	0·40 (3)	0·76 (3)	38 (3)
"Fe^{2+}"	0·69 (2)	3·02 (2)	14 (1)
"S"	0·6 (1)	1·4 (1)	5

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In an applied magnetic field D, Fe^{2+} and S are all quadrupole doublets, indicating that they arise from iron atoms coupled to S = O or integer electronic systems. At 1.5K, even in the absence of a magnetic field, component M is a magnetically coupled spectrum indicative of a half-integer electronic spin system. When the temperature is raised to 30K, the M component collapses into a quadrupole doublet. At the same temperature the EPR spectrum of the protein is unobservable, being broadened by spin relaxation.

Incubation of the molybdenum–iron protein with the iron protein, MgATP, and dithionite eliminates the EPR spectrum by reducing the EPR-active state to an EPR- silent one. Under those conditions the M component is the only component of the Mossbauer spectrum to change as it collapses into a pair of quadrupole doublets. For these reasons component M was assigned to the cluster of metal atoms that give rise to EPR signal of the semi-reduced molybdenum iron protein.

Mossbauer investigations of the molybdenum-iron protein from *Klebsiella pneumoniae* and *Clostridium pasteurianum* gave quantitatively similar experimental results which, in the latter case, were viewed similarly as well. The former experiments were at first interpreted differently, however, existence of four Fe_4S_4 clusters was an attractive suggestion.

If thirty six iron atoms were present in the protein, the Mossbauer analysis allows sixteen to be D irons and four to contribute to Fe^{2+}. The supposition of four Fe_4S_4 clusters composed of D iron atoms and two

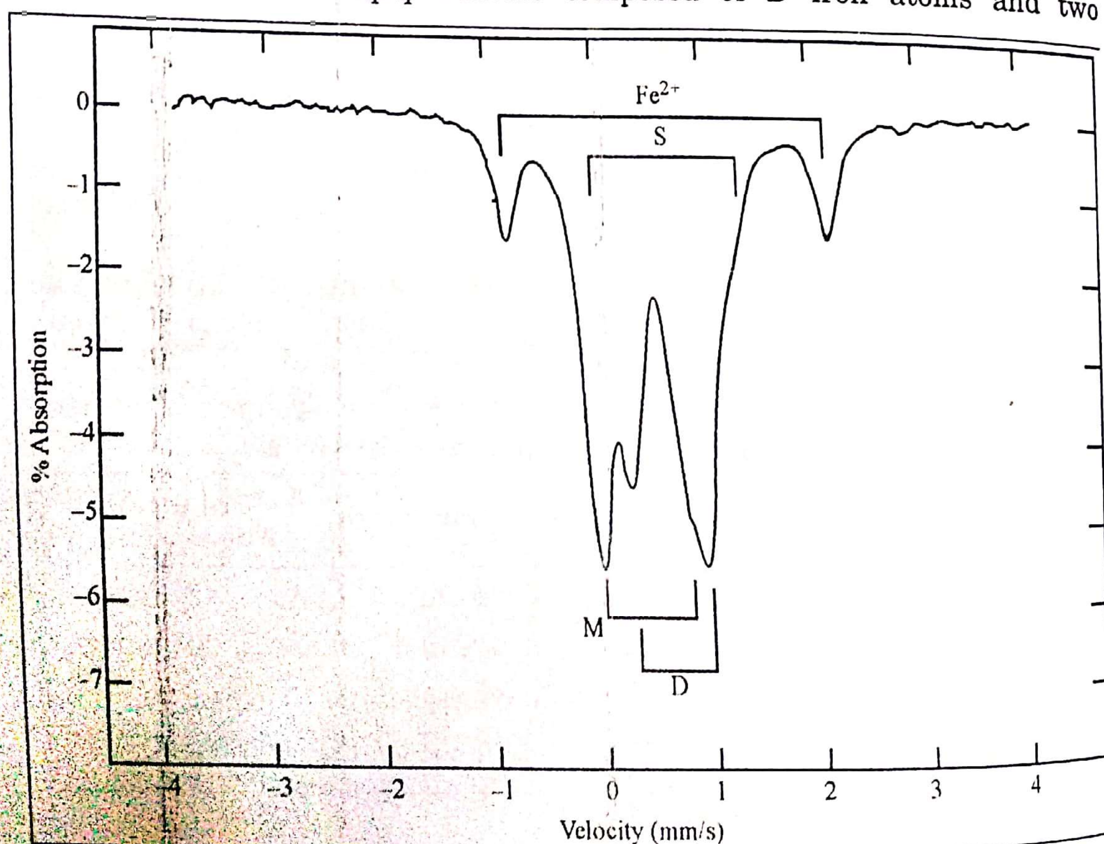

Fig. 6.7. Mössbauer spectrum of *C. pasteurianum* nitrogenase FeMo protein, indicating the various components (quadrupole doublets) and their assignments. The doublet labeled M is the cofactor signal: those labeled D. S. and Fe^{2+} are attributed to the P-clusters.

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diamagnetic Fe_2S_2 clusters composed of spin-coupled Fe^{2+}-type iron atoms eliminates the necessity of a complicated spin-coupling scheme to render *P* clusters diamagnetic.

The Fe_2S_2 clusters could be extruded from the molybdenum-iron protein by treatment with p-(trifluromethyl) benzenethiol. In later studies in which clusters were extruded with O-xylyl–α, α'–dithiol and identified after ligand exchange with p-(trifluoromethyl) benzenethiol demonstrated 3·4 to 4·0 Fe_4S_4 clusters and no Fe_2S_2 clusters per molybdenum iron protein. Thus only Fe_4S_4 clusters were detected in molybdenum-iron protein from *Clostridium Pasteurianum*. Recently, the Mossbauer data from molybdenum iron proteins from *Klebsiella* have been re-examined and explained best by four P clusters model.

Iron-molybdenum cofactor : Precipitation of the molybdenum iron protein by acid treatment, followed by neutralization, removal of water, and extraction with N-methyl formamide yields a dark brown solution of the nitrogenase iron-molybdenum cofactor. Addition of partially purified cofactor to an inactive, cofactorless molybdenum iron protein from the mutant strain of *Azotobacter vinelandii* restores nitrogenase activity.

The cofactor has been shown to contain all the molybdenum of the molybdenum iron protein, as well as six to eight iron and upto six sulfide ions per molybdenum.

The isolated cofactor exhibits an EPR spectrum indicative of a site more anisotropic than that in the protein, with g values of 4·6, 3·3 and 2·0. The spectrum of the cofactor is substantially broader than that of the protein, but sharpens appreciably upon addition of one equivalent of thiophenylate. The Mossbauer spectrum of the isolated cofactor is broadened with respect to that of the protein. For these reasons the iron-molybdenum cofactor is considered to be essentially the M cluster of the intact protein, though in the isolated form the spread of environments of the metal atom is substantially broader than in the protein bound form.

An apparent derivative of the cofactor has been extracted into methyl-ethyl ketone from acid precipitated molybdenum iron proteins. The product called "MoFe cluster" lacks the EPR spectrum of the MoFe protein unless cluster is taken into N-methylformamide. Even then significant differences exist between the spectra. More importantly, the MoFe cluster will not reconstitute the molybdenum iron protein.

Consequently, much effort has been associated with substrate reduction, to determine the structure of the protein and cofactor in the immediate vicinity of the molybdenum and associated iron atoms.

EXAFS : Spectroscopy has revealed details of the environment of that metal in both the holoprotein and the isolated cofactor.

The recent data from the proteins have been interpreted to reveal three to four sulphur atoms 2·36Å from the molybdenum, and two to three iron atoms at 2·68Å. The isolated cofactor appears to be very similar in the vicinity of the Molybdenum with three to four sulfurs at 2.35Å and two to three irons at 2.66Å, and in contrast to the protein, two or three nitrogen (or oxygen) atoms at 2·10Å.

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These EXAFS results have led to the formulation of numerous models for the molybdenum site of the protein, based on an MoS_4 core attached to an iron sulphur cluster.

Comparison of the EPR signals arising from the molybdenum iron protein from *Azotobacter vinelandii* revealed no detectable line broadening resulting from the isotopic substitution. Either, Mo is isolated from the spin system of the M cluster or the hyperfine coupling constant between the spin and the Mo is unobservably small. The latter conclusion was confirmed by observation of a broad Mo peak in the **ENDOR** spectrum of protein.

The small hyperfine coupling constant (~ 8MHz) limits the Mo to even formal oxidation states, while the small quadrupole coupling constant (≤ 3/8 MHz) and consequent small electric field gradient at the nucleus suggests octahedral Mo (O), tetrahedral Mo (II) or Mo (IV). Five sets of magnetically distinct, non-exchangeable protons were observed coupled to the spin system. Thus **ENDOR** spectroscopy of the isolated cofactor is of great interest.

EPR spectra of *clostridium pasteurianum* nitrogenase are given :

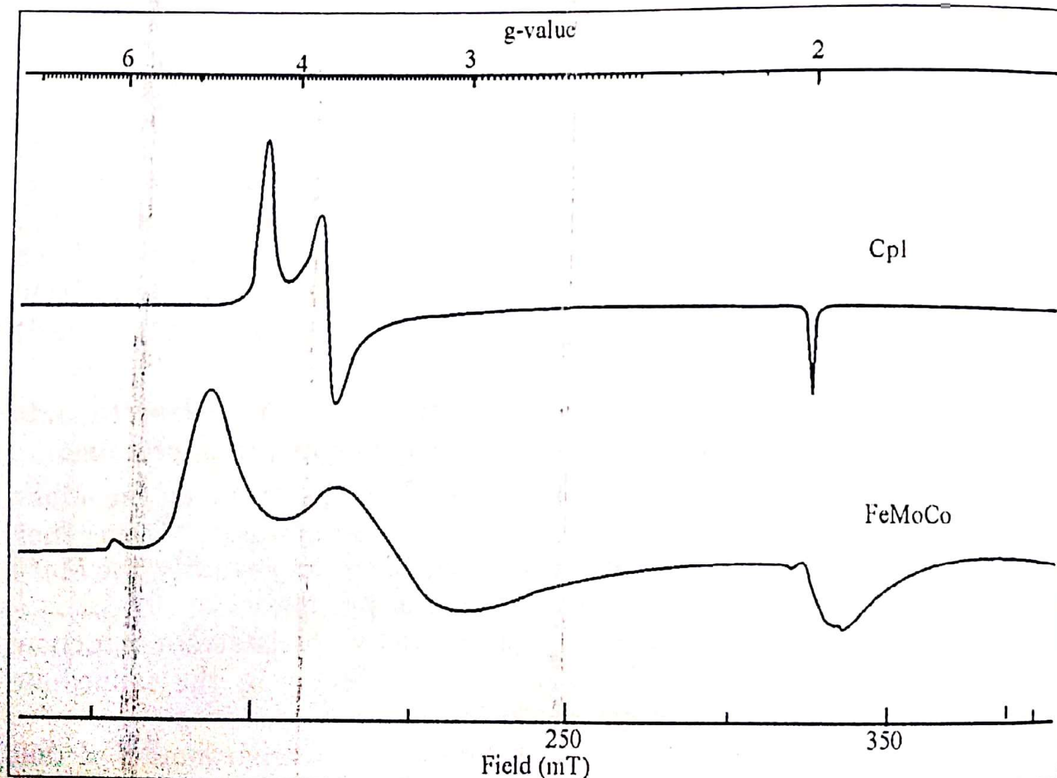

Fig. 6.8. EPR spectra (A) the S = 3/2 M center in *C. Pasteurianum* nitrogenase FeMo Protein and (B) the FeMoCo.

P clusters : The unique nature of the P cluster was established by Mossbauer and MCD spectroscopies. According to the analysis of the Mossbauer spectrum of the native protein, these Fe_4S_4 clusters contain one distinguishable iron atom. The diamagnetic nature of the clusters indicates

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that they are in an even charge state; either + 2 (in oxidized ferredoxins), 0 or + 4. MCD studies of the MoFe protein from *Azotobacter vinelandii* show difference between the P clusters and known Fe_4S_4 clusters in the + 1, + 2, + 3, oxidation state in the long wavelength region.

The Mossbauer spectral parameters would not favour a (+4) state, leaving the possibility that the native P clusters exist in the 0 oxidation state (*i.e.* all Fe (II)), a state which has been attained electrochemically in model compounds.

EPR signals observed during inhibition by carbon monoxide of substrate reduction have been interpreted as arising from Fe_4S_4 clusters in + 1 and + 3 states, leading to the intriguing possibility that P clusters may transit four oxidation states (0 to + 3) during turnover.

Oxidation of MoFe protein by six or seven electrons produces P clusters each with a magnetic moment equivalent to $S = 5/2$. The oxidized clusters may be reduced into the native diamagnetic state by the iron protein, MgATP and dithionite, or, more slowly, by methylviologen (MV) and dithionite.

Oxidation and Reduction of the MoFe Protein :

The EPR Spectrum of native molybdenum-iron protein disappears during oxidation. The mid point potential of this oxidation has been determined potentiometrically for Mo-Fe protein. They include (all NHE) : *Azotobacter vinelandii* (– 42 mV) *clostridium pasteurianum* (0 mV), *Bacillus polymyxa* (– 95 mV), *Azotobacter croococcum* (– 42 mV) and *Klebsiella pneumoniae* (– 180 mV).

Six electron oxidation of the MoFe protein from *Azotobacter vinelandii* followed by controlled potential electrolysis (re-reduction) showed two regions of redox activity; one at – 290 mV and one at – 480 mV. In each region the oxidized protein accepted three electrons one at –290 mV and one at –480 mV. In each region the oxidized protein accepted three electrons. Examination of samples reduced at constant potential revealed that the EPR signal reappeared during reduction in the – 290 mV potential region.

This indicates that all the three higher potential electrons are required for EPR signal.

Other evidence comes from studies in which the native Mo – Fe protein was titrated with low potential phenothiazine derivative thionine. The EPR spectrum of the partially oxidized protein was observed as a function of the number of electron equivalents removed. Until approximately four electrons were removed, the signal remained constant. It disappeared as the last two electrons were removed, indicating that the EPR-activity depends on only two of the higher potential electrons in the protein. These results suggest the interpretation that the first four electrons are removed from P clusters and thus induce no change in the EPR spectrum, while the last two come one each from the M clusters and bleach the EPR signal.

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Recently, two groups have been reported oxidative titrations of the MoFe protein using methylene blue as the oxidant. Removal of more than three electrons from the native protein resulted in loss of the EPR signal, again implying a requirement of three electrons for EPR activity. The reconciliation of these disparate results may lie in the kinetics of the redox reactions used to probe the protein. This is reported in the oxidation reduction cycle of the *Azotobacter vinelandii* MoFe protein, this results in formation of different metastable states during oxidation and reduction.

Methylene blue may be able to oxidize the M clusters only with concomitant oxidation of one of the P clusters. This, in itself, implies that one of the P clusters is physically distinguishable from the others.

No such physical distinction has been seen by other techniques. The resolution of these paradoxes may hold much understanding of the mechanism of electron transfer within the nitrogenase complex.

Other nitrogenase model systems :

Several structural models of the cofactor are based on tetra-thiomolybdate cores. EXAFS studies of molybdenum in both the iron-Mo cofactors and the MoFe protein have implied that four sulphurs are within bonding distance of the molybdenum. Thiomolybdate also detected in solutions of acid denatured MoFe protein. These two findings have prompted synthesis of MoFe sulphur complexes based on tetrathiomolybdate. Within this class of structural models for the M cluster there are presently two subtypes : linear compounds and compounds based on cubane structures (Fig. 6.9).

The linear structures are the result of synthetic procedures based on the chemistry of the thiomolybdate anion. A variety of complexes with thiomolybdate as terminal or bridging ligand have been synthesized. For example, treatment of thiomolybdate with $[Fe(SPh)_4]^{2-}$ yields $[PhS_2FeS_2MoS_2]^{-2}$, in which molybdenum is coordinatively unsaturated. This compound is paramagnetic (S = 2), but the formal oxidation states of the metals (Fe II)/Mo (VI) or Fe (III)/Mo (V)) could not be determined.

Addition of $FeCl_3$ yields $[Cl_2FeS_2MoS_2FeCl_2]^{-2}$, with a bridging MoS_4 unit. In polar solvents or in the presence of MoS_4^{-2}, this compound decomposes to give $[Cl_2FeS_2MoS_2]^{-2}$.

Weak antiferromagnetic coupling between the iron atoms was detected in the MoS_4 bridged complex. The complex in which iron bridges two tetrathiomolybdate ligands $[S_2MoS_2FeS_2MoS_2]^{-3}$, is also prepared. From studies of these complexes, several suggestions regarding the function of the MoS_4 unit in nitrogenase are made.

This ligand acts as an electron sink. In model complexes electron density is accepted from the iron into low-lying molybdenum d orbitals. For example; Mossbauer parameters of the $[S_2MoS_2FeS_2MoS_2]^{-3}$ complex suggests that iron is present as Fe (III) instead of the Fe (I). The trianion can be reduced even further at –1.8 V. The best reduction of substrates by nitrogenase occurs in two electron events. The ability to store those

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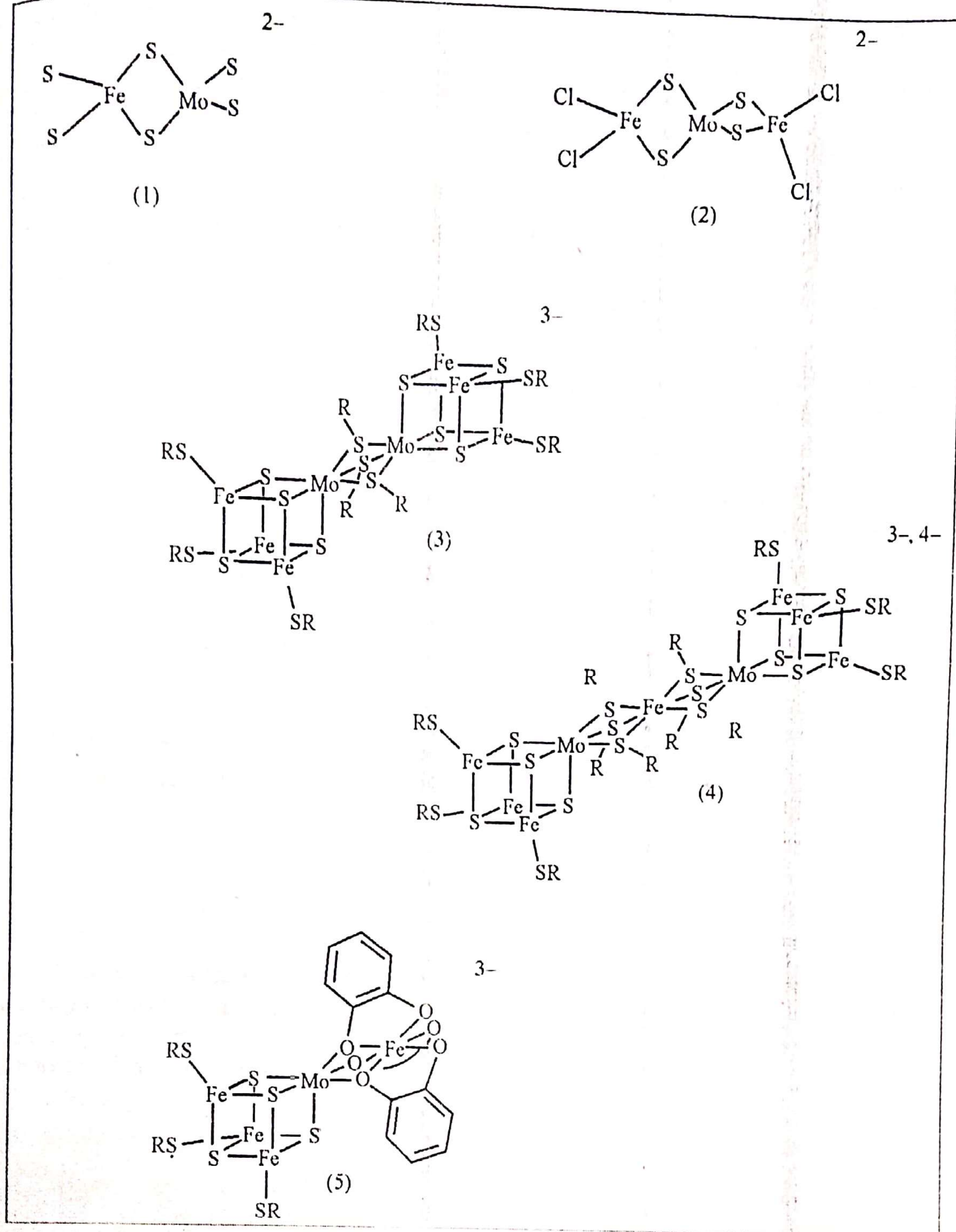

Fig 6.9. Synthetic Models of the Molybdenum Site of Nitrogenase. some of the types of molybdenum-iron-sulfur clusters synthesized as structural models of the molybdenum site of nitrogenase. 1, 2, 3, R may be alkyl or aryl 4, R may be alkyl or aryl (the 5-state will reduce protons to dihydrogen 5. "catechol-capped" cluster (one catecholate is omitted for clarity).

electrons in the thiomolybdate portion of the cofactor site may be of great importance to the mechanism of those reactions.

The second class of structural model for the cofactor site has modification of the Fe_4S_4 cubane structure present in four-iron ferredoxins.

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Mixtures of MoS_4^{-2}, $FeCl_3$ and RS^- yield a variety of dimeric cubane clusters of formula $[Mo_2\,Fe_6\,S_8\,(SR)_9]^{3-}$, $[Mo_2\,Fe_6S_9\,(SR)_8]^{-3}$ and $[Mo_2\,Fe_7\,S_8\,(SR)_{12}]^{-3}$, whose structures are shown in Fig. (6.9).

These compounds have been characterized thoroughly, and, in the molybdenum vicinity, are structurally similar to the cofactor site as seen in the molybdenum EXAFS experiments.

An attempt to cleave the dimeric iron-bridged cluster to yield a monomeric iron-Mo-sulphur cluster led to an unusual "catechol capped" cluster bridged to a single iron.

This complex may be the forerunner of a series of monomeric, cubane based, iron-molybdenum sulfur clusters that structurally will model the cofactor site.

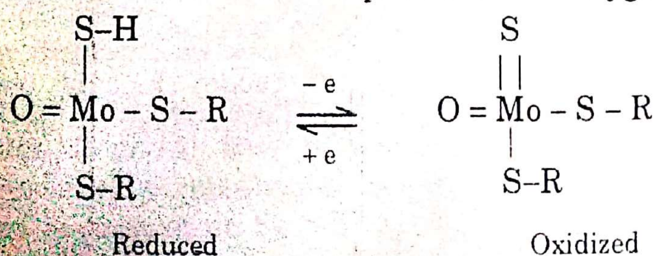
Two reactions that may be relevant to nitrogenase chemistry are exhibited by these types of clusters. The $[Mo_2\,Fe_6S_8\,(SPh)_9]^{-5}$ cluster, in the presence of benzenethiol as proton source, reduces protons to–hydrogen. Use of stronger acids results in non-productive oxidation of the cluster. The mechanism of this reaction was not explored, but it was concluded that a low potential two electron reductant was required for the reaction.

In a non-catalytic and non-stoichiometric reaction, the molybdenum-less cluster $[Fe_4S_4\,(p\text{–}Me\,PhS)_4]^{-3}$ reduces acetylene to ethylene in the presence of acetic acid and acetic anhydride. The stereochemistry of the hydrogen addition is cis in analogy to that by the protein. A ligand substitution reaction involving reduction of a $[Fe_4S_4(SR)_3(C_2H_2)]^{2-}$ cluster was suggested as the mechanism. No reduction of acetonitrile or dinitrogen was observed.

Further examination of reduction of dinitrogen bonded to molybdenum and the synthesis of structural models of the cofactor site should contribute to the understanding of the protein system. The model chemistry becomes invaluable for designing and interpreting experiments to understand the chemistry of this complex system.

Xanthine oxidase :

During metabolism the purines, adenine and guanine are broken down to uric acid via xanthine. One biosynthetic process involved in the overall metabolic cycle is the oxidation of xanthine. This is achieved with the aid of an enzyme, xanthine oxidase. This enzyme is large; its molecular weight is around 300 kDa, and complex involving two molybdenum atoms, four Fe_2S_2 clusters and two flavin adenine dinucleotide (FAD) molecules. It is believed that one of the intermediates in the oxidation cycle involves molybdenum associated with three sulphur and one oxygen atom;



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During the course of oxidation process, molybdenum converts from +6′ to +4 oxidation state. The overall electron flow may be considered as being from xanthine molybdenum to Fe_2S_2 to FAD and then to molecular oxygen (Fig. 6.10).

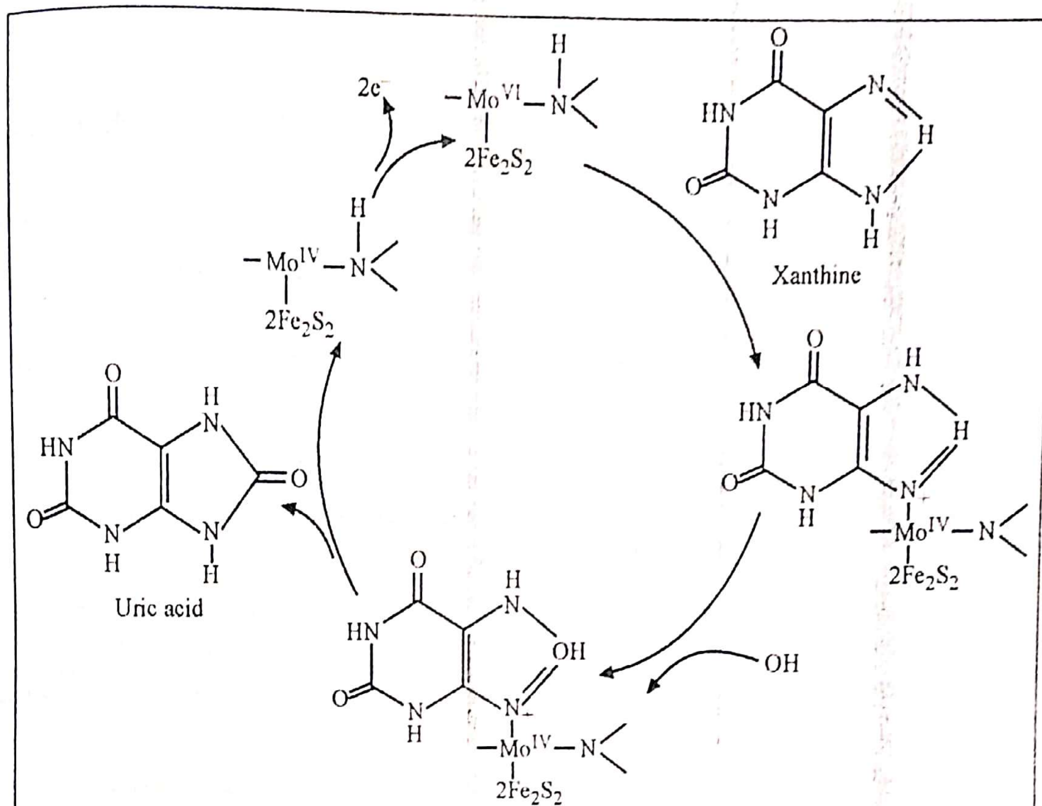
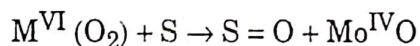


Fig. 6.10. Xanthine oxidation.

Molybdenum plays a role in biological systems. It undergoes two electron transfer reactions between Mo (VI) and Mo (IV) states. It transfers an oxo atom to a substrate.



$$M^{VI}(O_2) + S \rightarrow S = O + Mo^{IV}O$$

There are two specific cofactors : one is FeMoCo and second found in oxidases, reductases and dehydrogenases involved in oxo transfer. In this cofactor the molybdenum binding moeity is a substituted Pterin, which coordinates metal via two sulphur atoms.

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