Bioinorganic Chemistry

Unit IV: Metalloenzymes (15 h)

Contents: Zinc enzymes: Useful characteristics of Zn (II) ion, Metal substitution in Zn enzymes, Carbonic anhydrase – structure, function, Co & Cu substitution studies, catalytic mechanism. Carboxypeptidase – structure of active site, catalytic mechanism. Monooxygenase: Cytochrome P 450 –structure, mechanism, porphyrin-based model systems. Nitrogen fixation: Nitrogenases – structure, spectroscopy, N_2 activation and fixation, V-Nitrogenase, Model systems. V-peroxidase, Ni-hydrogenase

Metalloenzymes – Introduction

- Metalloenzymes are metal activated enzymes
- Metalloenzymes have two structural components: protein portion known as apoenzyme and a small non-protein prosthetic group may be simple or complexed metal ion.
- Enzyme containing both protein and non-protein part is called holoenzyme
- The selectivity and catalytic efficiency largely depend on protein structure.
- The metal does not change its oxidation state nor, generally, its protein ligands.
- Changes in the coordination sphere may occur on the side exposed to solvent
- The substrate interacts with protein residues inside the active cavity and/or with the metal ion in order to be activated, so that the reaction can occur.
- Under these circumstances, the catalyzed reactions involve bond-breaking and/or bond making process.

Enzyme	Metal (s)	Function
Carboxypeptidase	Zn(II)	Hydrolysis of C-terminal peptide residues
Thermosylin	Zn(II), Ca(II)	Hydrolysis of peptides
Alkaline phosphatase	Zn(II), Mg(II)	Hydrolysis of phosphate esters
Carbonic anhydrase	Zn(II)	Hydration of CO_2
ATPase	Mg(II)	Hydrolysis of ATP
Alcohol dehydrogenase	Zn(II)	Hydride transfer from alcohols to NAD^+
DNA Polymerase	Mg(II), Mn(II)	Polymerization of DNA with formation of phosphate esters
Superoxide dismutase	Zn(II), Cu(II)	Disproportionation of O_2^-
Phosphatase	Mg(II), Zn(II)	Hydrolysis of phosphate esters
Catalase	Fe(III)	Disproportionation of O_2^{2-} (catalyses peroxide decomposition)

Characteristics of Zn(II) ion

- Zn(II) has a specific role in bioinorganic processes due to its unique coordination behavior.
- First, Zn(II) can easily be tetra, penta or hexa coordinated, without any considerable preference for hexa-coordination.
- With $3d^{10}$-configuration, Zn(II) has no ligand field stabilization energy
- M-L distances: M-L (four-coordinate) < M-L (five-coordinate) < M-L (six-coordinate)
- The ligand repulsion (both steric & electronic) also increases in the same order.
- In enzymes, Zn(II) usually has coordination numbers smaller than six, so that they have available binding sites in their coordination spheres.
- Substrate binds to Zn(II) by substituting for a coordinated water or by increasing the coordination number (Zn(II) is the most common Lewis acid in bioinorganic chemistry)
- Zn(II) can thus substitute for protons in polarizing a substrate bond, e.g., carbonyl C-O bond of peptides & esters, by accepting a substrate atom (oxygen) as a ligand
- Secondly, as a catalyst, Zn(II) in zinc enzymes is exposed to solvent (usually water).
- A coordinated water molecule exchanges rapidly, because ligands in Zn(II) complexes are kinetically labile.
- This accounted for its lack of preference for a given coordination number.
- A six-coordinate complex can experience ligand dissociation, giving rise to a five-coordinate complex with little energy barrier.
- Moreover, four-coordinate complexes can add a fifth ligand with little energy barrier followed by dissociation of another ligand.
- The pKa of coordinated water is considerably lower than free water, and is controlled by the coordination number and total charge of the complex (decreases with decrease in coordination number and increase in positive charge.

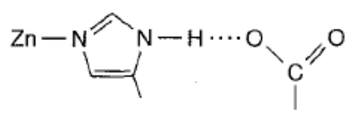
- Zn(II) ion with its positive charge has greater attraction for the oxygen lone pair, thereby lowering the pKa.
- Charged ligands affect the pKa of water *pKa* more than the number of ligands.
- The pKa in metalloproteins is further controlled by the presence of charged groups from protein side chains inside the cavity or by the binding of charged cofactors.
- Finally, Zn-complexes show facile four- to five-coordinate interconversion.
- The low energy barrier between for the interconversion is important for substrate binding to the coordination sphere by replacing the solvent or to get coordinated along with the solvent.
- Faster the interconversion, faster is the catalysis

In summary,

- Zn(II) is a good Lewis acid, especially in complexes with lower coordination numbers
- Zn(II) lowers the pKa of coordinated water and is kinetically labile
- The interconversion among its four-, five-, and six-coordinate states is fast.
- All of these properties make zinc quite suitable for biological catalysis

Groups to which Zn(II) is bound

- Zn(II) is an ion of borderline hardness and displays high affinity for N, O and S donors
- Zn(II) ion can bind histidine, glutamate or aspartate, and cysteine.
- In catalytic process, Zn(II) ion is exposed to solvent, and generally one water molecule completes the coordination, with the dominating histidine ligand.
- The binding of Zn(II) ion is often determined by entropy factors.
- Water molecules are released when Zn(II) enters its binding position, thus providing a large entropy increase.

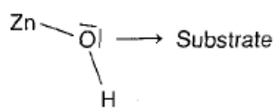


Reactivity of Zn(II) in cavities

- Reactivity of Zn(II) ion cannot be described solely on the basis of above properties considering Zn(II) as an ion
- Catalysis occurs in cavities whose surfaces are constituted by protein residues.
- Catalytic Zn(II) is bound to a water molecule, which often is H-bonded to other residues in the cavity.
- The structure of the water molecules in the cavity differs from the structure of bulk water.
- The substrate interacts with the cavity residues through either hydrophilic (H-bonds or electric charges) or hydrophobic (London dispersive forces) interactions.
- Thus, the overall thermodynamics of the reaction pathway is quite different from that expected in bulk solutions.

$$H_3O^+ < H_2O{-}M^{2+} \simeq H_2O < HO{-}M^+ \simeq HO^-$$

Order of nucleophilicity



The investigation of zinc enzymes

- Direct electronic or EPR spectroscopic studies of Zn enzymes is difficult, because Zn(II) is colorless and diamagnetic
- Its NMR-active isotope, ^{67}Zn (abundance 4.11%) has a small magnetic moment, and hence NMR spectroscopy at low concentration (10^{-3} M) is not useful.
- Although, the enzymes could be reconstituted with ^{67}Zn, nuclear quadrupolar moment of ^{67}Zn provides efficient relaxation times (in slow-rotating proteins and low-symmetry chromophores), making the line very broad.
- ^{1}H NMR can be useful for the investigation of the native enzymes. Due to high molecular weight of proteins, full signal assignment of ^{1}H NMR signals are difficult.
- X-Ray data is most reliable and is available for carboxypeptidase & alcohol dehydrogenase
- Zn(II) can be replaced by other metal ions (with partial/full retention in enzymatic activity).

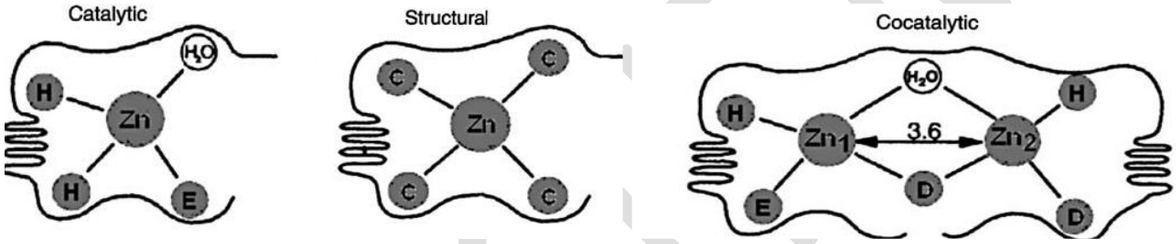
Biochemical role of Zn(II) ion/Zn-enzymes

- Zn(II) is the second most abundant trace element, plays both catalytic and structural role

- Zn(II) stabilizes the structure of proteins and nucleic acids, contributing to their overall stability and function.
- Zn(II) participates in antioxidant defence mechanisms, helping to protect cells from oxidative stress and damage.
- Zn(II) is essential for proper immune function, contributing to the development and activation of immune cells.
- Zn(II) is involved in neurotransmission and synaptic function in the central nervous system.in neurotransmission and synaptic function in the central nervous system.
- Zn(II) can bind to DNA, influencing its structure and stability, and is essential for the proper functioning of DNA-binding proteins.

Summary of function of Zn(II) ion/Zn-enzymes

- Catalytic role
- Structural stability
- Antioxidant properties
- Cell signaling
- Immune function
- Neurotransmission
- Metalloenzyme
- DNA binding
- Wound healing



Mononuclear Zinc enzymes

- Carbonic anhydrase and carboxypeptidase, both are mononuclear zinc enzymes characterized by a central catalytically active Zn^{2+} atom.
- Mono-zinc enzymes share a common tetrahedral structure.
- The catalytic properties are influenced not only by the nature of donor ligands but also by the distance separating them in the amino acid.
- The mechanism of action for mono-zinc enzymes centres revolves around the zinc-bound water molecule.
- The Zn^{2+}-OH_2 centre in mono-zinc enzymes participates in the catalytic cycle by providing properties such as an activated water molecule for nucleophilic attack.
- Majority of zinc enzymes are involved in hydrolytic reactions, often associated with the cleavage of peptide bonds.

Metal substitution in Zn-enzyme

- Metal substitution in Zn-enzyme is used for monitoring the protein and its function by means of spectroscopic techniques.
- Dependence of reactivity of the enzyme on the nature of the metal ion & its coordination properties can be scrutinized by metal substitution
- Apart from Zn-enzymes, metal substitution is also used in magnesium-activated enzymes and other metalloenzymes
- Dialyzing a protein solution against chelating agents (EDTA, 1,10-phenanthroline, 2,6-dipicolinic acid) at moderately acidic pH or by reversibly unfolding the protein with denaturing agents, Zn(II) ion can be released giving the inactive apoprotein.
- Removal of Zn(II) ion occurs slowly, the conformation of apoprotein is retained by the crystal packing forces & denaturation is avoided.
- Chelating agent is dialyzed out against a high-salt (ClO_4-) buffer to reduce nonspecific binding, and a new metalloprotein can be obtained by addition of suitable metal salt.

Few metal-substituted Zn-enzymes. % activities with respect to the native Zn-enzymes are shown in parenthesis

Enzyme	Substituted metals
Alcohol dehydrogenase	Co(II) (70), Cu(II)(8), Cd(II) (30), Ni(II)(12)
Superoxide dismutase	Co(II) (90), Hg(II) (90), Cd(II) (70), Cu(II) (100)

Carboxypeptidase A	Mn(II) (30), Fe(II) (30), Co(II) (200), Ni(II) (50), Cd(II) (5)
Thermosylin	Co(II) (200), Mn(II) (10), Fo(II) (60), Mg(II) (2), Cr(II) (2), Ni(II) (2), Cu(II) (2), Mo(II) (2), Pb(II) (2), Cd(II) (2), Nd(III)(2), Pr(III)(2)
Alkaline phosphatase	Co(II) (30), Cd(II) (1), Mn(II) (1)
Carbonic anhydrase	Cd(II) (2), Ni(II) (2), Co(II) (50), Mn(II) (18), Cu(II) (0)

Cobalt (II) substitution

- Co(II) substituted Zn proteins show almost similar activity as the native zinc enzyme. This is because, the coordination chemistry and ionic radii of Co(II) is very similar to that of Zn(II)
- Co(II) derivatives generally display useful electronic spectra.
- High spin Co(II) ions are paramagnetic with three unpaired electrons (S =3/2); giving rise to EPR spectra
- The electronic relaxation time (average lifetimes of the unpaired electrons in a spin state) are very short (10^{-11} to 10^{-12} s) at room temperature
- To detect EPR spectra, the sample temperature is decreased to liquid He-temperature (-269 ºC), thereby increasing the electronic relaxation times and sharpening the EPR line widths.
- Whereas, broadening of the NMR lines in such system is inversely related to the electronic relaxation times, ^{1}H NMR spectra of Co (II) complexes can be easily detected at room temperature
- Thus, Co (II) is an exceptional probe to monitor the structure & reactivity of Zn enzymes.
- Understanding the functioning of the Co-enzyme would be critical for understanding the kinetic properties of the native enzyme.

Copper (II) substitution

- Cu(II) substituted Zn-proteins are generally inactive with respect to the natural & artificial substrate.
- In model compounds, Cu(II) is usually four-coordinate, with two more metal-ligands distances longer than normal coordination bond at most
- Like Zn, Cu(II) cannot switch between four- and five-coordinate species with low energy barrier and usual metal-donor distances.
- Compared to Zn, the binding at the four principal coordination positions is generally stronger for Cu. Substrates may have slow detachment kinetics. These properties are unfavorable for catalysis.
- Cu(II) can be easily studied by electronic spectroscopy.
- The EPR spectra can be recorded even at room temperature because of the long electronic relaxation times (of the order 10^{-9} s)
- Protein (macromolecule) rotates slowly & the EPR spectra in solution at RT is similar those of crystalline powders or frozen solution (powder like spectra).
- ENDOR (Electron-nuclear double resonance, measures hyperfine and quadruple splitting of nuclear spins that are coupled to the electron spins) spectra are also easily obtained for Cu-proteins at low temperature (at this temperature, the relaxation times are longer, and saturations of EPR lines is easily achieved, prerequisite to obtain ENDOR spectra)
- The long electronic relaxation times make the broadening effects on the NMR lines of nuclei sensing the metal ion too severe and hence the lines cannot be detected (unlike Co(II) complexes)
- If there exist a fast exchange between a free species in large excess and a bound species in the nucleus, the line may be observed
- The nuclear relaxation parameters contain precious structural and/or dynamic information

Carbonic anhydrase

- CO_2 is the starting point for photosynthesis and it is the end product of respiration
- Carbonic anhydrase (CA, 1932) catalyzes the reversible CO_2 hydration (interconversion of CO_2 and HCO_3^-/CO_3^{2-}) in blood.
- Presence of Zn in the enzyme CA was recognized in 1939.
- CA is present in animals, plants, and several bacteria (known as ubiquitous).
- CA obtained from different sources have homologous structures, are termed as isoenzymes.
- Human carbonic anhydrase (HCA) has more than one isoenzyme for a particular function.
- Solubility of CO_2 in water is 0.003 molL^{-1} at room temperature and 1atm pressure), but its hydration in physiological condition (pH) is slow in absence of any catalyst ($k=10^{-1}$ s^{-1})

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$

- In presence of the isoenzymes of CA, the reaction is fast ($k_{cat}=10^6$ s^{-1}) at physiological condition (pH).
- CA has the turnover number (the number of substrate molecules transformed per unit time by each molecule of enzyme) of 10^6 s^{-1}, one of the highest known biological rates.

- At physiological pH, the rate of spontaneous dehydration of HCO_3^- ion is 10^{-4} s^{-1} while the CA catalysed rate for the same is 15 s^{-1}
- Above pH = 9, the uncatalyzed reaction become fast, owing to direct attack of OH^- (better nucleophile than H_2O) ($k = 10^4$ s^{-1})

$$CO_2 + OH^- \rightleftharpoons HCO_3^-$$

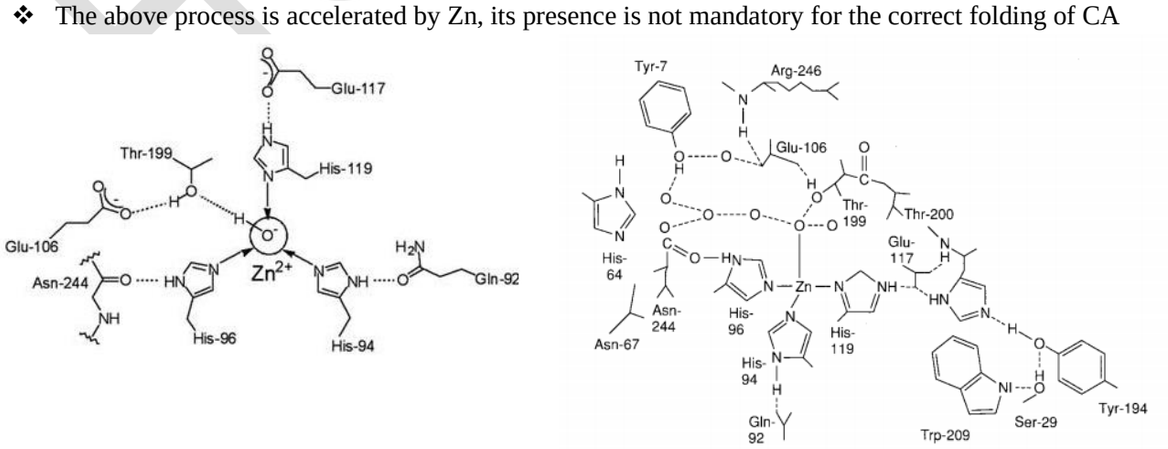
- In addition to above, the enzyme also catalyzes the hydration of carbonyl compounds and hydrolysis of esters.

pH dependence of CA activity:

- The catalytic activity of CA in CO_2 hydration depends on the working pH.
- Above pH = 8, the rate constant in the presence of CA (k_{cat}) is independent of pH (attains the maximal rate)
- The deprotonated form of the enzyme is the active species in the enzymatic activity.
- The pKa value of the deprotonation process is close to 7.0

Structural features

- CA is classified as high-activity form (CA II, $k_{cat}=10^6$ s^{-1}), low-activity form (CA I, $k_{cat}=10^5$ s^{-1}), and very-low-activity form (CA III, $k_{cat}=10^3$ s^{-1})
- In human, they are described as HCA I and HCA II (high resolution structure refined)
- Isoenzymes are all structurally similar, and are single-chain polypeptides (~30kDa) with one Zn(II) ion per molecule.
- In HCA II, Zn(II) is in distorted tetrahedral environment, coordinated to three histidine-N sites (Nε of His-94, Nε of His-96 and Nδ of His-119)
- The fourth coordination site is occupied by H_2O molecule or OH^- ion
- At the active site, several amino acid residues are present (probably stabilize the transition state or holding the substrate or proton transfer)
- The three histidine NH protons are engaged in H-bonding with amino acid residues
- His-94 & His-96 are H-bonded with Gln-92 and carbonyl backbone of Asn-244 respectively
- His-119 is H-bonded with a Glu-117 (controls the basicity of the metal ligands).
- The coordinated H_2O molecule is H-bonded with Thr-199 which is again H-bonded with Glu-106. The same is also coordinated to His-64 via H-bonding (proton shuttle).
- Near the active site, a hydrophobic pocket for accommodating non-polar CO_2 is produced by amino acid residues Trp-209, Leu-198, Val-121 and Val-123. First three comprise the mouth while Val-143 comprises the base cavity.
- The hydrophobic pocket is important for substrate (CO_2) binding with the active site
- The hydrophobic pocket dissolves the substrate & properly orients the electrophilic CO_2 for nucleophilic attack by the Zn-OH group
- H-bonding network is important for understanding the subtle structural changes that occur with pH changes (possibly account for the pH-dependent properties)
- The tertiary structure of CA is maintained in the absence of Zn; even the denatured apoprotein can refold spontaneously to a native-like conformation
- The above process is accelerated by Zn, its presence is not mandatory for the correct folding of CA



Active site of HCA II. Hydrogen bonds (---) & ordered water molecules (O) are indicated

Figure 1

- Anions are attracted in the metal cavity by the positive $Zn(N_3OH_2)^{2+}$ moiety, and get effectively bound to Zn in CA
- At high pH, Zn in CA is four-coordinate with an OH^- group in the fourth coordination site.
- At low pH, CA contains water, can be tetra (one water) or penta (two water) coordinated
- Occurrence of low-pH species depends on the pKa of the complex acid-base equilibria.

Characteristic of the Enzymatic Activity

Prosthetic group

- In absence of Zn(II), the apoenzyme becomes inactive & activity is restored on Zn uptake.
- The corresponding Co(II) is equally active compared to native Zn(II) enzyme
- The spectral properties of Co(II) CA are useful in elucidating the coordination environment around Zn(II)
- This supports the distorted tetrahedral environment around the metal centre.
- Electronic spectra of Co-CA are considerably pH dependent.
- Sharp change of molar extinction coefficient at 640 nm with varying pH indicates the pKa of the metal bound H_2O molecules ~ 7.5
- For Co-CA, the ε_{640} vs pH profile is very much like the rate vs pH profile for CO_2 hydration catalysed by Co-HCA.
- It indicates that deprotonation of the coordinated water molecule is critical of the enzymatic activity and the hydroxy species is the catalytically active species

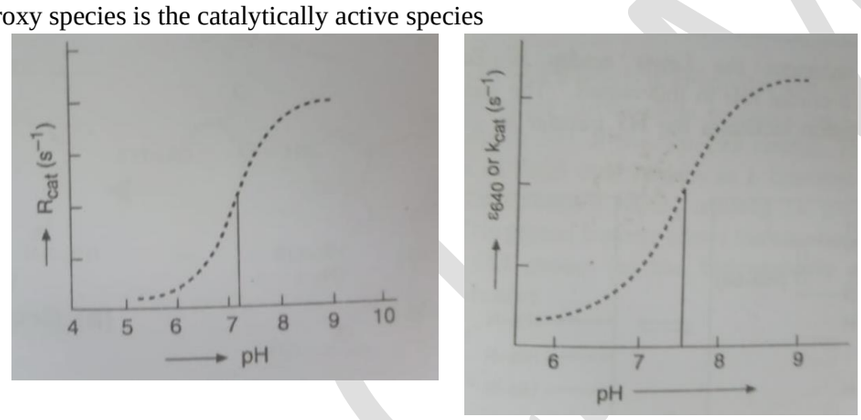


Figure 2

- The active site is highly structured in nature and because of this, there found to be retardation in the complexation (with small chelating ligands) with the apoenzyme
- The tertiary structure of the apoenzyme does not change on binding Zn(II) (from spectra)
- Zn(II) does not to stabilize the protein structure but controls the enzymatic activity.

Formation of Zn(II)-OH to offer a better nucleophile

- Hydration of CO_2 by OH^- is faster than that by H_2O (OH^- is better nucleophile than H_2O).
- But, at physiological pH, availability of OH^- is unlikely.
- The CA enzyme $(N_3)Zn\text{-}OH_2$ generates the metal bound OH-group through deprotonation, the most crucial step in the catalytic activity of CA

$$(N_3)Zn\text{-}OH_2 \leftrightarrow (N_3)Zn\text{-}OH + H^+$$

- The pKa value of $[Zn(OH)_2]_6^{2+}$ is ~ 9.0 while for CA it is ~ 7.0
- The Lewis acidity of Zn(II) in the enzyme is enhanced (back bonding) by three imidazole moieties (π-acceptor ligand) from histidine residues
- The imidazole moiety of the His-64 residue present in suitable position facilitates the H^+ transfer to generate the active Zn(II)-OH center
- The proton transfer from the Zn(II)-bound water molecule to His-64 occurs through a H-bonded network of two H_2O molecules acting as proton wire for the proton shuttle
- The Zn(II) bound OH-group is H-bonded with the Thr-199 residue and this H-bonding interaction favors catalytic activity in two ways

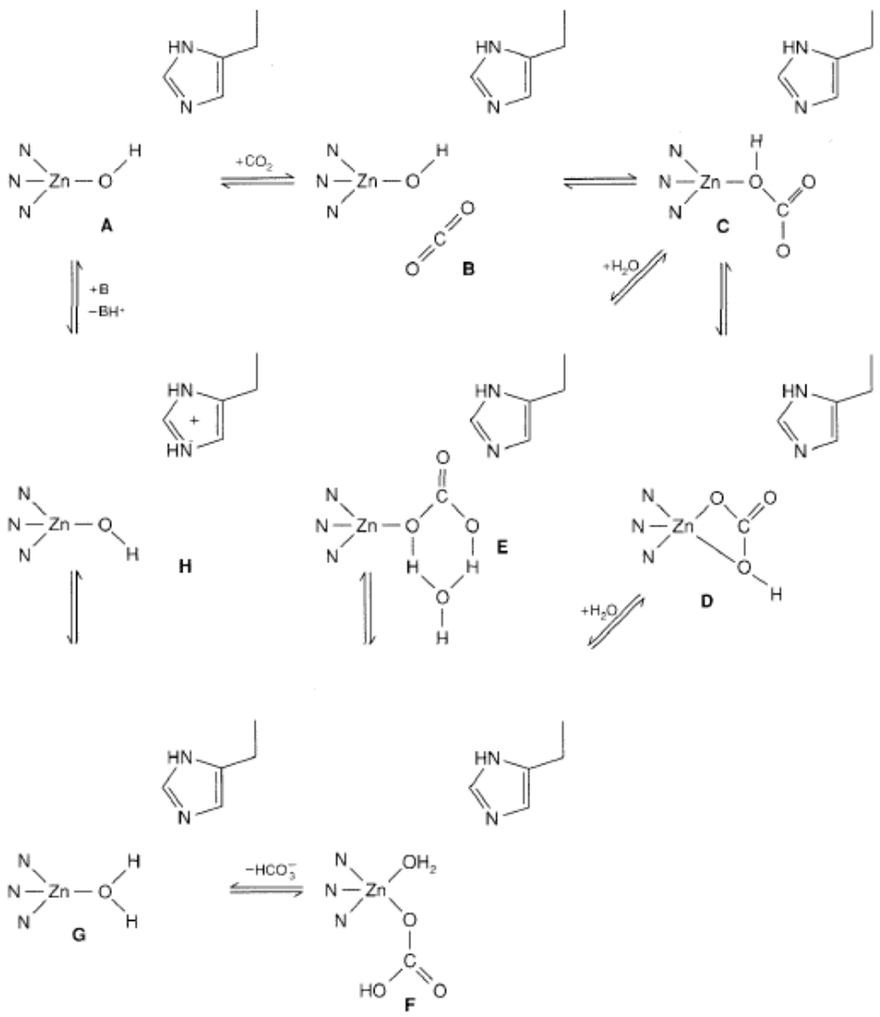
(a) The H-bonding interaction introduces some oxo-character to the metal bound OH-group and this makes the metal bound OH-group a better nucleophile (because oxo-O is more electron rich than hydroxo-O)

(b) The H-bonding interaction fixes the nucleophilic lone pair in a proper orientation required for the nucleophilic attack on the substrate CO_2

Plausible mechanism of enzymatic activity of CA

- At physiological pH, the enzyme is essentially in the Zn-OH form (step **A**).

- At the active site, the hydrophobic pocket acts as a docking or binding site for CO_2. The hydrophobic cavity favors the desolvation of the substrate (CO_2) and orients CO_2 properly towards Zn(II) bound hydroxido group.
- The substrate CO_2 does not directly interact with Zn(II) center (spectral studies). The driving force behind the reaction is the generation of metal bound OH group which is H-bonded with Thr-199 residues. This H-bonded metal bound OH group is a potential nucleophile to attack the C-center of CO_2.
- The concentration of CO_2 in the cavity is higher than in bulk solution (step **B**). The positioning of CO_2 between Zn(II) and the peptide NH of Thr-199 would be ideal for the OH attack.
- The HCO_3^- ion produced within the coordination sphere of Zn(II) (step **C**) facilitate the proton transfer to a terminal O-atom either via a bidentate HCO_3^- intermediate (step **D**) or via a hydrogen-bond network (step **E**).
- This proton transfer allows the bicarbonate to coordinate to the metal center in such a way that possibly keeps the OH group as far as away from the Zn center. The HCO_3^- derivative is presumably in equilibrium between four- and five-coordinate species (step **F**), (from electronic spectra of the Co-derivative)
- A five-coordinate intermediate has been suggested in the associative pathway of displacement of HCO_3^- by H_2O (step **G**). This associative pathway has low activation barrier (energetically more favorable) for the displacement of HCO_3^-
- The nucleophile H_2O is properly positioned through H-bonding interaction with the free OH group of coordinated HCO_3^-, and executes nucleophilic attack on the Zn-center with the change of coordinating mode of HCO_3^- (bidentate to unidentate; carboxylate shift).
- At this stage the second substrate, which is H^+, must be released (step **H**). The water proton transfers to a group inside the cavity. The presence of buffer can assist in proton transfer at this stage, in such a way that the internal proton transfer becomes the rate-limiting step.



Proposed catalytic cycle of CA.

Inhibition of enzyme activity

- The enzyme activity inhibited in presence of anions (I^-, SH^-, N_3^-, CN^-, CNO^-) and neutral substances (suphonamide, imidazole)
- Zn(II) is a borderline acid and hence the displacement of the harder base OH^- by above soft bases is thermodynamically feasible.
- In CA, the apoenzyme softens (symbiosis) the Zn(II) ion due to presence of imidazole moieties (enzyme affinity $I^- > Br^- > Cl^- > F^-$, Zn(II) ion affinity $F^- > Cl^- > Br^- > I^-$)
- The anions like NO_3^-, CNO^- and N_3^- strongly bind with the enzyme (electron cloud of these anions possess some softness) and are isoelectronic or isostructural with the reactant and products of the enzymatic reaction (CO_3^{2-}, HCO_3^-, CO_2)
- These inhibitors are positioned in a pocket close to Zn(II) thereby blocking the active site.
- Zn(II) probably binds to either O or N-atom of sulphonamides and blocking the active site
- Sulphonamides are used in the treatment of glaucoma to reduce the intracellular pressure through the inhibition of CA
- Imidazole acts as a competitive inhibitor, it binds to Zn(II) and gives the pentacoordinate intermediate keeping H_2O molecule still within the coordination sphere.

Steady-State and Equilibrium Kinetics of CA-Catalyzed CO_2/HCO_3^- interconversion

- The $CO_2 \leftrightarrow HCO_3^-$ interconversion catalyzed by CA is extremely fast.
- The kinetic parameters are described in terms of turnover number or kinetic constant for the reaction (k_{cat}), and the Michaelis constant (K_m).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

- K_m is given by $K_m = k_1/(k_{-1}+k_2)$, If k_2 is small, $k_{cat} = k_2$ and $K_m = k_1/k_{-1}$, K_m corresponds to the thermodynamic affinity constant of the substrate for the enzyme.
- K_m is pH-independent while k_{cat} increases with pH, reaching a plateau above pH 8
- For HCO_3^- dehydration (the reverse), H^+ is a co-substrate of the enzyme.
- The pH dependence of k_{cat}/K_m for HCO_3^- dehydration is due to k_{cat}
- The pH dependence of k_{cat} reflects at least two ionizations in the absence of anions
- The exchange of the H^+ with the solvent is the rate-limiting step along the catalytic pathway if buffer system does not provide relatively high concentrations of H^+-acceptors and H^+-donors
- At high buffer concentration, k_{cat} shows an isotope effect, with the occurrence of an internal H^+-transfer as the new rate-limiting step
- At equilibrium, an O-atom can pass from HCO_3^- to CO_2 and vice versa several times before getting released to H_2O (^{13}C NMR spectroscopy is useful in studying substrate interconversion rates)
- In the absence of enzyme (slow exchange) two ^{13}C signals (one for CO_2 and one for HCO_3^-) and in presence of enzyme (fast exchange) only one ^{13}C signal is observed
- In case of slow exchange (in absence of enzyme), the increase in linewidth *(dv)* of the substrate (A) and product (B) signals (caused by exchange broadening) depends on the exchange rate and on the concentration of each species
- The exchange rate (τ_{exch}^{-1}) can be calculated by the following relationship

$$\Delta\nu_A[A] = \Delta\nu_B[B] = \tau_{exch}^{-1}.$$

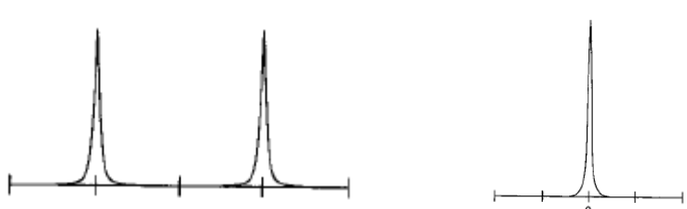


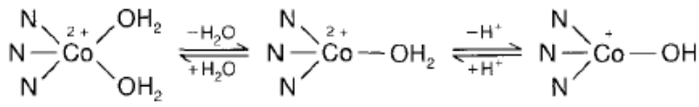
Figure 3

- For the high-activity enzyme the maximal exchange rates are larger than the maximal turnover rates under steady-state conditions. The ratio between k_{exch} of the high-activity (type II) and low-activity (type I) enzyme 50, i.e., larger than the ratio in k_{cat}
- The rate-limiting step in the steady-state process is an intra molecular H^+ transfer in the presence of buffer for type II enzymes, whereas it may not be so in case of type I enzymes.
- The exchange is pH-independent (in pH range 5.7-8.0), and does not show a proton-deuteron isotope effect.

What Do We Learn from Cobalt Substitution?

(a) Acid-base equilibria

- Electronic spectra Co-substituted CA enzyme is pH-dependent (easy to measure)
- A sharp absorption at 640 nm is present at high pH and absent at low pH.
- CoHCA I almost entirely exists in the low-pH (pH = 5.7), not true for CoBCA II isoenzyme
- The acid-base equilibrium for Co-substituted CA (deprotonation of the metal-coordinated water) involves three species



- The five-coordinate species have been proposed to exist in HCA I at low pH
- Fig. 4 shows isosbestic points (frequency at which two species in equilibrium have same absorption) between 16000-18000 cm^{-1} (single acidic group could account for this experimental data)
- CoHCA I has a pKa ~ 8, CoBCA II and CoHCA II pKas ~ 6.5, and CoBCA III a pKa ~ 5.5
- The pH dependence analysis of absorbance shows that two apparent pKa's can be extracted from the electronic spectra of CoCA I and II (Fig. 5)

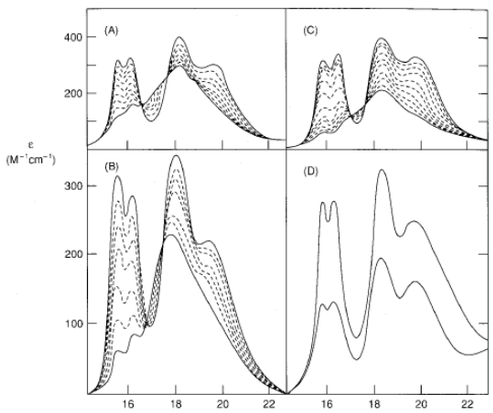


Fig. 4 pH-variation of the electronic spectra of Co(II)-substituted BCA II (A), HCA II (B), HCA I (C), and BCA III (D).

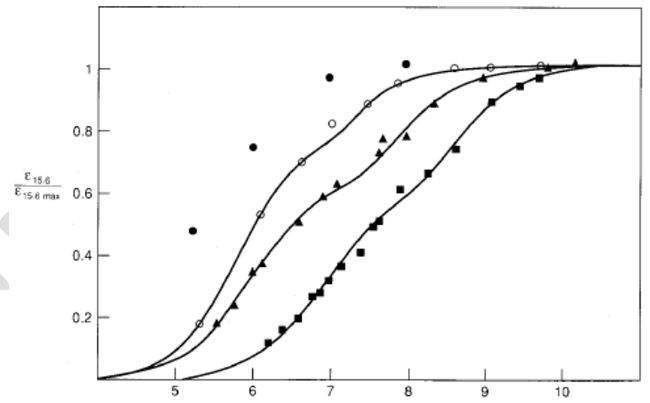


Fig. 5 pH dependence for Co(II)-substituted BCA III (o), HCA II (•), BCA II (▲), and HCA I **(■)** isoenzymes.

- These isoenzymes contain at least another histidine in the cavity (represents another acidic group) with pKa ~ 6.5 in its free state. The interaction between such an acidic group and metal-coordinated water (via H-bonds) validates the observed experimental results.
- Two apparent (because they do not represent actual acid dissociations) acid dissociation constants Ka can be obtained from the fitting of the curves of Fig. 2.8
- When two acidic groups are interacting with each other, the system must be described in terms of four constants (micro-constants), because the dissociation of each of them is described by two different pKa's (Fig. 6)

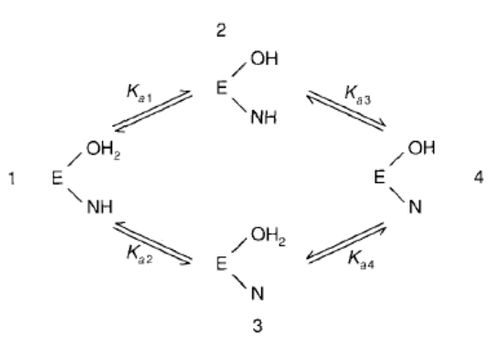


Fig 6: General scheme for two coupled acid-base equilibria applied to carbonic anhydrase. The two acid-base groups are the metal-coordinated water molecule and a histidine residue present in the active-site cavity.

- The changes in the electronic spectra of Co(II) (Fig. **4**) are solely due to the ionization of the coordinated water.
- This assumption not only accounts for the observation of approximate isosbestic points but also useful in obtaining the four microconstants

- ❖ Similar values of microconstants have also been obtained on ZnHCA II by analyzing the pH dependence of the maximum velocity of the hydration reaction, V_{max} (the two hydroxo-containing species are assumed to have the same activity)
- ❖ Thus, the species (**2**) and (**3**) of Fig. **6** are distinguishable, although their interconversion may be fast.
- ❖ Metal coordination lowers the pKa of coordinated water, which arises from the contribution from

(1) the charge of the chromophore (+2) (although it is possibly lowered by the H-bonding between a coordinated histidine and a negative glutamate residue)

(2) the coordination number (which is four), since a higher value leads to a larger electron density on the metal ion ligands;

(3) the presence of other acidic groups with which the coordinated water interacts;

(4) the presence of positively charged residues inside the metal binding cavity that favors one H^+ removal from the cavity.

- ❖ This last factor is presumably operating for CA III, which contains several arginine residues in the cavity

(b) Coordination Geometries

- ❖ The binding of inhibitors is also pH-dependent. Fully inhibited systems can be obtained by adjusting the inhibitor concentration and pH (the limit spectra of CoCA derivatives are obtained in this way, Fig. **7**).
- ❖ The differences in molar absorbance are larger than expected for changing only one coordinated atom.
- ❖ Gray et al., have suggested larger maximal absorption for four-coordinate species than five-coordinate species, due to greater mixing of p and d-metal orbitals in the former, allowing the d-d transitions partially (neglecting the covalency of the coordination bond, nephelauxetic effects (cloud expanding), or vicinity of charge transfer bands).
- ❖ Further measurements towards near-infrared region have indicated the low-intensity spectra with a weak absorption between 13,000 and 15,000 cm^{-1}.
- ❖ The latter band was assigned to the highest in energy of the F → F transitions (transition between two F-term in the free ion and split by the ligand field), which increases in energy with the coordination number.
- ❖ Both the low intensity of the bands (ε_{max} < 200 $M^{-1}cm^{-1}$) and the presence of the F → F transition at high energy were taken as evidence for five coordination.
- ❖ Spectra showing high maximal absorption (ε_{max} > 300 $M^{-1}cm^{-1}$) were assigned as arising from four-coordinate species.
- ❖ The corresponding chromophores are $CoN_3In(OH_2)$ and CoN_3In; In = inhibitor.
- ❖ Intermediate maximal absorptions may indicate an equilibrium between four- and five-coordinate species.
- ❖ Bicarbonate, which is a substrate of the enzyme, gives rise to an equilibrium between four- and five-coordinate species.

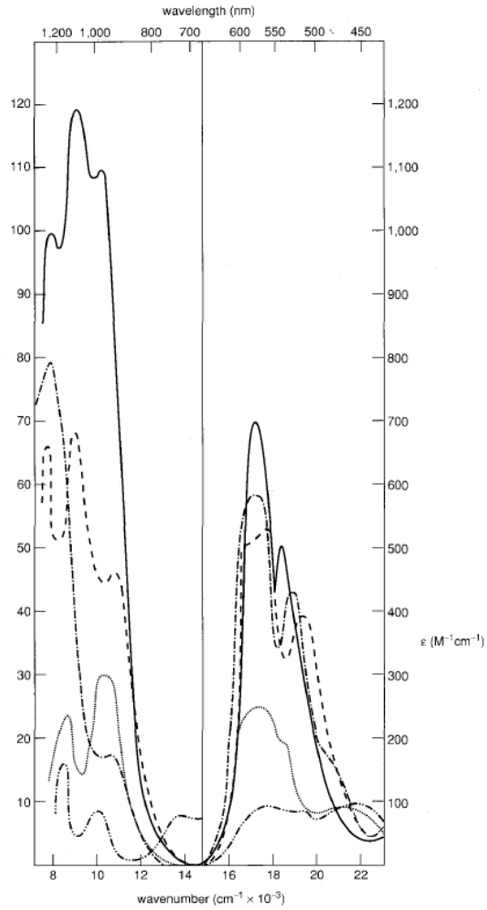


Figure 2.10
Electronic spectra of cyanide (———), cyanate (–·–·–), acetazolamide (– – –), azide (· · · · ·), and thiocyanate (–··–) adducts of cobalt(II)-substituted bovine carbonic anhydrase II.[59]

Figure 7

- CD and MCD spectra both provide useful information about the coordination number.
- In MCD spectra, pseudo tetrahedral species give a sizably positive band in the high-energy region, whereas five-coordinate species show a much weaker positive band and six-coordinate complexes have only weak negative bands (Fig. **8**)
- A further criterion is based on how much of the splitting of the S = 3/2 ground state is caused by spin-orbit coupling (zero-field splitting).
- This splitting can be indirectly measured from the temperature dependence of the electronic relaxation times of the Co-complexes, in turn estimated from their ability to saturate the EPR lines of the complexes at low temperatures.
- The splitting increases in the order four coordination < five coordination < six coordination.

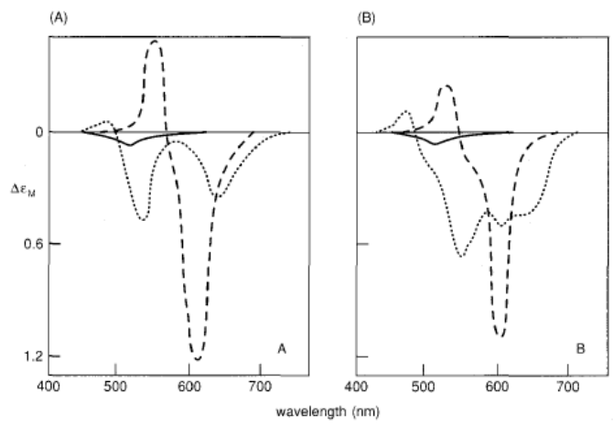


Figure 2.11
(A) MCD spectra of model six-coordinate ($Co(Gly\text{-}Gly)_2$, ———), five-coordinate (($Co\text{-}Me_6tren)Br_2$, ... , and four-coordinate ($Co(py)_2Br_2$, – – –) cobalt(II) complexes and (B) MCD spectra of the cobalt(II) derivatives of pyruvate kinase (———), alkaline phosphatase (· · · · ·), and carbonic anhydrase in the presence of acetazolamide (– – –).[21,61]

Figure 8

- Three binding sites have been identified in the cavity of CA (Fig. **9**).
- The OH-binding site provides a tetrahedral structure around the metal ion (the A site)

- The hydrogen interacts via H-bonding with the oxygen of Thr-199. Thr-199 and Thr-200, together with their protein backbone, identify a hydrophilic region that probably plays a fundamental role in the energetic balance of ligand binding.
- On the back of the cavity there is a hydrophobic region formed by Val-143, Leu-198, and Trp-209.
- Although hydrophobic, the X-ray structure of cavity shows the evidence of a water molecule, H-bonded to the coordinated water.
- Ligands with a hydrophobic end could easily be located at B site.
- The coordinated water molecule would change its position to make suitable angles between coordinated groups (C site).
- The X-ray structure of the thiocyanate derivative of HCA II illustrates the latter case. The NCS^- ion is in van der Waals contact with Val-143, Leu-198, and Trp209.
- The water interacts with the hydroxyl group of Thr-199. The geometry of the five-coordinate derivative can be roughly described as a distorted square pyramid with His-94 in the apical position (Fig. **10 A**).

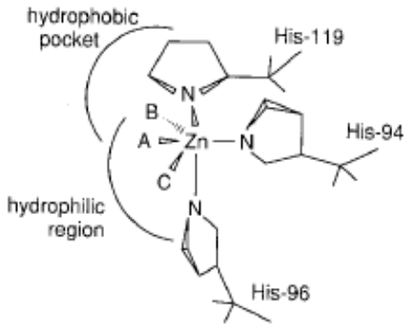


Fig. 9 Schematic drawing of the active cavity of HCA II showing the three possible ligand binding sites. Site A is the site of the OH – ligand in the active form; site B is the binding site of NCS^-, which gives rise to a five-coordinate adduct with a water molecule in the C site.

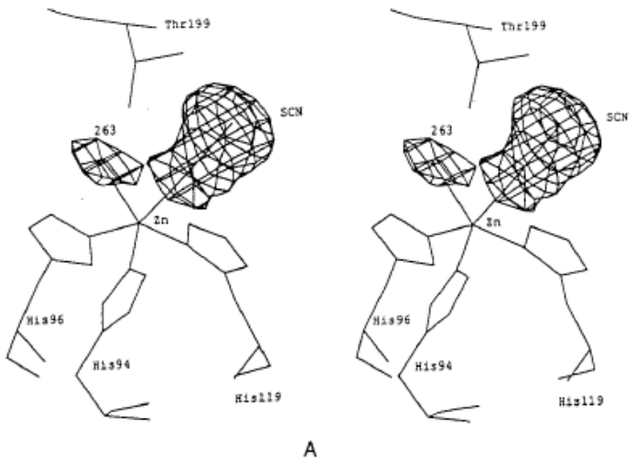


Fig. **10 A**: Stereo views of the NCS adduct of HCA II

- In aromatic sulfonamide ($Ar\text{-}SO_2\text{-}NH_2$) derivatives (probably bind as anions), the NH-group binds Zn in the A position, giving rise to an H-bond with Thr-199.
- The oxygens do not interact with the metal; one of them sits in the hydrophobic pocket. The chromophore around zinc is pseudo-tetrahedral (Fig. **10 B**).
- The energy involved in the coordination includes the coordination bond, the hydrophobic interactions of the aromatic sulfonamide ring, and the maintenance of the Zn-X-H-Thr-199 hydrogen bonding (X=N, O).
- Cyanate (spectroscopic studies) ion gives rise to tetrahedral derivatives, possibly due to the formation of H-bonds between the terminal oxygen and hydrophilic region of the cavity.
- ^{13}C NMR data on $N^{13}CO^-$ interacting with CoBCA indicate that the anion interacts directly with the metal ion.
- The balance between hydrophobic and hydrophilic interactions, and major steric requirements play important roles in the binding of inhibitors.
- Cyanide is the only ligand that may bind in 2: 1 ratio. The spin state of the bis-cyanide adduct is S = ½

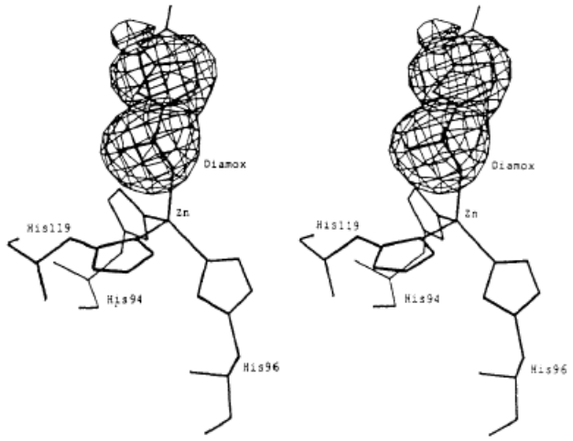


Figure 2.13
Stereo views of the NCS^- (A) and acetazolamide (B) adducts of HCA II.[40]

Figure 10 B Stereo views of the acetazolamide (B) adduct of HCA II

(c) Coordinated water and NMR

- NMR spectra are useful in understanding whether H_2O is coordinated to the metal ion in a metalloenzyme, & whether H_2O is still coordinated in the presence of substrates and inhibitors.
- The presence or absence of H_2O coordinated to a paramagnetic center can be monitored by solvent water ^1H NMR (from the magnetic interaction between the magnetic moments of the unpaired electrons & the nuclear magnetic moments of the water protons).
- When this interaction fluctuates with time, it causes a shortening of the water-proton relaxation times
- The longitudinal relaxation rate values, T_1^{-1}, of all the solvent water protons increase when even a single water molecule interacts with a paramagnetic center (condition: this bound water exchanges rapidly with free water molecules)
- Nuclear Magnetic Relaxation Dispersion, NMRD method contains information on correlation time & gives information on the number of protons and their distance to the metal.
- Short electronic relaxation times in paramagnetic compounds cause only minor broadening of ^1H NMR lines, whereas the isotropic shifts (the shifts due to the presence of unpaired electron, very large) are independent of the value of the electronic relaxation times.
- In ^1H NMR spectra of CoCA, the proton signals of histidines coordinated to the metal were found to be shifted well outside the diamagnetic region (Fig. **11**).
- Five-coordinate species give sharper signals than four-coordinate ones. The spectra in D_2O for both kinds of derivatives show three fewer isotropically shifted signals than in H_2O.
- These signals are assigned to histidine NH protons, are replaced by deuterons in D_2O.
- Five-coordinate species provide ^1H NMR spectra with many signals slightly shifted from the diamagnetic position. These complexes have relatively large magnetic anisotropy, which, summed up to the external magnetic field, provides further differentiation in shifts of the protons. Such shift contributions are called pseudocontact shifts. These shifts depend on the third power of the distance from the metal and on the position of the proton with respect to the molecular axes
- These signals belong to protons of non-coordinated residues from 5 to 10 Å from the metal.
- Their assignment in principle provides further information on the structure in the vicinity of the metal ion.
- The ^1H NMR spectra of Co(II) enzymes thus afford a powerful method for monitoring structure and reactivity of the metal-bound residues.
- ^1H NMR spectra of Co(II)-substituted bovine carbonic anhydrase II and some inhibitor derivatives. The three sharp downfield signals in each spectrum disappear in D_2O and are assigned to the exchangeable ring NH protons of the three coordinated histidines. The sharp signal labeled with an arrow is assigned to the Hδ2 proton of His-119, which is the only non-exchangeable ring proton in a meta-like rather than in an ortho-like position with respect to the coordinating nitrogen.

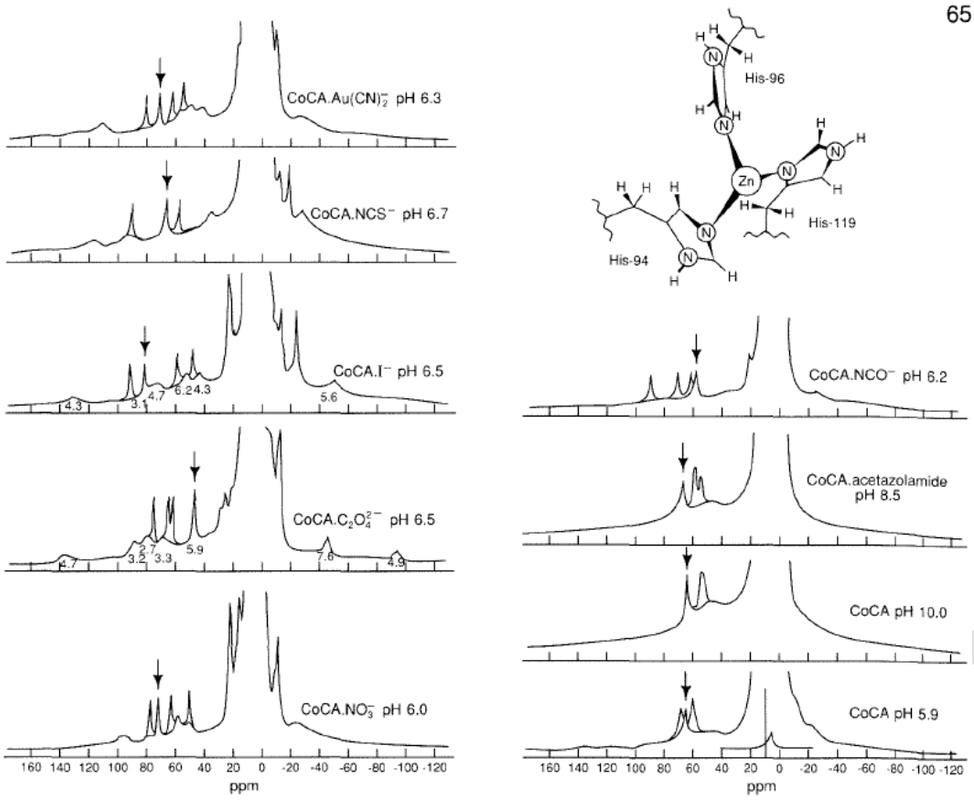


Figure 11: ^{1}H NMR spectra of Co(II)-substituted BCA II and some inhibitor derivatives. The three sharp signals in each spectrum disappear in D_2O and are assigned to the exchangeable ring NH protons of the three coordinated histidines. The sharp signals labelled with an arrow is assigned to the Hδ2 proton of His-119, which is the only non-exchangeable ring proton

pH dependence of inhibitor binding

- Electronic spectra (ease of studying) provide a simple way of determining the affinity constants of inhibitors for the cobalt-substituted enzymes (by comparing the spectra of the enzyme and that of the enzyme with increasing amounts of inhibitor).
- If solutions of the enzyme and inhibitor have the same pH, both absolute values and pH dependences of affinity constants obtained from electronic spectra are the same as those obtained from inhibition measurements, and are comparable to those obtained on the native enzyme.
- Affinity constant values depend on pH, buffer type, and buffer concentration
- Accordingly, three types of inhibitors are classified (Fig. **12**).
- Class I, the affinity constant (log K*)* decreases linearly with increasing pH. Anions such as Cl^-, N_3^-, CH_3COO^-, NO_3^-, and neutral ligand such as CH_3OH and aniline behave in this way

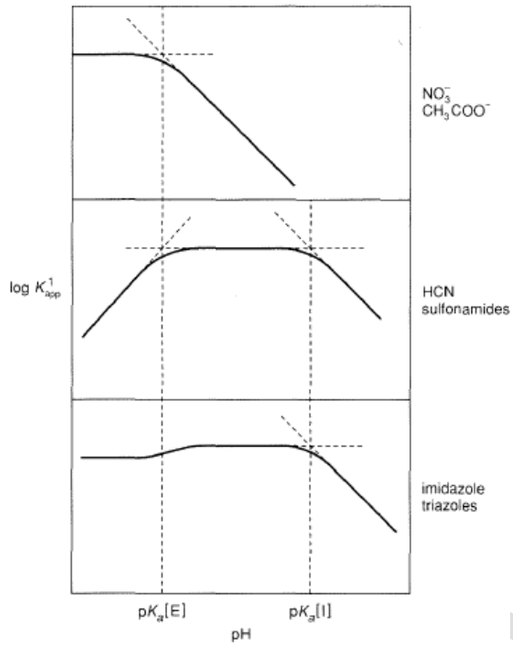
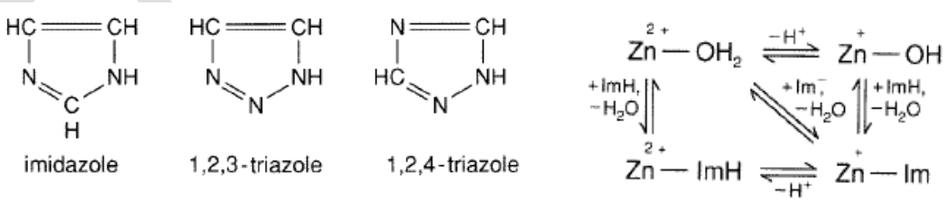


Fig. **12** Types of pH dependences observed for the affinity constants of inhibitors for Co(II)-substituted CA. pKa[E] represents the main pKa value of the enzyme, pKa[I] that of the inhibitor, if present.

- Thus, the ligand binds only the low-pH form of the enzyme where only one *pKa* value determines the species distribution in CA.
- If the species distribution calculated according to the scheme of Figure **6** is assumed to hold, and if it is assumed that only the two water-containing species (1) and (3) can be bound by the ligand, then actual affinity constants can be evaluated for both species (1) and (3).
- Such constants are similar for the three isoenzymes, whereas the apparent affinity constants at pH 7, for example, mainly depend on the *pKa'*s of the coordinated water according to the values of Table 2.5 (***see book***).
- Therefore, the low-activity species CAI has larger affinity for anions like nitrate (and bicarbonate) than do the high-activity forms at pH 7.
- Class II, A second type of behavior occurs for weak acids like HCN, H_2S, and aromatic sulfonamides ($ArSO_2NH_2$). If the anions (conjugated bases) bind the low-pH species of the enzyme, the bell-shaped plot of log K versus pH (Fig. **12**) can be accounted for.
- In fact, at low pH, the inhibitors are in the protonated form, which is not suitable for metal binding. At high pH the concentration of the low-pH species of the enzyme decreases. The maximal apparent affinity is experimentally halfway between the pKa of the inhibitor and the pKa of the enzyme, treated as if it were only one.
- The same type of curve is also expected if the high-pH species of the enzyme binds the weak acid. Indeed, kinetic measurements seem to favor this hypothesis for sulfonamides



- Class III, A third type of behavior obtains for inhibitors like imidazole and triazoles, which bind the enzyme with similar affinities over a large range of pH (Fig. **12**) because both the imidazolate anion and the neutral imidazole can bind to the aquo forms of the enzyme with essentially the same affinity, and the reaction of imidazole with the Zn-OH species cannot be distinguished thermodynamically from the reaction of imidazolate with the aquo forms:
- It is possible that the non-coordinated nitrogen can interact with a group in the protein via hydrogen bonding.
- A candidate could be the NH group of His-200 in HCA I or the -OH group of Thr-200 in HCA II.
- Only imidazole and triazoles with two nitrogens in 1,3-positions, seem to have this ability

In summary,

- Coordination geometry of the high- and low-pH forms by means of electronic spectroscopy;
- The values of the pKa's from the pH dependence of the electronic spectra;
- The four and five coordination of the various derivatives with exogenous ligands;
- The affinity constants of exogenous ligands and their pH dependence;
- A fingerprint in the ^{1}H NMR spectra that can be used to monitor structural variations.
- Most of these conclusions can be safely transferred to the native zinc enzyme, although minor differences can occur. For example, in the position of the equilibrium between four- and five-coordinate species.

What Do We Learn from Copper Substitution?

- The coordination chemistry of CuCA is not fully understood (since the electronic spectra are not very pH-sensitive).
- The affinity of anions is pH-dependent (like CoCA), the affinity of anions, including HCO_3^- is higher than that of CoCA.
- Water is usually present in the coordination sphere, along with the anion (from water ^{1}H NMRD).
- The steric requirements of the three histidines and of the cavity allow the anion and the water molecule to arrange in an essentially square pyramidal geometry (Figure **13**). This is consistent with the electronic and EPR spectra

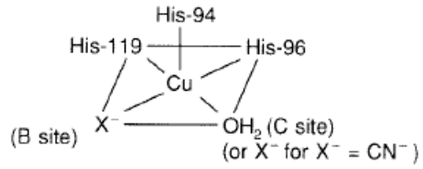


Figure 13 Schematic representation of the suggested coordination geometry for the anion adducts in CuCA

- The EPR spectra are all axial, with g-values decreasing from 2.31 in the non-ligated enzyme to 2.24 in the various anion adducts.
- The water molecule would be in the C site or hydrophilic binding site, and the anion would be in the B site or hydrophobic pocket.
- His-94 would be in the apical position of the square pyramid.
- EPR spectroscopy has shown that at low temperature two cyanide anions bind to copper. The donor atoms are two cyanide carbon and two histidine nitrogen atoms in the basal plane, and the third histidine nitrogen in the axial position.
- The hyperfine splitting is observed only with nuclei in the basal plane (both with ^{13}C nuclei of ^{13}C-enriched CN^- and with the two ^{14}N of two histidines). The second cyanide may thus displace the coordinated water
- Oxalate and sulfonamides displace water from the coordination sphere. For the oxalate ion this may occur through bidentate behavior.
- Coordination to an oxygen of the sulfonamide cannot be ruled out, although the electronic and EPR spectra of the sulfonamide complex are more consistent with a pseudotetrahedral chromophore.
- The SO_2 moiety would in any case point toward the B binding site.
- It is likely that sulfonamides bind as in ZnCA. Bicarbonate also shows less water relaxivity than other monodentate anions
- ^{13}C NMR spectroscopy has been used to investigate the location of the two substrates, CO_2 and HCO_3^-, with respect to the metal ion in CuCA. The interconversion between the two species is slow on the NMR timescale in the absence of catalysts. Therefore, two signals are observed (Figure **14**).
- In the presence of the catalytically active CoCA, only one signal is observed at suitable enzyme concentrations, and individual information on CO_2 binding cannot be obtained
- In the presence of inactive CuCA, two signals are again observed, which are broadened to different extents.
- The Cu-C distance would be 2.5 Å if the unpaired electron were completely on the copper ion. Electron delocalization on the bicarbonate ligand may account for such a short distance (possibly a bidentate type of ligation)
- The dissociation rate, which is very low, by itself accounts for the lack of activity of the derivative.
- CO_2 has no affinity for Cu, yet the paramagnetic effect is paradoxically high
- Substrate does not bind to a specific site, but probably binds in the hydrophobic region.
- The effect of the cavity is to attract CO_2 by interaction either with the metal ion or with a hydrophobic part of the cavity itself.

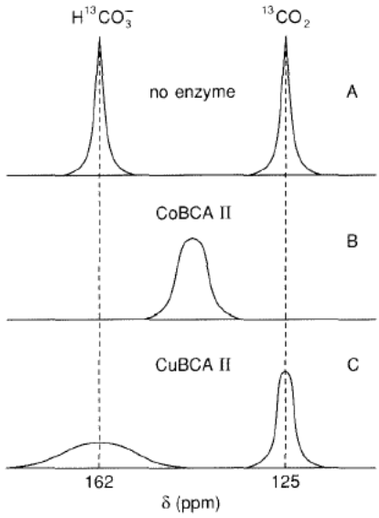


Fig **14** Schematic representation of the ^{13}C NMR spectra of the CO_2/HCO_3^- system (A) in pure water, (B) in the presence of CoCA, and (C) in the presence of CuCA

In summary,

- The main information concerning the catalytic cycle obtained from the copper derivative is the structural and kinetic characterization of both CO_2 and HCO_3^- species when they are not interconverting but present within the cavity.
- In this way, HCO_3^- is bound to the metal and that CO_2 is attracted inside the cavity either by hydrophobic interactions or by the metal ion or both.
- The data obtained on the geometry around Cu are consistent with those obtained on Co

Carboxypeptidase: Peptide hydrolysis

- The enzyme which catalyzes the peptide bond hydrolysis are called peptidases or proteases
- Peptidases are biological catalyst involved in the degradation of proteins into its primary constituents (amino acids) through the cleavage of peptide linkages
- Depending on the position of peptide linkage to be attacked, the peptidases are classified.
- Endopeptidases (Thermosylin, trypsin, chymotrypsin, pepsin etc.) catalyze the hydrolysis of non-terminal peptide bonds while exopeptidases (carboxypeptidase A, B, C, leucine aminopeptidase etc.) catalyze the hydrolysis of terminal peptide bonds.
- Exopeptidases are of two types: carboxypeptidase involved in the hydrolysis of C-terminal peptide bond (carboxy terminal residue) and aminopeptidase involved in the hydrolysis of N-terminal peptide bond (amino terminal residue)

$H_2NCH(R^1)CO\text{-}NH$ (*aminopeptidase*) $CH(R^2)$ CO-NH (*endopeptidase*)…… CO-NH(*carboxypeptidase*)$CH(R^n)COO^-$

- Peptidases are metalloenzymes and most of them contains Zn(II) in their active site
- Carboxypeptidase is a protease (metalloprotease) enzyme (exopeptidase).
- It catalyzes the hydrolysis of carboxy terminal (C-terminal) amino acids containing an aromatic or bulky aliphatic side chain.

$$R\text{-}CO\text{-}NH\text{-}R^{/} + H_2O \leftrightarrow RCOO^- + R^{/}NH_3^+$$

- Uncatalyzed reaction is very slow ($k=10^{-11}\ s^{-1}$) & catalyzed reaction is very fast ($k=10^4\ s^{-1}$)
- Carboxypeptidases are synthesized as inactive precursors in the pancreas for secretion into the digestive tract (source - bovine pancreas).
- The inactive form is converted to the active form by the enzyme enteropeptidase.
- The enzyme can also show the esterase activity (ester hydrolysis)

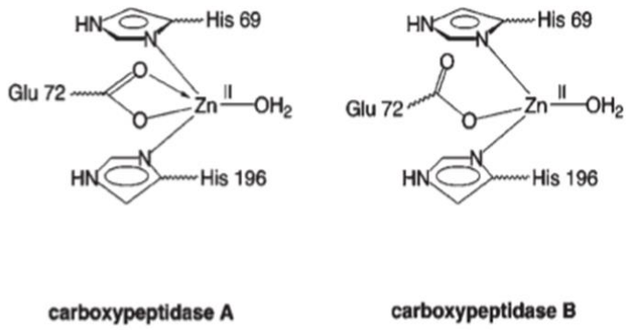
Carboxypeptidase and its classification

- The enzyme carboxypeptidase is generally activated by different metal ions like Zn(II), Mn(II), Co(II) etc.
- Based on substrate preference, carboxypeptidase can be classified as
- **Carboxypeptidase A** (CPA): Carboxypeptidases that have a stronger preference for those amino acids containing aromatic or branched hydrocarbon chains are called Carboxypeptidase A (A for aromatic or aliphatic).
- **Carboxypeptidase B** (CPB): Carboxypeptidases that cleave positively charged amino acids (arginine, lysine) are called Carboxypeptidase B (B for basic).

- CPA & CPB are released from their inactive precursor or zymogens (procarboxypeptidase) in the pancreas for the digestion of proteins.
- But, yeast carboxypeptidase C (CPC) is not a metalloenzyme.

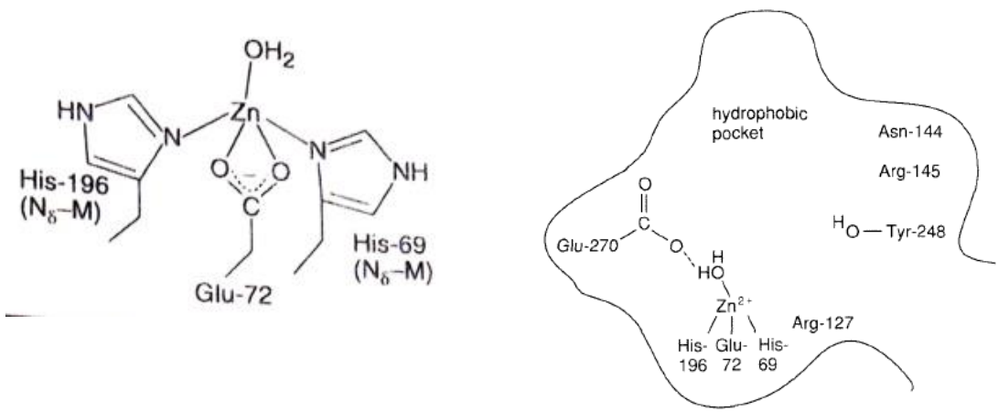
Structural features of CPA and CPB

- They consist of one single peptide chain with molecular weight ~ 34600 D.
- CPA and CPB are both similar from their structural and enzymatic activity aspects
- CPA (307 amino acid residues) and CPB (308 amino acid residues) require one Zn(II) per molecule
- Zn^{2+} has a distorted penta-coordination and is coordinated to two histidine residues (His69 and His196), a glutamate-CO_2^- (bidentate) (Glu72) residue and an H_2O molecule (or OH^-).



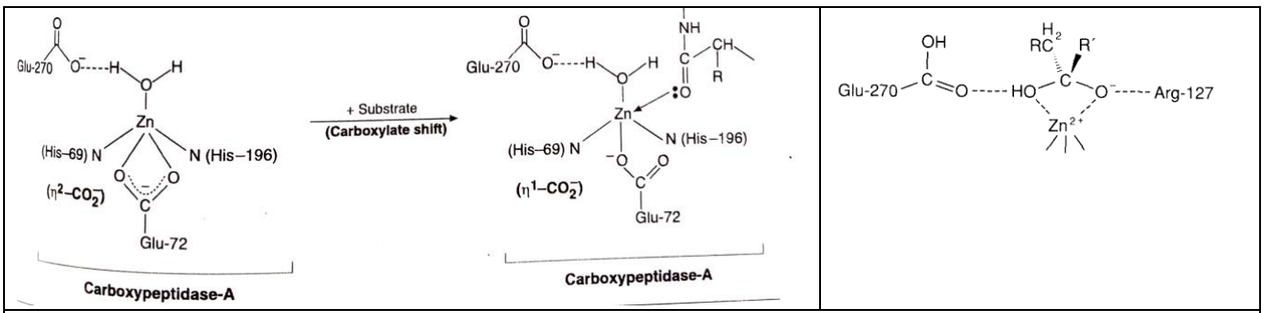
Structural features of CPA

- CPA exists in different forms viz. CPA-α (307 amino acid residues), CPA-β (305 amino acid residues) and CPA-γ (300 amino acid residues) depending on the size of the fragment lost from the zymogen
- CPA-α is commonly known as CPA with MW ≈ 34600 D.
- The prosthetic group of CPA contains a Zn(II) site and bears 307 amino acid residues
- CPA looks egg shaped and the active site is situated in cleft in the protein structure
- The Zn(II) center is coordinated by two N-sites (His-69 and His-196), one carboxylate group (unidentate) of the glutamate (Glu-72) and a water molecule (fourth coordination site). It provides a distorted tetrahedral geometry around Zn(II)
- The metal is thus solvent-accessible and, can activate the deprotonation of a water molecule to form a hydroxide ion, or polarize the carbonyl oxygen of the substrate by coordinating it in the place of the solvent molecule, or both.
- At resting condition, carboxylate group of Glu-72 may also act as bidentate (CN=5) ligand and on substrate binding it becomes unidentate (maintains the CN 5 and stereochemistry).
- Such arrangement is possible for a kinetically labile Zn(II) center (no CFSE). This is known as carboxylate shift (a characteristic feature of Glu-72 residues in CPA; carboxylate shift occurs on substrate binding)
- Near the active site, the three amino acid residues viz. protonated guanidyl moiety of Arg-145 (H-bonded to terminal carboxy group), Arg 127 (H-bonded to carbonyl oxygen), carboxylate end of Glu-270 are present and play important in enzyme activity
- Besides these three, phenolic-OH of Tyr-248 & and Asn-144 (H-bonded to terminal carboxy group) may also facilitate enzyme activity.
- A chiral hydrophobic cavity produced by the apoenymatic portion and is important in housing the hydrophobic group of the terminal residue (phenyl alanine, leucine) of the substrate



Schematic drawing of the active-site cavity of carboxypeptidase A. Only the residues believed to

play a role in the catalytic mechanism are shown.



Coordination environment around Zn(II) in CPA and illustration of carboxylate shift of Glu-72 (bidentate to unidentate) on substrate binding to maintain the constancy of CN around Zn(II)

Characteristic Features of CPA activity

(a) Metal substitution in CPA

- Zn(II) can be removed from the enzyme using the stronger chelating agents like 1,10-phenanthroline. The apoenzyme can be isolated through dialysis against the chelating agent, and is inactive
- Activity can be restored by adding Zn(II) and other metal ions like Co(II), Ni(II) and Mn(II)
- Co(II)-CPA is more active than native Zn(II)-CPA, but nature had to select Zn(II) rather than Co(II) due to non-bioavailability of Co(II) and possibly easy oxidation of Co(II) and Co(III) (d^6-LS, high LFSE)
- The reactivity order of hydrolysis of glycyltyrosine by metal substituted CPA is Mn(II) < Zn(II) < Co(II)
- With increase in M-O bond strength (supported by their stretching frequency) the carbocationic character of carbonyl carbon increases and thereby increasing the reactivity

(b) Distorted tetrahedral geometry around Zn(II) - entatic state in CPA

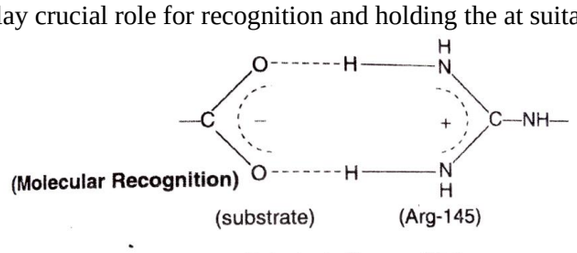
- Substitution of Zn(II) by Co(II) allows (d^7) spectral studies (absorption, circular dichroism, magnetic circular dichroism) of the substituted enzyme
- Co(II) substituted enzymes furnishes valuable information about the metal environment
- The electronic spectrum of Co(II)-CPA indicates the distorted tetrahedral symmetry around the metal center (also supported by X-ray studies)
- Vallee has termed the distorted condition as the entatic state which is believed to lower the activation energy to attain the transition state

(c) Chiral hydrophobic pocket at the active site of CPA

- The pocket is created by the polypeptide chain of the enzyme residues in the vicinity of the active site to house the hydrophobic group (θ) of the terminal residue of the substrate of appropriate stereochemical orientation (L-configuration)
- This chiral nature of the hydrophobic pocket gives the enantioselectivity (can accommodate aromatic or aliphatic hydrophobic moiety of the side chain of C-terminal amino acid residues) (eg., phenyl alanine, leucin)
- In contrast, CPB has aspartate (Asp-255). The anionic side chain of Asp-255 favorably interacts with the cationic side chain (eg., lysin, arginine)

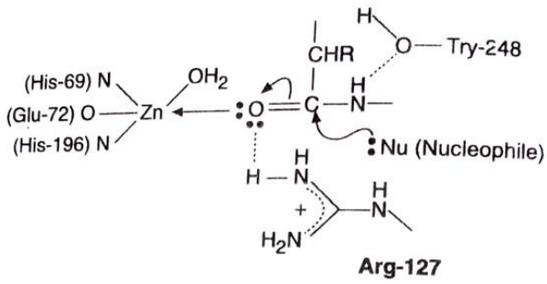
(d) Role of Arg-145 in substrate docking and recognition in CPA

- The terminal carboxyl group of the substrate forms a salt bridge (ionic interaction) with the protonated guanidyl group of Arg-145
- It keeps the substrate in a proper position and orientation required for the catalytic process
- Arg-145 is the ideal docking position of the C-terminal end (anionic nature) of the polypeptide chain
- This is why the enzyme is specific for the terminal peptide linkage at the carboxyl end (this is an example of molecular recognition)
- This interaction also helps the rupture of the N-C bond of the peptide linkage
- Arg-145 in CPB also functions similarly. Other amino acid residues like Asn-144, Tyr-248 and a chiral the hydrophobic pocket play crucial role for recognition and holding the at suitable place



(e) Generation of carbocationic character by the joint action of Zn(II) and Arg-127

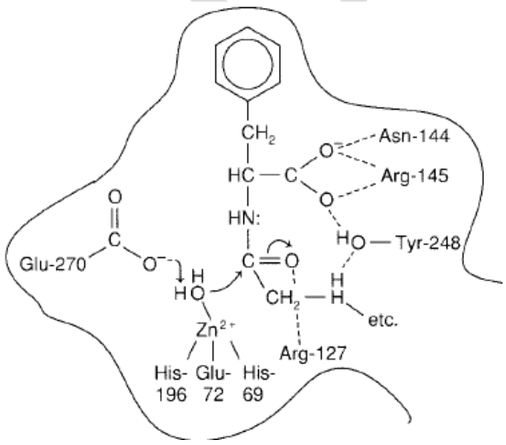
- ❖ Zn(II) of the enzyme can bind with the carbonyl oxygen as a ligand site with the concomitant change in the coordinating behavior of Glu-72 around the kinetically labile Zn(II) center
- ❖ It will maintain the constancy in coordination number and stereochemistry around Zn(II)
- ❖ The Lewis acid character of Zn(II) (enhanced by weak π-acid character of imidazole group) will polarize the C=O bond to develop a carbocationic character on the C-centre
- ❖ Besides this, the H-bonding interaction between the Arg-127 residue and the peptide carbonyl O-atom will also facilitate the carbocationic character.
- ❖ These interactions of the carbonyl O-atom with Zn(II) and Arg-127 residues are the main factor to generate the carbocationic character to welcome nucleophilic attack



(f) Firm holding of the substrate at the active site in CPA

- ❖ The involved non-covalent supramolecular interactions are

(a) Coordination by the carbonyl-O of the peptide bond to Zn(II)
(b) H-bonding interaction of Arg-127 with the carbonyl-O of the peptide linkage
(c) Electrostatic interaction between the carboxylate group of C-terminal amino acid residue and the cationic guanidinium moiety of Arg-145 (substrate specificity)
(d) The H-bonding interaction between the carboxylate group of C-terminal amino acid residues and Asn-144 residue (substate holding)
(e) The mobile Tyr-248 can participate in H-bonding interaction with the docked substrate
(f) Chiral hydrophobic moiety can accommodate the (aromatic or aliphatic) non-polar side chain of the terminal amino acid residue (substrate specificity and holding)



(g) Role of Glu-270 to generate potential nucleophile (OH-group bound to Zn)

- ❖ Nucleophilic attack on the peptide bond is known as the push effect of hydrolytic cleavage
- ❖ The carboxylate group of Glu-270 may function in different possible ways.

(a) The carboxylate group of Gly-270 can itself act as a good nucleophile to attack the carbonyl carbon center of the peptide bond giving acid anhydride
(b) Glu-270 may hold the nucleophile (H_2O) in the right position through H-bonding to attack the target carbonyl carbon
(c) Glu-270 may interact with the H_2O molecule coordinated to Zn(II). Proton abstraction (base catalysis) by carboxylate group of Glu-270 lowers the pKa of H_2O coordinated to Zn(II).
(d) Besides this, the electron withdrawal from Zn(II) due to π-acid character of imidazole moieties also lower the pKa of the same.
(e) Both interactions occur jointly to lower the pKa of coordinated water to Zn(II) around 7.0
(f) However, lowering of pKa through proton abstraction by Glu-270 is the most crucial event of the enzymatic activity of CPA

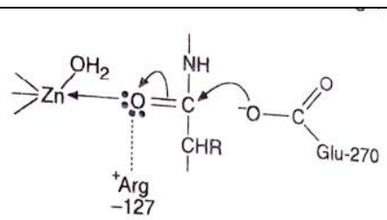
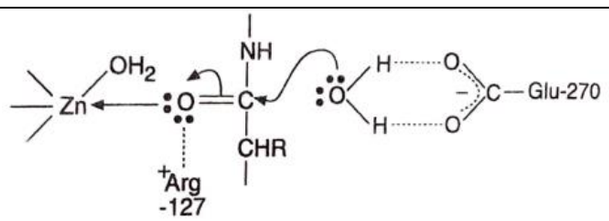
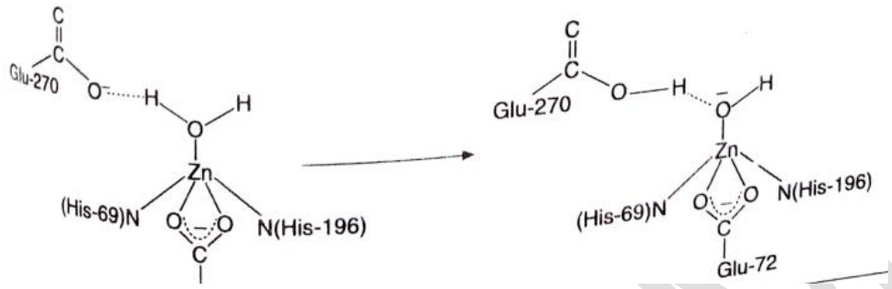


Illustration of the possibility of nucleophilic attack by the carboxylate group of Glu-270 to produce an acid anhydride



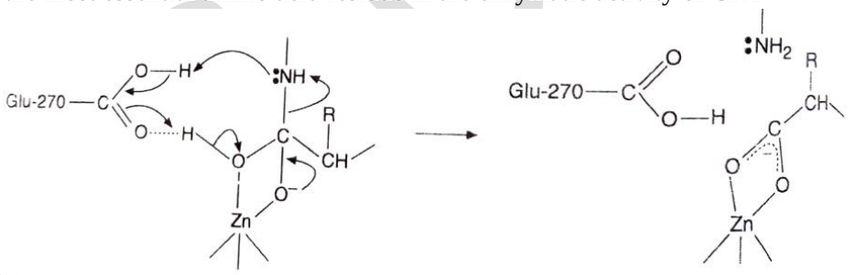
Correct positioning of the nucleophile (H2O) through H-bonding with Glu-270



Generation of the potential nucleophile (metal bound OH) through the base catalysis of carboxylate group of Glu-270 and favored by π-acidic histidine

(h) Protolysis of the leaving group and role of Glu-270

- Protolysis of the leaving –NH group favors its departure (pull effect)
- The Phenolic-OH group of Tyr-248 is possibly involved in protolysis of leaving group (X-ray structural analysis)
- Site directed mutagenesis studies indicated that replacement of Tyr-248 by Phe-248 does not alter the reactivity significantly
- Thus, alternatively it is proposed that protonated Glu-270 participates in protolysis (proton donation to leaving group)
- Glu-270, the most essential amino acid residue in the enzymatic activity of CPA



Protolysis of the leaving group by Glu-270 in the enzymatic activity of CPA

(i) L-Benzylsuccinic acid as potential reversible inhibitor for CPA

- The Arg-145 residue and the Zn(II) center are essentially important for substrate binding
- The phenyl group of the inhibitor is accommodated int the hydrophobic chiral pocket near the active site.
- The anionic carboxylate moiety interacts with the cationic side chain of Arg-145 and another carboxylate group coordinates the vacant coordination site of Zn(II)
- Thus, the inhibitor binds at the active site of CPA buy it cannot be hydrolyzed because it does not have any peptide linkage
- Thus, CPA being bound to L-benzylsuccinic acid becomes inactive

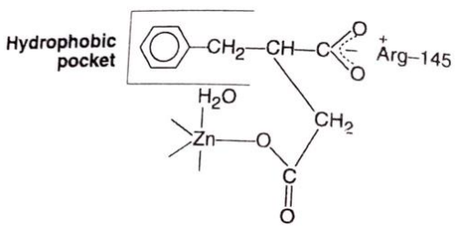
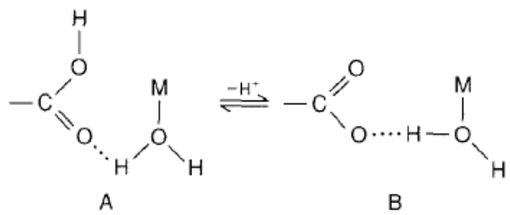


Illustration of blocking of the active site of CPA by inhibitor L-benzylsuccinic acid

(j) Kinetics:

- k_{cat}/K_m pH profiles are bell-shaped, characterized by an acid pKa limb around 6 and an alkaline pKa limb around 9: k_{cat} increases with the pKa of 6 and then levels off, and K_m increases with a pKa of 9.

- The pKa = 6 corresponds to the ionization of the Glu-270-coordinated H_2O moiety:



- Tyr-248 favors substrate binding three to five times more than the mutagenized Phe-248 derivative.
- The three possible candidates for this pKa are the coordinated water, Tyr-248, and the metal-coordinated His-196, whose ring NH is not H-bonded to any protein residue.
- The X-ray data at different pH values show a shortening of the Zn-O bond upon increasing pH. This favors the ZnOH hypothesis

(k) Anion binding:

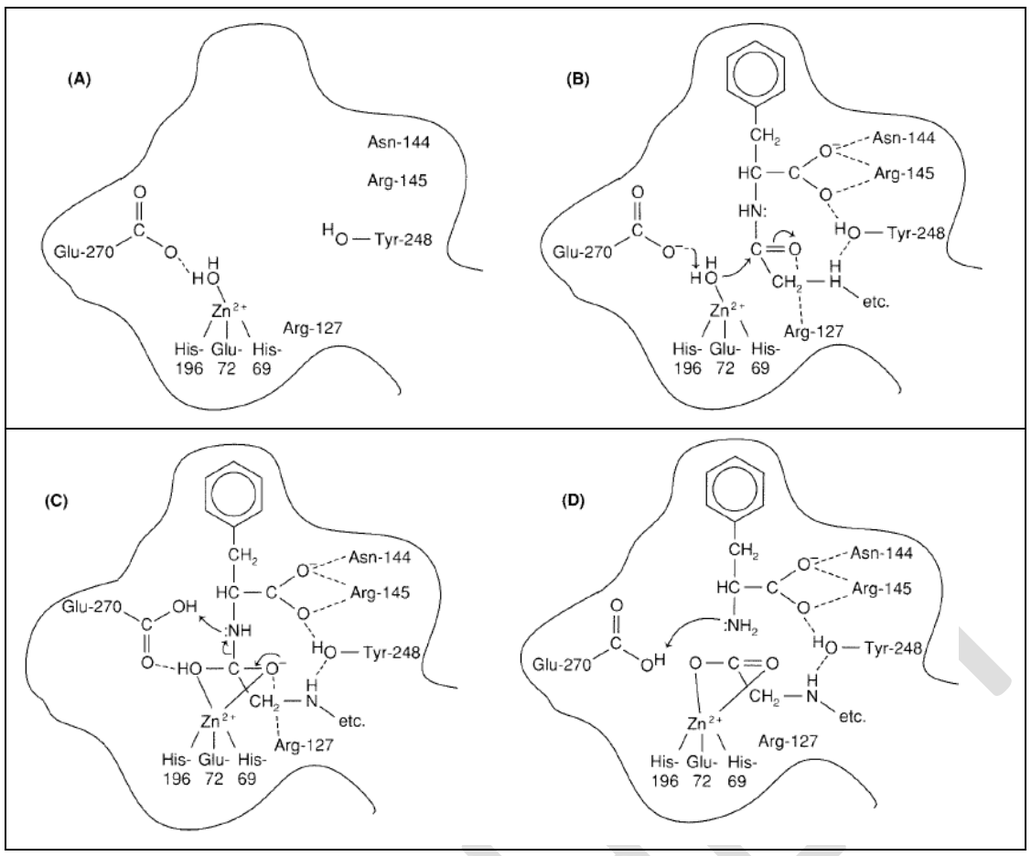
- The metal binds anionic ligands only below pH 6, i.e., when Glu-270 is protonated, when Glu-270 is chemically or genetically modified, or when aromatic amino acids or related molecules are bound in the C-terminal binding domain (Arg-145 + hydrophobic pocket)

(l) Intermediates:

- An anhydride intermediate involving Glu-270 for a slowly hydrolyzed substrate may have been identified.
- Some other intermediates have been observed spectroscopically at subzero temperatures with the Co(II) derivative.
- Peptides bind in a fast step without altering the spectroscopic properties of Co(II), following which a metal adduct forms and accumulates.
- Thus, if an anhydride intermediate is formed, it is further along the catalytic path.

Mechanism (Christianson and Lipscomb)

- The incoming peptide interacts with Arg-145 residues through its C-terminal carboxylate group via electrostatic and H-bonding interaction.
- The peptide would smoothly slide to its final docking position at Arg-145, while the R residue (hydrophobic) moves to the hydrophobic pocket (B).
- The carbonyl oxygen forms a strong H-bond with Arg-127. Additional stabilization comes from H-bonding of Tyr-248 to the penultimate peptide NH (possibly the first intermediate).
- At this point the metal-bound hydroxide (assisted by Glu-270) performs a nucleophilic attack on the carbonyl carbon activated by Arg-127 and further possibly by an electrostatic interaction of the carbonyl oxygen with the metal ion.
- Carbonyl binds in bidentate fashion. Five coordination is maintained by switching the Glu-72 metal ligand from bidentate to monodentate, because the metal moves toward Arg-127 (C) (possibly the second intermediate).
- The system then evolves toward breaking of the C-N bond, caused by addition of a H^+ (from Glu-270) to the amino nitrogen, thereby returns to the ionized state
- The breaking of the peptide bond could be the rate-limiting step.
- The second H^+ required to transform the amino nitrogen into an NH_3^+ group could come from the coordinated carboxylic group of the substrate bearing one excess H^+, again through Glu-270 (D). It is a ternary complex with a carboxylate ligand and an amino-acid zwitterion, bound synergistically (D).
- Finally, the metal moves back to regain a bidentate Glu-72 ligand, and the cleaved peptide leaves, while a further water molecule adds to the metal ion and shares its proton with the free carboxylate group of Glu-270.
- After hydrolysis, the cleaved amino acid still interacts with Arg-145 and the hydrophobic pocket, whereas the amino group interacts with Glu-270.
- The carboxylate group of the new terminal amino acid interacts with Zn
- This mechanism underlines the role of the Zn-OH moiety in performing the nucleophilic attack (as in CA)



Possible catalytic cycle of CPA.

Oxygenase

- The oxygenase enzymes catalyze reactions of dioxygen with organic substrates in which oxygen atoms from dioxygen are incorporated into the final oxidized product.
- These can be divided into two categories viz. dioxygenases and monooxygenases
- Dioxygenase directs both atoms of oxygen into the product
- Monooxygenases direct one atom of oxygen from dioxygen into the product and the other is reduced to water

$$\text{Dioxygenase: substrate} + {}^*O_2 \longrightarrow \text{substrate}({}^*O)_2$$

$$\text{Monooxygenase: substrate} + {}^*O_2 + 2H^+ + 2e^- \longrightarrow \text{substrate}({}^*O) + H_2{}^*O$$

- Dioxygenase enzymes are known that contain heme Fe, nonheme Fe, Cu, or Mn
- The substrates whose oxygenations are catalyzed by these enzymes are very diverse, as are the metal-binding sites (no detailed mechanism is not available)
- However, some of the intradiol catechol dioxygenases isolated from bacterial sources have structural and mechanistic information available

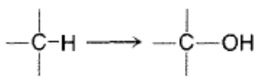
Monooxygenases

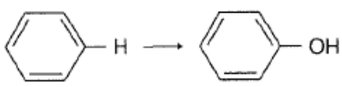
- Metal-containing monooxygenase enzymes are known that contain heme Fe, nonheme Fe, or Cu at their active sites.
- For most of these enzymes, there is only limited information about the nature of the active site and the mode of interaction with dioxygen or substrates.
- There are three monooxygenase enzymes that strongly resemble well-characterized reversible dioxygen-carrying proteins.
- This suggests that dioxygen binding to the metalloenzyme in its reduced state is an essential first step in the enzymatic mechanisms, presumably followed by other steps that result in oxygenation of the substrate.
- **Cytochrome P-450**, a heme-containing protein whose active site resembles the dioxygen-binding sites of Mb or Hb in many respects, except that the axial ligand to iron is a thiolate side chain from cysteine rather than an imidazole side chain from histidine
- **Tyrosinase**, which contains two copper ions in the vicinity of its active site and has deoxy, oxy, and met states that closely resemble comparable states of hemocyanin in their spectroscopic properties; and
- **Methane monooxygenase**, which contains two nonheme iron ions in proximity, and which resembles hemerythrin in many of its spectroscopic properties.

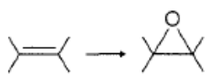
- In addition to these three, there are also monooxygenase enzymes containing single nonheme Fe or Cu ions, or nonheme Fe plus an organic cofactor such as a reduced pterin at their active sites.
- The enzyme for which the most information available is cytochrome P-450

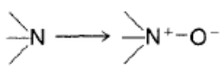
Cytochrome P-450

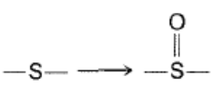
- Cytochrome P-450 enzymes (source: plants, animals, and bacteria) are a group of monooxygenase enzymes that oxygenate a wide variety of substrates
- Hydroxylation of aliphatic compounds (Reaction 5.59);
- Hydroxylation of aromatic rings (Reaction 5.60);
- Epoxidation of olefins (Reaction 5.61);
- Amine oxidation to amine oxides (Reaction 5.62);
- Sulphide oxidation to sulfoxides (Reaction 5.63); and
- Oxidative dealkylation of heteroatoms (for example, Reaction 5.64).
- Some of these reactions have great physiological significance, because they represent key transformations in metabolism (lipid metabolism and biosynthesis of corticosteroids)
- Cytochrome P-450 is also known to catalyze the transformation of certain precarcinogens (benzpyrene) into their carcinogenic forms


(5.59)


(5.60)


(5.61)

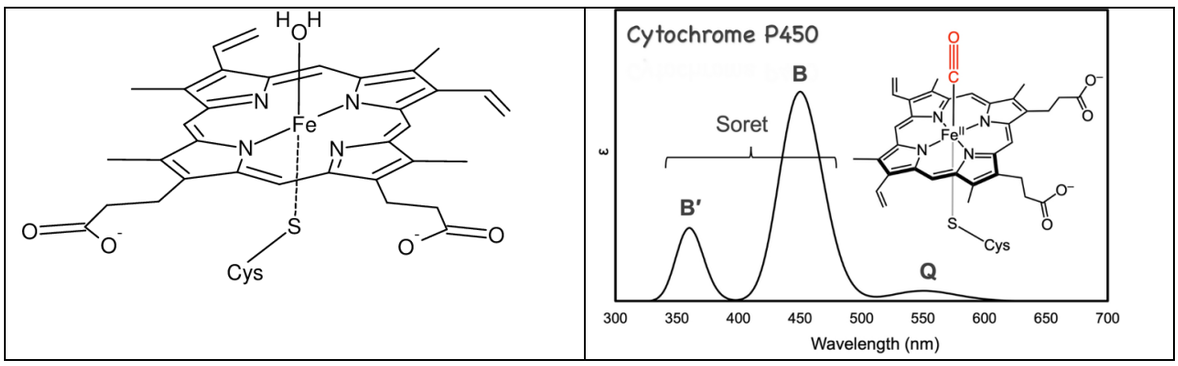

(5.62)


(5.63)

(5.64)

Cytochrome P-450 – Structure

- P-450 enzymes are difficult to characterize, because they are membrane-bound and so are relatively insoluble in aqueous solution.
- Cytochrome $P\text{-}450_{cam}$ (a component of the camphor 5-monooxygenase system isolated from the bacterium *Pseudomonas putida*), is soluble and, is well characterized (spectral and X-ray crystallography), and mechanistic studies are also performed.
- The enzyme consists of a single polypeptide chain, mainly α-helical, with a heme *b* group (Fe-protoporphyrin IX) sandwiched in between two helices, with no covalent attachments between the porphyrin ring and the protein.
- Fifth coordination site (axial) is occupied by a thiolate side chain of cysteine residues
- In the resting state, the Fe is predominantly low-spin Fe(III), probably with a water as the other axial ligand.
- When substrate binds to the resting enzyme, the spin state changes to high-spin Fe(III), and the non-cysteine axial ligand (water) is displaced.
- The enzyme can be reduced to a high spin Fe(II) state (like deoxy Hb or Mb in spectral properties) and in this state it binds to O_2 giving a diamagnetic oxy complex (similar to oxymyoglobin) or carbon monoxide to make a carbonyl form.
- The CO derivative has a Soret band (high-energy π- π^* transition of the porphyrin ring, attributed to axial thiolate ligand) at 450 nm. This spectroscopic feature aids in the isolation of the enzyme and is responsible for its name (P - Fe protoporphyrin IX, 450 - Soret band)

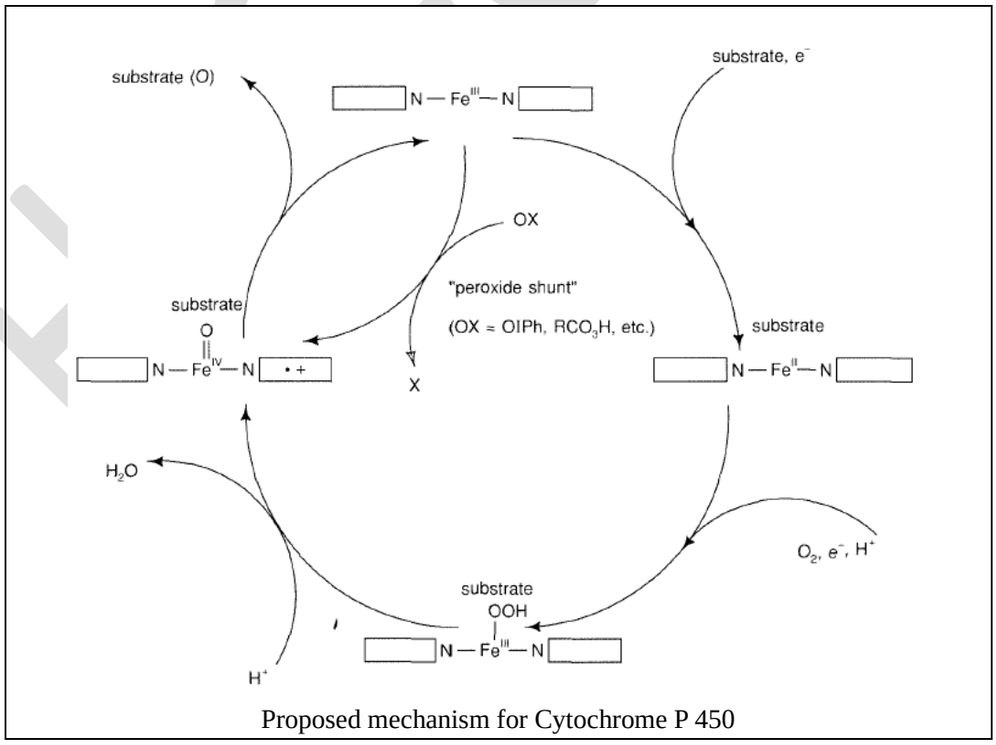


- "Active Oxygen"- Camphor 5-monooxygenase is a three-component system, consisting of cytochrome P-450_{cam}, two electron-transfer proteins, a flavoprotein, and an Fe-S protein.
- The role of the electron-transfer proteins is to deliver electrons to the P-450 enzyme (in vitro, flavoprotein may be replaced by other reducing agents (shown in Figure)

Proposed mechanism for Cytochrome P 450

Part I:

- Substrate binds to the enzyme at a position close to the iron center, but it is not directly coordinated to the metal ion.
- The enzyme-substrate complex is then reduced to the ferrous form.
- Dioxygen then binds to form an oxy complex (not shown). The oxy complex is then reduced by another electron and protonated, giving a ferric-hydroperoxy complex shown at the bottom of the cycle. The ligand bound here to the FeIII center is HO_2- (deprotonated H_2O_2).
- The ferric hydroperoxy form of the enzyme-substrate complex then undergoes heterolytic O-O bond cleavage, giving a high-valent Fe IV oxo center, with the porphyrin ligand oxidized by one equivalent.
- This species then transfers a neutral oxygen atom to the bound substrate, which is then released, giving the oxygenated product and regenerating the resting form of the enzyme.
- The "peroxide shunt" refers to the mechanism proposed for the cytochrome P 450-catalyzed oxygenation of substrates by single-oxygen-atom donors.
- It is believed that the same high-valent iron-oxo intermediate is generated in these types of reactions as well



Proposed mechanism for Cytochrome P 450

Part II

Active oxygen

- For understanding the mechanism for cytochrome P-450, one must have clear idea about the activation of dioxygen and its reaction with substrate.

- Dioxygen binds to the Fe(II) state of the enzyme-substrate complex, and the resulting oxy ligand is not sufficiently reactive to attack the bound substrate.
- The oxy form is then reduced and the active oxidant is generated (the nature of the active oxidant is not known)
- Three species are potential candidates for "active oxygen," the oxygen-containing species that attacks thesubstrate, in cytochrome P-450.

1. a ferric peroxo (la) or hydroperoxo complex (1b) is formed from one electron reduction of the oxy complex (Reaction 5.65);
2. an iron(IV) oxo complex (2) is formed by homolytic O-O bond cleavage of a ferric hydroperoxo complex (Reaction 5.66); and
3. a complex at the oxidation level of an iron(V) oxo complex (3) is formed by heterolytic O-O bond cleavage of a ferric hydroperoxo complex (Reaction 5.67).

$$Fe^{II}P + O_2 \longrightarrow FePO_2 \xrightarrow{e^-} \underset{\textbf{1a}}{[Fe^{III}P(O_2^{2-})]^-} \xrightarrow{H^+} \underset{\textbf{1b}}{Fe^{III}P(O_2H^-)} \qquad (5.65)$$

$$\underset{\textbf{1b}}{Fe^{III}P(O_2H^-)} \longrightarrow \underset{\textbf{2}}{Fe^{IV}P(O)} + HO\cdot \qquad (5.66)$$

$$\underset{\textbf{1b}}{Fe^{III}P(O_2H^-)} \longrightarrow \underset{\textbf{3}}{[Fe^{V}(P^{2-})(O)^+ \longleftrightarrow Fe^{IV}(P^-)(O)^+]} + HO^- \qquad (5.67)$$

(P^{2-} = porphyrin ligand; P^- = one-electron oxidized porphyrin ligand)

- The highly reactive hydroxyl radical (HO·), although capable of attacking P-450 substrates is an unlikely candidate for "active oxygen" (because of poor selectivity).
- An iron(V) oxo complex (or a related species at same oxidation state) (3) is formed via Reaction (5.67) and is favorable for "active oxygen" in cytochrome P-450 (evident from the reactions of the enzyme with alkylhydroperoxides and single-oxygen-atom donors)
- Ferric cytochrome P-450 can catalyze oxygenation reactions using organic peroxides or single-oxygen-atom donors in place of dioxygen and reducing agents.
- Usually, the same substrates would give the identical oxygenated product. This reaction pathway was referred to as the "peroxide shunt" (see Figure).
- This is believed to be responsible for generation of “active oxygen” i.e., O-O bond cleavage.
- The mechanism suggested for this reaction was Reactions (5.68) and (5.69).

$$Fe(III)P^+ + OX \longrightarrow \mathbf{3} + X \qquad (5.68)$$

$$\mathbf{3} + \text{substrate} \longrightarrow Fe(III)P^+ + \text{substrate(O)} \qquad (5.69)$$

Metalloporphyrin Model Systems

- The chemistry and biology of cytochrome P450 model compounds have a rich history.
- Many intermediates have been identified or proposed to exist in the catalytic cycles proposed by studying cytochrome P450 biomimetic (model) chemistry.
- These include (1) ferric-peroxo (Fe(III)-O_2^{2-}) complexes, (2) oxoiron(IV)porphyrin π- cation radical, (3) oxidant-iron(III) porphyrin adducts, (4) oxoiron(V)porphyrins (isoelectronic with oxoiron(IV) porphyrin π-cation radicals), and (5) oxoiron(IV) porphyrins
- It is desirable to mimic the enzymatic oxygenation reactions of substrates using iron porphyrins, dioxygen, and reducing agents
- But iron-porphyrin-catalyzed reactions were unable to produce results that can be corelated to the P-450 mechanism (because enzyme system has to provide electrons into the iron-dioxygen-substrate complex, generating the active oxidant within the active site which is in the vicinity of the bund substrate, without these restrictions, iron porphyrins oxidize the reducing agent without effecting the substrate)
- Formation of oxoiron(IV) porphyrin π-cation radicals requires a two - electron oxidation from the Fe(III) porphyrin resting state, whereas the oxoiron(IV) porphyrins require a one electron oxidation.
- Oxoiron(IV) porphyrin π-cation radicals have been proposed as the sole oxidant in P450 chemistry, whereas other researchers have proposed multiple oxidizing species in these oxygen transfer reactions

A Cytochrome P450 Model Compound: Functional

- The first article on the use of a synthetic iron(III) porphyrin complex is [Fe(TPP)Cl]; (TPP = tetraphenyl porphyrin)
- This complex, in the presence of the oxidant iodosylbenzene, PhIO, used as a source of oxygen atoms, was found to catalyze alkene epoxidation and alkane hydroxylation reactions.

- Groves enunciated the "oxygen rebound" mechanism to explain the reactions.
- In the hydroxylation mechanism, the oxoiron(IV) porphyrin π-cation radical intermediate, $[Fe^{IV}=O(TPP^{+\bullet})]^{+}$, forms in the presence of PhIO, and it is this intermediate that abstracts a hydrogen atom from the substrate (R-H) to form the "caged" carbon radical and $[Fe^{IV}=OH(TPP)]$.
- The cage ensures that the intermediates do not diffuse away from each other. The two species then recombine in the rebound step to produce the hydroxylated product and the resting state of the Fe(III)-porphyrin complex (Figure 7.19).

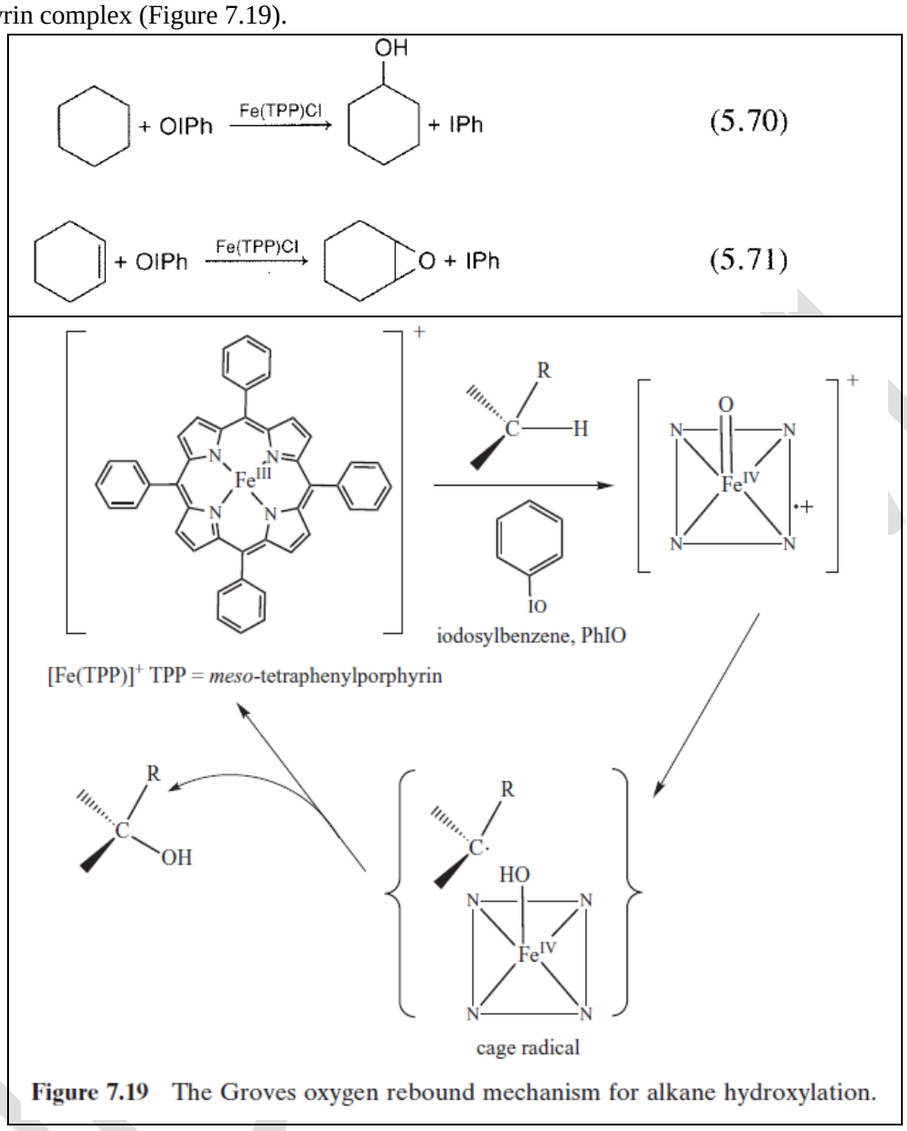
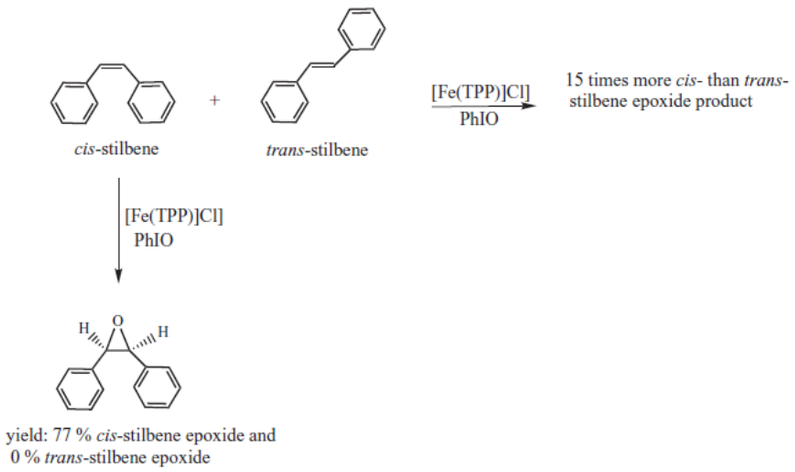
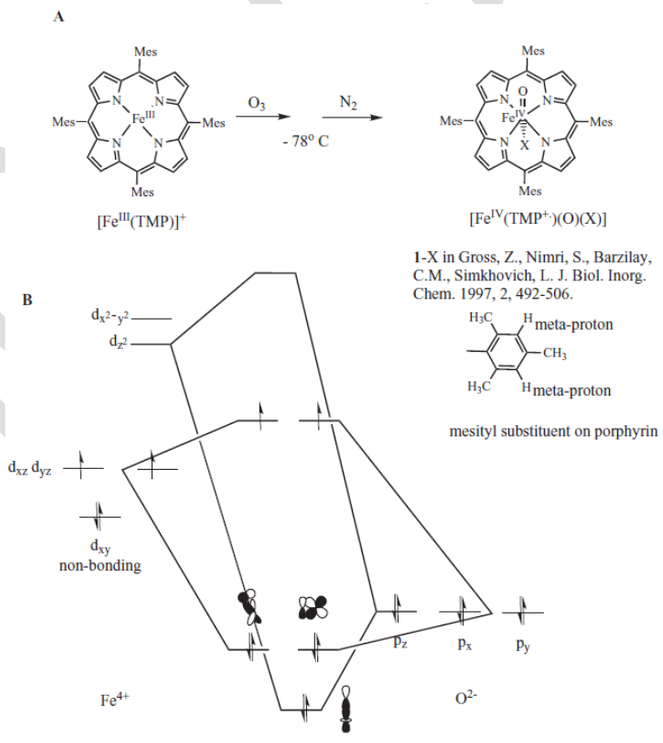


Figure 7.19 The Groves oxygen rebound mechanism for alkane hydroxylation.

- The oxygen rebound mechanism was supported by experimental evidence including (1) high kinetic isotope effects, (2) partial positional or stereochemical scrambling, and (3) allylic rearrangements.
- For instance, in the presence of [Fe(TPP)Cl] and PhIO, *cis* - stilbene was stereospecifically epoxidized. In addition, it was found that *cis* - stilbene was 15 times more reactive than *trans* - stilbene in competitive epoxidations (Figure)
- *cis*-stilbene preference to steric interference between the phenyl groups of *trans*-stilbene and the phenyl groups on the $[Fe^{IV}\text{-}O(TPP^{+\bullet})]^{+}$ intermediate



- The Groves research group also gathered the first spectroscopic indications of a synthetic, compound I analog.
- The $[Fe^{IV}=O(TMP^{+\bullet})]^{+}$ complex (TMP equals the dianion of *meso*-tetramesitylphenylporphyrin) was prepared by the *m*-CPBA (3-chloroperoxybenzoic acid) oxidation of $[Fe^{III}$-O(TMP)Cl]. (Figure)
- The $[Fe^{IV}=O(TMP^{+\bullet})]^{+}$ complex exhibited a characteristic bright green color and corresponding visible absorbance in its UV-Vis spectrum.
- In its NMR spectrum, the meta - proton doublet of the porphyrin mesityl groups were shifted more than 70 ppm downfield from tetramethyl silane (TMS) because they were in the presence of the cation radical, while the methyl protons shift between 10 and 20 ppm downfield.
- In Mossbauer spectroscopy, the isomer shift, δ_{Fe} = 0.06 mm/s and ΔE_Q = 1.62 mm/s, and were similar to those for other known Fe (IV) complexes.
- Electron paramagnetic resonance (EPR), resonance Raman (RR), and EXAFS spectroscopies provided additional indications of an Fe^{IV}=O π- cation radical intermediate.



one 2c-2e σ-bond (p_z-d_{z^2}) and two 2c-3e π-bonds (p_x-d_{xz}, p_y-d_{yz}), bond order = 2

Figure 7.21 (A) Formation of the $[Fe^{IV}=O(TMP^{+\bullet})]^{+}$ complex using the TMP ligand. (B) Molecular orbital diagram describing bonding in the $[Fe^{IV}=O(TMP^{+\bullet})]^{+}$ complex.

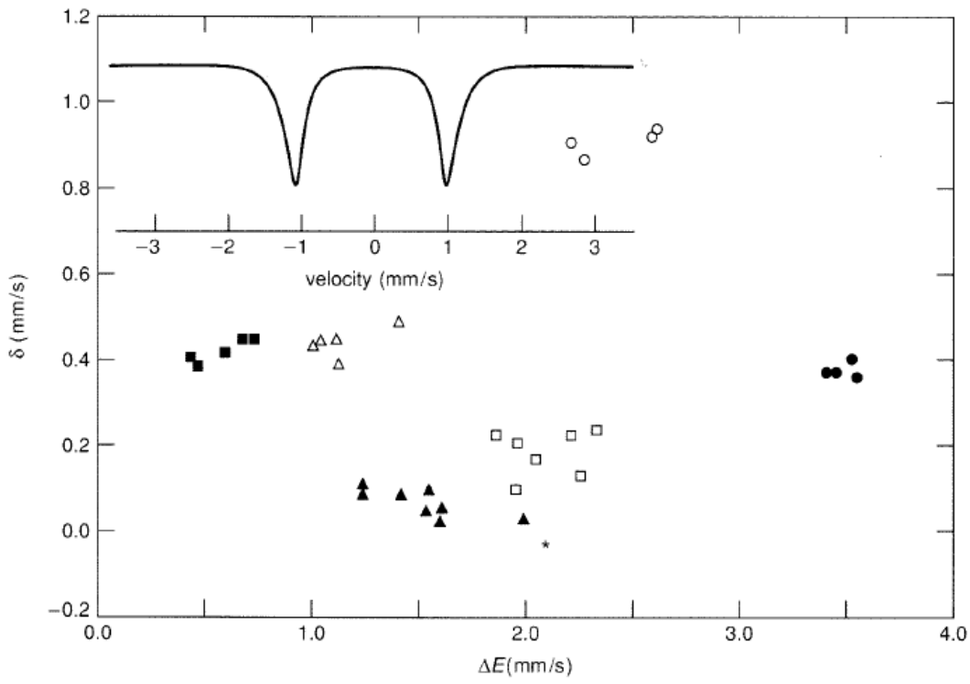
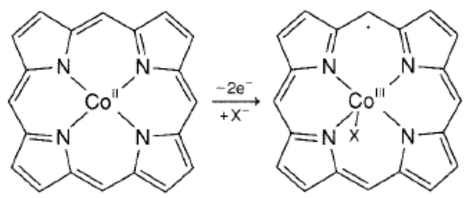


Figure 5.11
Comparative Mössbauer data for iron-porphyrin complexes: ■ high-spin Fe^{III}, □ low-spin Fe^{III}, ● intermediate-spin (admixed) Fe^{III}, ○ high-spin Fe^{II}, △ low-spin Fe^{II}, ▲ oxo- and imido-Fe^{IV}, * dimethoxyiron(IV)TMP. Insert shows the zero-field Mössbauer spectrum of ^{57}Fe-dimethoxyiron(IV)TMP at 4.2 K. (From Reference 83.)

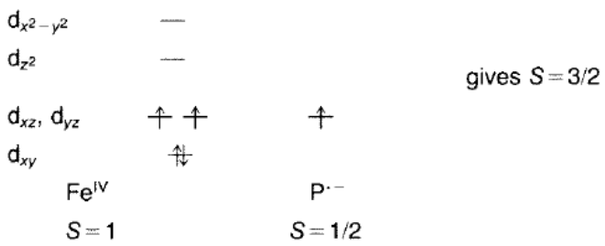
- For instance, EXAFS data indicated an Fe=O bond length of 1.65 ± 0.05 Å, similar to that found for compound I intermediates in several enzymes such as catalases, horseradish peroxidase, cytochrome c peroxidase, and chloroperoxidases
- This short Fe-O distance is consistent with the formulation of the complex as a "ferryl" complex, i.e., Fe^{IV}=O.
- In such a complex, the oxo ligand, O_2^-, is bonded to the Fe (IV) center by a combination of σ and π bonding, the latter because of overlap of the filled ligand p-orbitals with the partially filled d_{xz} and d_{yz} orbitals of the metal.
- Studies by other researchers showed that substituents on the porphyrin ligand affected formation of the $[Fe^{IV}=O(P^{+\cdot})]^+$ radical. Electron deficient iron(III) porphyrin complexes were found to be better catalysts in hydrocarbon oxygenation reactions.
- Spectroscopic evidence for the $[Fe^{IV}=O(TMP^{+\cdot})X]$ moiety included that from NMR, EPR, and RR.
- The EPR spectra of frozen solutions complexes at 15 K showed a characteristic S = 3/2 spectrum with g_z = 2, g_y = 3.5 – 3.7, and g_x = 4.2 – 4.4. The spin state of 3/2 arises from the two unpaired electrons for a Fe^{4+}–O^{2-} bond plus the unpaired electron from the porphyrin π - cation radical
- The NMR data indicated that the axial ligand, X, does affect the shifts of the mesityl and pyrrole protons.
- Resonance Raman (RR) data also show differences for the $\nu_{Fe=O}$ stretching frequency, depending on the axial ligand. For X = F^- and Cl^-, $\nu_{Fe=O}$ = 801–806 cm^{-1}, whereas for CH_3OH and ClO_4- $\nu_{Fe=O}$ = 831 –835 cm^{-1}
- Visible absorption spectra of porphyrin complexes are due largely to π-π* transitions of the porphyrin ligand.
- The bright green color is unusual for iron porphyrin complexes, are usually red or purple (Oxoiron(V) porphyrins).
- The unusually long-wavelength visible absorption bands that account for the green color result from the fact that the porphyrin ring has been oxidized by one electron.
- Similar visible absorption bands can be seen, for example, in other oxidized porphyrin complexes, such as $Co^{III}(P^{\cdot-})^+$, formed by two-electron oxidation of $Co^{II}(P^{2-})$ (Fig 5.73)
- Oxidized porphyrin ligands also give characteristic proton NMR spectra, which are seen for the green porphyrin complex as well



(5.73)

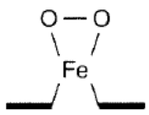
$Co^{II}(P^{2-})$ $Co^{II}(P'^{-})X]^{+}$

- Magnetic measurements indicate that the green porphyrin complex contains three unpaired electrons.
- Detailed analysis of the Mossbauer spectra has indicated that the two unpaired electrons on the Fe(IV) ion are strongly ferromagnetically coupled to the unpaired electron on the porphyrin, accounting for the resulting S = 3/2 state.



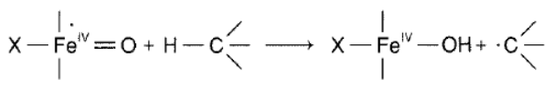
(5.74)

- Studies of the reactions of this species with P-450-type substrates demonstrate that this species is reactive enough to make it an attractive candidate for" active oxygen" in the enzymatic mechanism
- Synthetic analogues for two of the other candidates for "active oxygen" have also been synthesized and their reactivities assessed.
- For example, Fe^{III} and Mn^{III}-porphyrin peroxo complexes analogous to **1a** in Reaction (5.65) have been synthesized.
- The X-ray crystal structure of the Mn complex shows that the peroxo ligand is bound to the metal in a triangular, side-on fashion (see 5.75).
- The Fe complex is believed to have a similar structure.

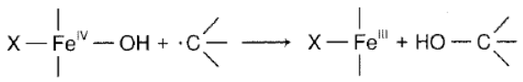


(5.75)

- Studies of this species indicate that **1a** in Reaction (5.65) would not have the requisite reactivity to be a candidate for "active oxygen" in the cytochrome P450 mechanism.
- These reactivity studies, and the observation of the peroxide shunt described above, indicate that $Fe^{V}(P^{2-})(O)^{+}$ or $Fe^{IV}(P^{-})(O)^{+}$ is the most likely candidate for "active oxygen."
- These two formulations are, of course, isoelectronic, and it is tempting to conclude that the latter is the more likely formulation of the enzymatic intermediate.
- However, it is important to remember that the model systems lack the axial cysteinyl ligand present in cytochrome P-450.
- The effect of the relatively easily oxidized sulfur ligand on the electron distribution within that intermediate is not known, since model systems for high-valent iron-oxo complexes containing axial thiolate ligands have not been synthesized.
- The mechanism of reactions of the high-valent oxo complex 3 in Reaction (5.67) with a variety of substrates is an area of active interest.
- Such studies are generally carried out by generation of the species *in situ* from the reaction of a ferric porphyrin with a single-oxygen-atom donor, such as a peracid or iodosylbenzene.
- In hydroxylation reactions of aliphatic hydrocarbons, the initial step appears to be abstraction of a hydrogen atom from the substrate to form a substrate radical and an Fe^{IV} hydroxide complex held together in a cage created by the enzyme active site so that they cannot diffuse away from each other (Reaction 5.76).
- This step is then followed by recombination of the OH fragment with the substrate radical to make the hydroxylated product (Reaction 5.77). This mechanism is referred to as the "oxygen rebound" mechanism

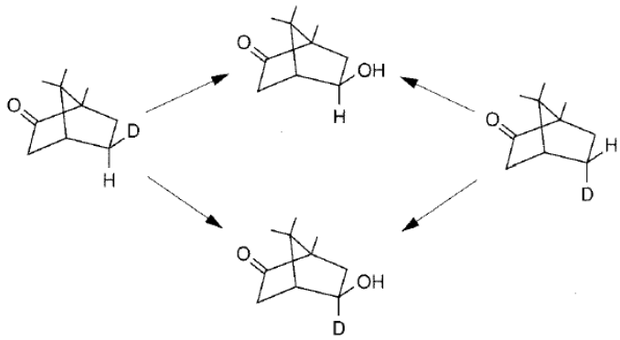


(5.76)



(5.77)

- The radical character of the intermediates formed in this reaction is supported by the observation that such reactions carried out using synthetic porphyrins and single-oxygen-atom donors in the presence of BrCCh give substantial amounts of alkyl bromides as products, a result that is consistent with radical intermediates
- In the enzymatic reactions, there is also strong evidence to support a stepwise mechanism involving free-radical intermediates.
- Example, cytochrome P-450$_{cam}$ gives hydroxylation of d-camphor only in the *5-exo* position, but deuterium-labeling studies show that either the *5-exo* or the *5-endo* hydrogen is lost (Reaction 5.78)



(5.78)

- The crystal structure of reduced cytochrome P-450$_{cam}$ with CO bound to the iron and the substrate camphor bound adjacent to it has been examined and compared with the crystal structure of the oxidized enzyme with camphor bound.
- The former is expected to be similar in structure to the less-stable oxy complex.
- The comparison shows that the substrate camphor is closer to the iron center in the oxidized enzyme.
- It is therefore possible that a similar movement of the substrate occurs during the catalytic reaction after either a *5-exo* or a *5-endo* hydrogen is abstracted, and that the new position of the camphor molecule then restricts the hydroxylation step to the *5-exo* position.
- It is interesting to note that the *5-exo* position on the camphor that is hydroxylated is held in very close proximity to the Fe(III) center, and therefore to the presumed location of the oxo ligand in the high-valent oxo intermediate in the structure of the ferric enzyme plus camphor derivative (Figure 5.12).
- Mechanisms for olefin epoxidations catalyzed either by the enzyme or by model porphyrin complexes are not as well understood as those for hydroxylation of aliphatic hydrocarbons. (Represented schematically).

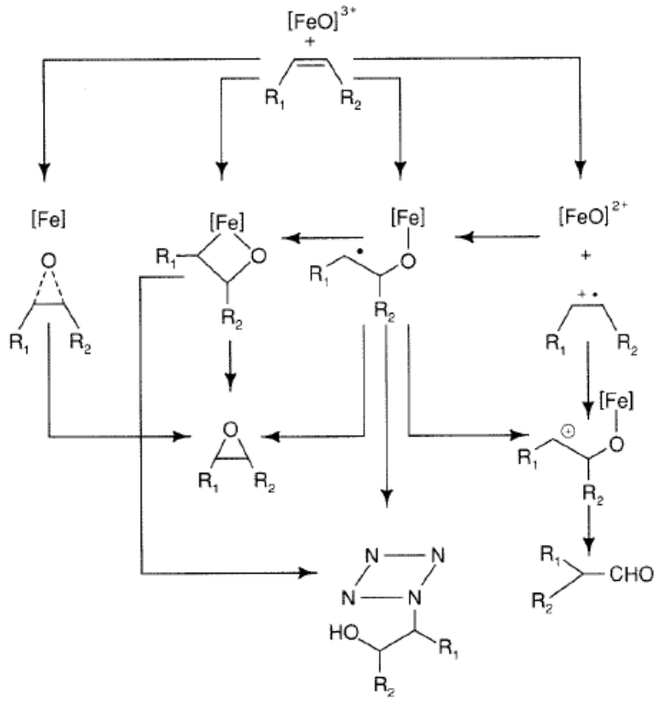


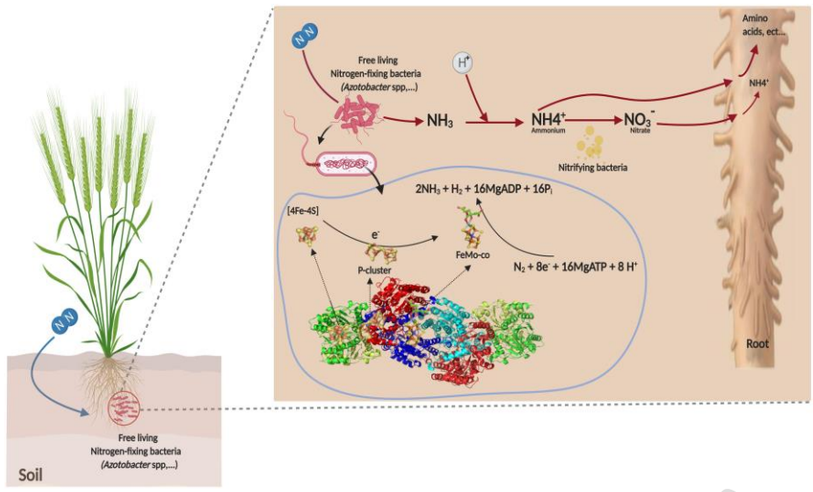
Figure 5.13
Schematic representation of possible mechanistic pathways for olefin epoxidation by **3**. The mechanisms described are, from right to left, concerted addition of oxygen to the double bond, reaction via a metallocylic intermediate, reaction via a ring-opened radical intermediate, and reaction proceeding via an initial electron-transfer step.[91]

c. O-O Bond Cleavage:

- The evidence is persuasive that the "active oxygen" species that attacks substrate in cytochrome P-450 is a high-valent iron-oxo complex.
- However, the mechanism of formation of that species in the catalytic reaction with dioxygen is less well-understood.
- Heterolytic O-O bond cleavage of a ferric porphyrin hydroperoxide complex, 1b (Reaction 5.67), is the logical and anticipated route, but it has not yet been unequivocally demonstrated in a model complex.
- The catalase and peroxidase enzymes catalyse heterolytic O-O bond cleavage in reactions of hydrogen peroxide, but in them the active sites contain amino-acid side chains situated to facilitate the developing charge separation that occurs in heterolytic cleavage (see Section VI).
- The crystal structure of cytochrome P-450$_{cam}$ shows no such groups in the active site cavity, nor does it give any clue to the source of a proton to protonate the peroxide ligand when it is produced.
- Also, we have little experimental evidence concerning possible roles that the cysteinyl sulfur axial ligand might play in facilitating O-O bond cleavage. These issues remain areas of active interest for researchers interested in cytochrome P-450 mechanisms

Nitrogen Fixation

- Nitrogen fixation (conversion of atmospheric N_2 to other N-containing species) is a key reaction of the biological nitrogen cycle
- Fixed nitrogen is essential for the synthesis of amino acids and nucleic acid.
- Nature cannot provide adequate amount of fixed nitrogen for agriculture/animal husbandry.
- Industrial (like Haber's Bosch) processes have been developed to fix nitrogen chemically.



Haber-Bosch Process

- Dinitrogen is almost chemically inert (bond energy ~ 945 kJmol^{-1}) and activation energy is prohibitively high (kinetically unfavourable).
- Breaking and reducing N-N triple bond (1.098 Å, $\nu_{N\equiv N}$ = 2331 cm^{-1}) is a challenging task.
- Requires about 300-500 ºC and 300 atmospheric pressures with Haber's catalyst, usually metallic Fe or its oxide (thermodynamically favourable) and Mo as the possible promotor.

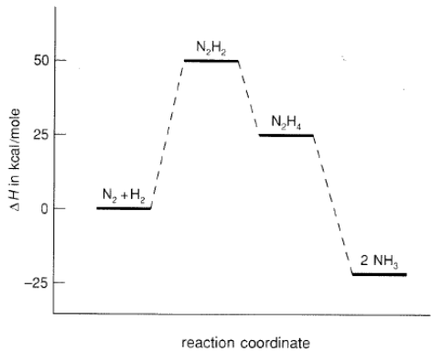
  $N_2 + 3H_2 \rightarrow 2NH_3$ ΔH = -50 kJmol^{-1}, ΔG = -16.6 kJmol^{-1}
- At pH=7, E^o value is easily accessible to biological reductant such as low potential ferredoxins.

  $N_2 + 8e^- + 6H^+ \rightarrow 2NH_4^+$ E^o = -280 mV
- In contrast, in the biological process, N_2 is reduced at room temperature and ~ 0.80 atm by the enzyme system called nitrogenase

Thermodynamic and Kinetic aspects of N_2-fixation

- N-N distance is 1.098 Å, $\nu_{N\equiv N}$ = 2331 cm^{-1}, and N-N bond energy is ~ 945 kJmol^{-1}.
- First IE of N_2 is 1503 kJmol^{-1}; Electron affinity is also high; thermodynamically unfavorable
- The HOMO is σ bonding (high IE; poor nucleophile) and LUMO is π-antibonding (reluctant to accept electrons; poor electrophile; few strong reducing agents can transfer electrons) in character
- The chemical inertness of N_2 towards its reduction in mainly due to kinetic barrier not due to the thermodynamic barrier (evident from the following equation)

  $N_2 + 3H_2 \rightarrow 2NH_3$ ΔH = -50 kJmol^{-1}, ΔG = -16.6 kJmol^{-1}



Energetics of N_2, NH_3, and some potential intermediates along the reaction pathway for their interconversion.

- The kinetic inertness of this thermodynamically favorable process can be understood by considering the steps involved in the 6e$^-$ reduction of N_2.

  $N_2 + 6e^- + 6H^+ \rightarrow 2NH_3$ E^o = -0.34 V (biological reductants such as Ferredoxins can reduce)
- A single step involving 6e$^-$, 6H$^+$ for direct reduction of N_2 to NH_3 is highly unlikely.
- The process is likely to pass through the formation of N_2H_2 (diimine, diazene, diimide) and N_2H_4 (hydrazine) intermediates (higher in energy than the reactants/products)

  $N_2 + 2H^+ + 2e^- \rightarrow N_2H_2$; E^o = -1.0 to -1.5 V

  $N_2 + 5H^+ + 4e^- \rightarrow N_2H_4$; E^o = -0.70 V

  E^o are sufficiently negative that the normal biological reductants cannot carry out the reduction on N_2 to these intermediates (the enthalpy of formation of these intermediates are thermodynamically unfavourable.

- To overcome the said barrier the intermediates may be stabilized through complexation at metal center. Alternatively, the formation of the unfavorable intermediates could be coupled with thermodynamically favored processes like ATP hydrolysis or the evolution of dihydrogen to make the overall process favorable.

Biological nitrogen fixation

- Nitrogen fixation with the help of microorganisms (nitrogen fixing bacteria).
- Two types - symbiotic and non-symbiotic
- **Non-symbiotic** - Fixation by free living organisms (aerobic, anaerobic and blue green algae)

  Free living aerobic: *Azotobacter vinelandii*
  Free living anaerobic: *Clostridium pasteurianum*
  Facultative aerobes: *Klebsiella pneumoniae*
  Free living photosynthetic: *Rhodobacter capsulatus*-purple,
  Blue green algae: *Anabaena cylindrica* (cyanobacterium)
- **Symbiotic** - Fixation by microorganisms in soil living symbiotically inside the plants.

  Nodule formation in leguminous plants: *Rhizobium* and *Bradyrhizobium*
  Nodule formation in non-leguminous plants: *Frankia*
  Non-nodulation: *Anabaena azollae*

Symbiotic Nitrogen Fixation

- Rhizobium (gram negative, aerobic) is present in nodules on the roots of legumes such as peas, beans, clover, alfalfa and soya (named after the host plant).
- Red colour inside the nodules is due to leghaemoglobin (a plant O_2-binding protein)
- The reaction takes place at 0.8 atmospheric pressure and ambient temperature.
- Intensive efforts have been made to determine the bacterial mechanism.
- The nitrogen fixing bacteria contains an enzyme called nitrogenase.
- Nitrogenase catalyses the reduction of N_2 to NH_3 in a reaction coupled to the hydrolysis of 16 ATP molecules and production of H_2.

  $N_2 + 16MgATP^{2-} + 8e^- + 8H^+ \rightarrow 2NH_3 + 16MgADP^- + 16H_2PO_4^- + H_2$
- Fe-Mo nitrogenase is mostly studied - reduction of N_2 probably occurs at Mo-site
- Fe-V nitrogenase - V plays (other metal can also) the role of Mo
- Fe-Fe nitrogenase - similar metals can also affect fixation

Biochemistry of nitrogen fixation

Basic requirements

- Nitrogenase and hydrogenase enzyme
- Protective mechanism against Oxygen (leghaemoglobin binds oxygen tightly and protects nitrogenase that cannot operate in presence of oxygen)
- Ferredoxin
- Hydrogen releasing systems or electron donor (pyruvic acid or glucose/sucrose
- Constant supply of ATP
- Coenzymes and cofactors TPP, CoA, inorganic phosphate and Mg^{2+}

Nitrogenase (molybdenum nitrogenase)

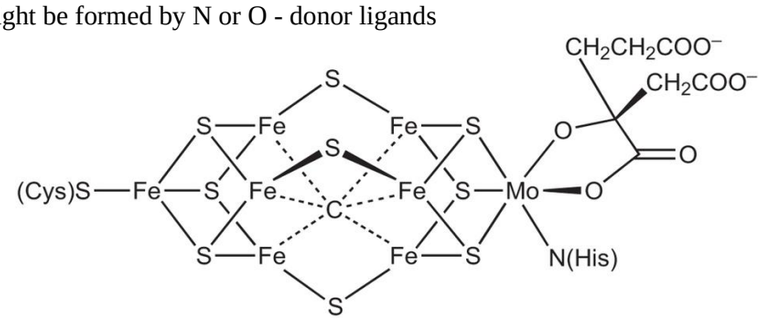
- Active in anaerobic condition
- The action of Mo-nitrogenase involves the functioning of two protein subunits (component I and II)
  [FeMo] protein: Dinitrogenase (larger)
  Non heme Fe-protein: (dinitrogen reductase, smaller), acts as a redox centre.
- Dinitrogenase is also called molybdoferredoxin, azofermo, or component I
- Dinitrogenase reductase is also called azoferredoxin or component II
- Fe-protein reacts with ATP and reduces second subunit which ultimately reduces N_2 to NH_3,

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

FeMo-protein – Structure

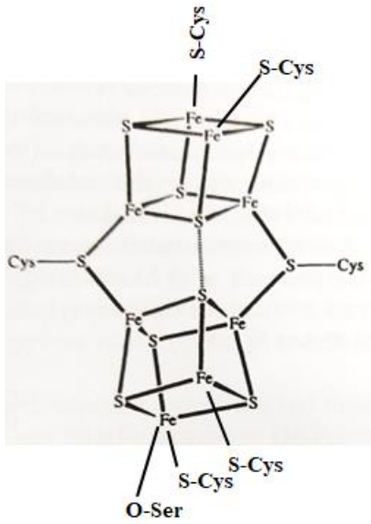
- FeMo-protein has a $\alpha_2\beta_2$ tetrameric structure with MW around 230 kD.
- *α unit is* coded by *nif* D and *β* unit is coded by *nif* K genes.
- In addition to protein, a total of 2 Mo, 30 Fe & 30 labile S^{2-} are also present
- It consists of cuboidal $[Fe_4S_4]$ and $[Fe_3MoS_3]$ fragments linked by two sulphide bridges
- The cluster is bound to the protein via a cysteine at Fe and a histidine at the Mo.
- The coordination sphere of the six coordinated Mo (probably in IV OS) is completed by the homocitrate anion.
- Two Fe-Mo cofactors are located at about 70 Å apart.

- A third bridge might be formed by N or O - donor ligands



Fe-protein- Structure

- P-cluster is the smaller protein with MW around 57-73 kD
- It contains a pair of $[Fe_4S_4]$ units linked by two bridging cysteine thiolate group
- A disulphide bridge formed between S atoms of each $[Fe_4S_4]$ cubane cluster.
- EPR studies suggests its oxidized form to be high spin with S=7/2 and Mossbauer spectra reveals inequivalent Fe-population indicating $[Fe_4S_4]$ cluster to be distorted or asymmetric.
- *In vivo*, the Fe protein is reducible by flavodoxin or ferredoxin
- The single Fe_4S_4 center undergoes a single one-electron redox process, wherein the reduced form is EPR-active and the oxidized form is diamagnetic.



- Redox potential of Fe-protein is dependent on the ATP or ADP level in the solution.
- For example, the Fe-protein from *Clostridium pasteurianum* shows E^o = -294 mV in the absence and -400 mV in the presence of MgATP.
- Two equivalents of MgATP and MgADP each bind to [Fe].
- The single Fe_4S_4 center can exist in two spin states, S = 1/2 (earlier consideration in EPR) and S = 3/2 (the EPR spin integration shows one paramagnetic site per Fe_4S_4 unit)

Properties of some representative nitrogenases.

Organism	Component	MW	Metal content
Azotobacter vinelandii	[MoFe]	234,000	2Mo, 34–38Fe, 26–28S
	[Fe]	64,000	3.4Fe, 2.8S
Azotobacter chrococcum	[MoFe]	227,000	2Mo, 22Fe, 20S
	[Fe]	65,400	4Fe, 3.9S
Clostridium pasteurianum	[MoFe]	221,800	2Mo, 24Fe, 24S
	[Fe]	55,000	4Fe, 4S
Klebsiella pneumoniae	[MoFe]	229,000	2Mo, 32Fe, 24S
	[Fe]	66,800	4Fe, 3.8S
Anabaena cylindrica	[MoFe]	223,000	2Mo, 20Fe, 20S
	[Fe]	60,000	
Rhodospirillum rubrum	[MoFe]	215,000	2Mo, 25–30Fe, 19–22S
	[Fe]	60,000	

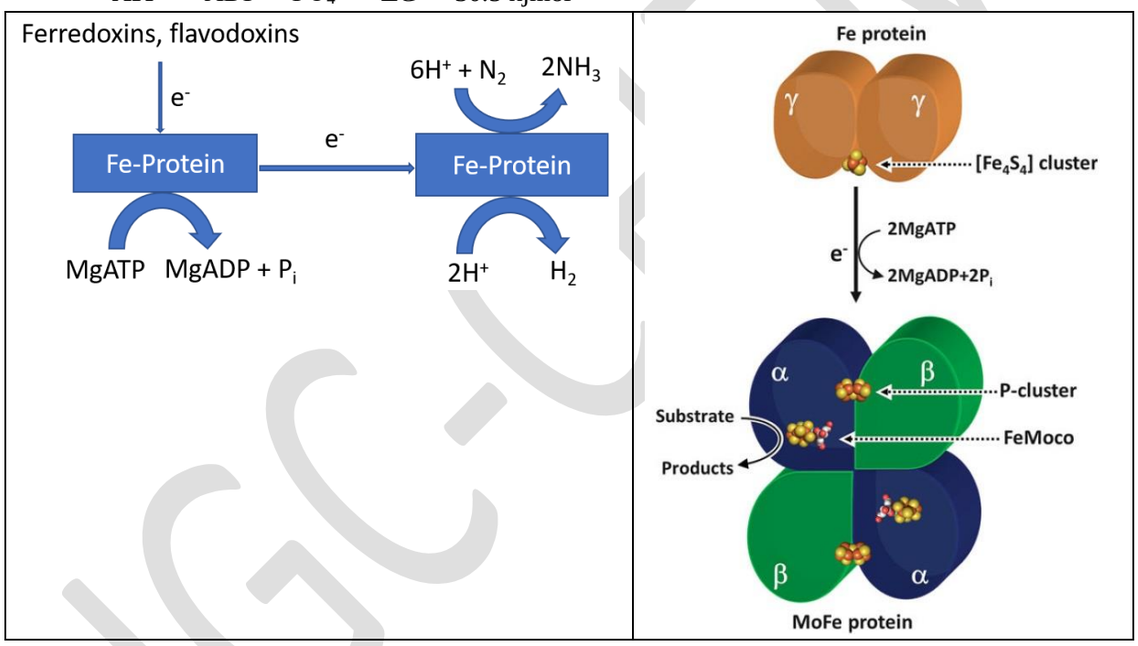
Dominant hypothesis for molybdenum nitrogenase

- The action of Mo-nitrogenase involves the functioning of two protein subunits viz. [FeMo] protein and [Fe] protein
- During enzyme turnover, the single Fe_4S_4 of the Fe-protein center transfers electrons to the FeMo protein in one-electron steps.
- The Fe protein binds two molecules of MgATP and, the cleft between the two subunits may serve as the ATP binding site.
- Two molecules of MgATP are hydrolyzed to MgADP and inorganic phosphate in conjunction with the transfer of each electron to the FeMo protein.
- Electrons required for the reaction are transferred to nitrogenase by reduced form of ferredoxins and flavodoxins.
- The source of these electrons is the oxidation of pyruvate (2-oxo-propionate).
- Fe-Mo-protein then transfer electrons to N_2 in a series of steps accompanied by H^+ transfer from H_2O to N_2 to produces NH_3, Mg-ADP and PO_4^{3-}
- Dominant Hypothesis designates [FeMo] as the protein responsible for substrate reduction
- Fe-Mo protein contains the site of N_2 binding (Fe-Mo cofactor) where the N_2 is reduced.
- P-cluster is believed to assist the reduction of N_2 by transfer of electrons.
- Overall reaction,

$$N_2 + 16MgATP^{2-} + 8e^- + 8H^+ \rightarrow 2NH_3 + 16MgADP^- + 16\ PO_4^{3-} + H_2$$

- The energy released from hydrolysis of ATP drives the reaction.

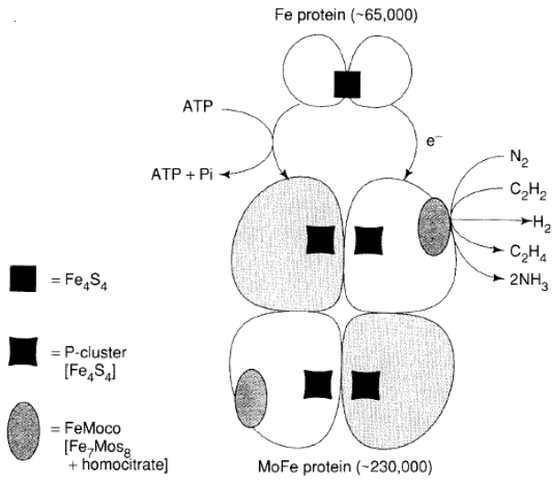
$$ATP \rightarrow ADP + PO_4^{3-} \quad \Delta G = -30.5\ kjmol^{-1}$$



Some important points

- FeMo protein can only be reduced to a catalytically active form in presence of Fe protein.
- Redox behavior between the S = 1/2 and S = 3/2 states of the protein is similar during enzyme turnover
- Even though [Fe] must be present for catalysis to take place, the Dominant Hypothesis designates [FeMo] as the protein immediately responsible for substrate reduction, and genetic/biochemical evidence supports this view.
- The ATP/2e- ratio should be minimum (4). Higher the value lower is the efficiency, can be attributed to "futile cycling," where transfer of electron back from [FeMo] to [Fe] raises the effective ratio.
- FeMo protein are designated P clusters and FeMoco (or M) centers
- Recombination of the cofactor with inactive nitrogenase restores the activity.

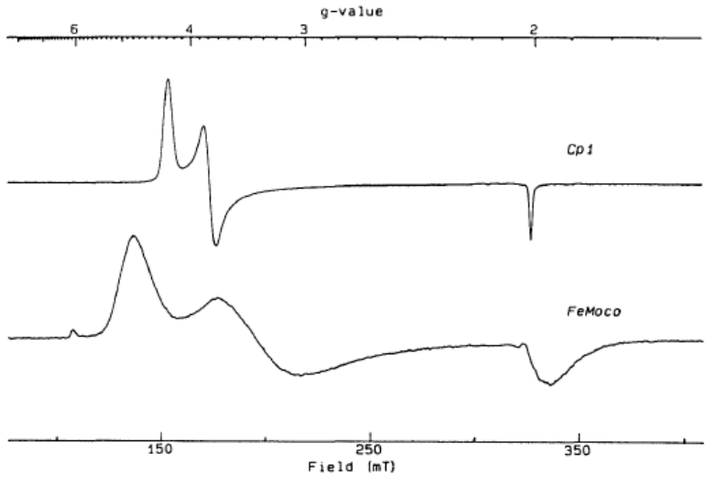
$$N_2 + 16MgATP^{2-} + 8e^- + 8H^+ \rightarrow 2NH_3 + 16MgADP^- + 16\ PO_4^{3-} + H_2$$



Schematic display of the nitrogenase proteins illustrating their mode of action according to Dominant Hypothesis

FeMoco (or M) centre

- Protein free cofactor is soluble and contains Mo and Fe (1 Mo, 6-8 Fe and 7-10 S^{2-} ions)
- The presence of the FeMo cofactor within the FeMo protein of nitrogenase, i.e., the M center, is revealed through spectroscopic and redox studies
- In the resting state of [FeMo], as isolated in the presence of dithionite, the M center has a distinct S = 3/2 EPR signal (see Figure)
- When the enzyme is turning over the EPR signal essentially disappears, leaving an EPR-silent state in which the FeMoco site is super-reduced to what is presumed to be its catalytically active form.
- In addition, a third state in which the S = 3/2 EPR signal disappears is produced upon oxidation under non-turnover conditions.
- Thus the M center within the protein shows three states of oxidation, and these appear to have been reproduced in the FeMoco extracted from the protein

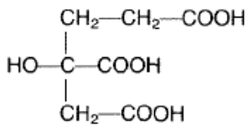


EPR spectra: (A) the $S = \frac{3}{2}$ M center in *Clostridium pasteurianum* nitrogenase FeMo protein; and (B) the FeMoco extracted into NMF from the protein.

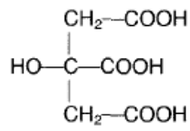
$$\text{FeMoco (oxidized)} \xrightarrow{e^-} \text{FeMoco (reduced)} \xrightarrow{e^-} \text{FeMoco (super-reduced)}$$

- Characterization of the FeMoco site has involved parallel studies of the site within the protein and in its extracted form.
- FeMoco was extracted (1976) from [FeMo] into N-methylformamide after the protein was acidified and then neutralized
- The acidification removes most of the acid-labile P clusters, and partially denatures the protein.
- Re-neutralization precipitates the protein (near its isoelectric point) and the precipitated denatured protein can then be extracted from organic solvents with suitable combinations of cations and anions.
- The role of the cation is to balance the charge of the negatively charged cofactor.
- The role of the anion is to displace the cofactor from anion-exchange columns (DEAE cellulose/ TEAE cellulose), to which the cofactor and/or its protein source had been adsorbed.

- The biochemical authenticity of FeMoco has been assayed by its ability to activate the FeMo protein from the cofactor-less mutant organism.
- The stoichiometry of the cofactor is $MoFe_{6-8}S_{7-10}$.
- The extracted cofactor resembles the M-center unit spectroscopically and structurally
- The differences are presumed to result from differences in the peripheral ligands of the metal-sulfide center between the protein and the organic solvent
- Strong evidence to support FeMoco as the site of substrate binding and reduction comes from the study of *nif* V (not vanadium) mutants
- FeMoco site is an important part of the substrate reactions of the nitrogenase enzyme complex
- Homocitrate (or a part of it) is a component of the cofactor center.
- Replacement of homocitrate by analogues that differ in structure or stereochemistry yields modified FeMoco sites that have altered substrate specificities.



Homocitrate



Citrate

- Both inorganic and organic components are present in FeMoco (like other cofactors).
- The biosynthesis of the cofactor and its insertion into [FeMo] apparently requires the presence of [Fe] and ATP in *A. vinelandii.*
- [Fe].MgATP binds to [FeMo], producing a state that is conformationally accessible for cofactor insertion.
- Cysteine residues are involved in binding FeMoco to the subunits of [FeMo].
- Moreover, these studies again implicate FeMoco in the substrate-reducing site.

The P-clusters

- Presence of four Fe_4S_4-like clusters in [FeMo] is designated as P-clusters.
- P-clusters are evident in electronic absorption and, especially, MCD and Mossbauer spectra of [FeMo]
- These spectra are different from those found in ferredoxins and in model compounds.
- In their oxidized forms, the P-clusters are high-spin, probably S = 7/2 according to EPR studies.
- Mossbauer spectra reveal decidedly inequivalent Fe populations, indicating that the putative Fe_4S_4 clusters are highly distorted or asymmetric
- The four P-clusters do not appear to behave identically, at least form two subsets.
- Furthermore, an additional Mossbauer signal sometimes designated as S may also be part of the P-cluster signal.
- However, the structural information of P-clusters available from spectral studies are not unequivocally (indicate the presence of three or four Fe_4S_4 clusters)
- Experimental result supports the Dominant Hypothesis, which designates the P centers as highly unusual Fe_4S_4 clusters (very close to each other).
- The P-clusters are thought to be involved in electron storage and transfer, and presumably provide a reservoir of low-potential electrons to be used by the M center (FeMoco) in substrate reduction (no direct evidence)

Comparison of the FeMo protein and isolated FeMoco.[a]

	FeMo protein (M center)	FeMoco (in NMF)	
EPR			
g' values	4.27	4.8	
	3.79	3.3	
	2.01	2.0	
EXAFS[a]			
Mo-S	2.36 (4)[b]	2.37 (3.1)[c]	
Mo-Fe	2.69 (3)[b]	2.70 (2.6)[c]	
Mo-O or N	2.18 (1)[b]	2.10 (3.1)[c]	
Fe-S		2.25 (3.4)[d]	2.20 (3.0)[e]
Fe-Fe		2.66 (2.3)[d]	2.64 (2.2)[e]
			3.68 (0.8)[e]
Fe-Mo		2.76 (0.4)[d]	2.70 (0.8)[e]
Fe-O or N		1.81 (1.2)[d]	
XANES			
MoO_3S_3 fits best[f]			

Spectroscopy techniques

- ❖ Nitrogenases (the enzymes responsible for nitrogen fixation), can be studied using various spectroscopic techniques.

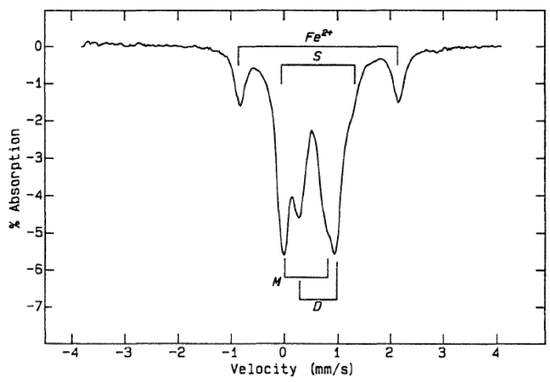
1. X-ray Crystallography
2. Electron paramagnetic resonance (EPR) spectroscopy
3. Nuclear magnetic resonance (NMR) spectroscopy
4. Infrared (IR) spectroscopy
5. UV-visible spectroscopy
6. Mossbauer spectroscopy
7. Resonance Raman spectroscopy
8. Fluorescence spectroscopy

EPR, ENDOR, and ESEEM studies

- ❖ The FeMoco or M center has been identified spectroscopically within the FeMo protein.
- ❖ It has a distinctive EPR signal with effective *g* values of 4.3, 3.7, and 2.01, and originates from an S = 3/2 state of the M center.
- ❖ The signal arises from transitions within the $\pm$ 1/2 ground-state Kramers doublet of the S = 3/2 system
- ❖ The isolated cofactor (FeMoco) gives a similar EPR signal (larger)
- ❖ Spectra from the C. *pasteurianum* nitrogenase and cofactor are shown above figure and comparative data are given in above Table
- ❖ The M-center EPR signal has proved useful in characterizing the nature of the site, especially when more sophisticated magnetic resonance techniques, such as ENDOR or ESEEM, are used.
- ❖ Extensive ENDOR investigations have been reported using protein samples enriched with the stable magnetic isotopes ^{2}H, ^{33}S, ^{57}Fe, ^{95}Mo, and ^{97}Mo.
- ❖ Individual hyperfine tensors of five coupled ^{57}Fe nuclei are apparent, and were evaluated (in details) by simulation of the polycrystalline ENDOR spectrum.
- ❖ ^{33}S gave a complex ENDOR spectrum, evidently with quite large hyperfine couplings
- ❖ ^{95}Mo was shown to possess a small hyperfine coupling, indicating that the Mo possesses very little spin density
- ❖ ENDOR studies have reported no nitrogen splitting.
- ❖ ESEEM (^{14}N modulations are observed in the ESEEM of the M center) spectroscopy has confirmed the involvement of nitrogen as a cluster component
- ❖ The observed ^{14}N is not from the substrate (N_2), or from an intermediate or product of nitrogen fixation, because enzyme turnover using ^{15}N as a substrate does not change the ESEEM spectrum.
- ❖ The isolated cofactor (FeMoco) does not show the modulation frequencies observed for the M center in the protein.
- ❖ These experiments suggest that the M-center ^{14}N ESEEM arises from a nitrogen atom (from histidine residues of amino acid side chain) that is associated with the M center
- ❖ Recent evidence from site-directed mutagenesis of the *Azotobacter vinelandii* protein provides strong support for the presence of histidine (His 195 of α-subunit) ligation

Mossbauer studies

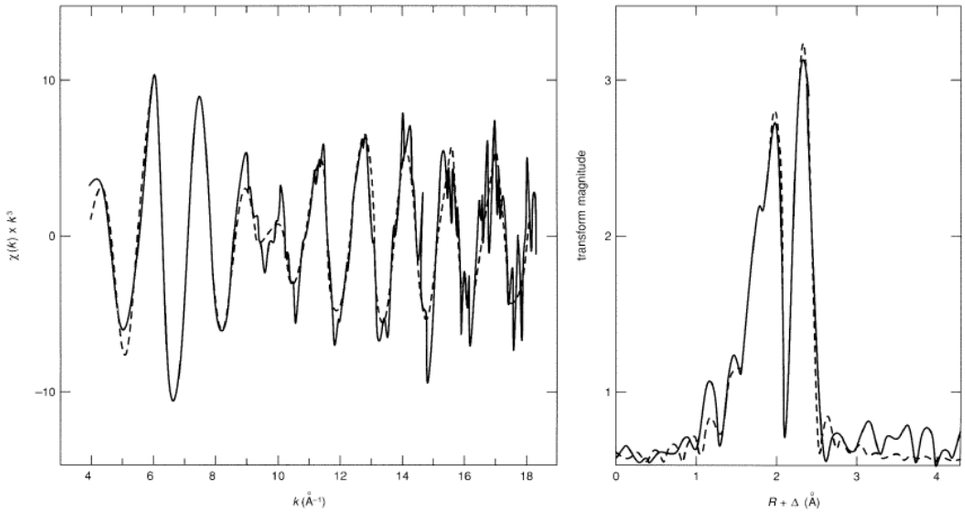
- ❖ Mossbauer studies have investigations of nitrogenase and FeMoco have been reported.
- ❖ Unlike EPR and EPR-based spectroscopies (used to investigate only the EPR-active S = 3/2 oxidation state), all three available M-center oxidation states are accessible to Mossbauer spectroscopy.
- ❖ The fully reduced site was found to be diamagnetic with S = 0 whereas the oxidized site was found to have $S \geq 1$.
- ❖ The zero-field spectrum of reduced C. *pasteurianum* nitrogenase is shown in Figure; the spectrum is comprised of four quadrupole doublets, one of which was concluded to originate from the M site.
- ❖ Mossbauer spectra taken in the presence of applied magnetic fields were used to deduce the presence of four types of ^{57}Fe hyperfine coupling; these were called sites A1, A2, and A3, which have negative hyperfine couplings, and B sites, which have positive hyperfine couplings.
- ❖ A and B sites were estimated to contain one Fe for each A and three Fe for B sites
- ❖ These conclusions were largely confirmed and extended by later ENDOR investigations, although the B sites were resolved as two inequivalent, rather than three equivalent sites.
- ❖ ENDOR is rather more sensitive to the nature of the hyperfine couplings than Mossbauer, although it cannot usually be used to count numbers of exactly equivalent sites.
- ❖ Thus, the number of iron atoms in the M center is minimally five (higher also possible)



Mössbauer spectrum of *C. pasteurianum* nitrogenase FeMo protein,[282] 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.

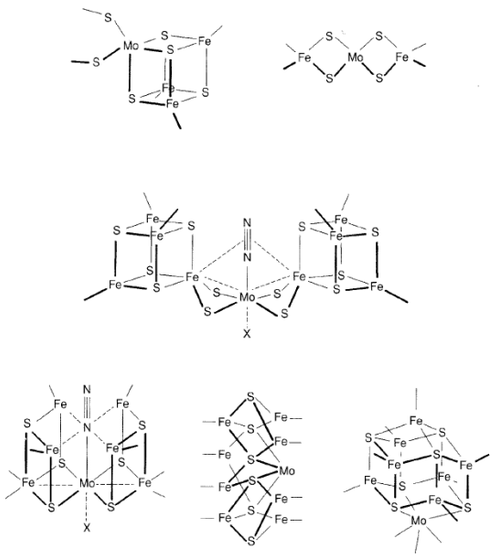
X-ray absorption studies

- X-ray absorption spectroscopy has indicated that the nitrogenase M center is an Mo-Fe-S cluster (the first *enzyme* to be studied by x-ray absorption spectroscopy)
- Early work on lyophilized protein samples indicated the presence of two major contributions to the Mo K-edge EXAFS, which were attributed to Mo-S ligands, plus a more distant Mo-Fe contribution.
- Most EXAFS studies to date have been on the molybdenum K-edge of the protein or of FeMoco, and indicate a very similar Mo environment in both (earlier Table, Figure)
- Best available analyses have indicated that Mo is coordinated by three or four sulfur atoms at 2.4 Å, one to three oxygens or nitrogens at 2.2 Å, with approximately three nearby iron atoms at 2.7 Å
- Although EXAFS studies indicated weak O/N contribution, comparison of Mo-K edge and Mo-L edge XANES spectra with model compounds indicates strong similarities with $MoFe_3S_4$ thiocubane model compounds possessing MoS_3O_3 coordination, and argued in favor contribution from O/N ligands.



Mo K-edge EXAFS spectrum (left panel) and EXAFS Fourier transform (right panel) of *Klebsiella pneumoniae* nitrogenase MoFe protein. The solid line is the processed experimental spectrum and the dashed line a calculated one.[287]

- The iron EXAFS of FeMoco has indicated that the iron is coordinated largely to sulfur at about 2.2 Å, with more distant Fe-Fe interactions at about 2.6 Å doubtful over the presence of short (l.8 Å) Fe-O interactions (possibly due to extraneous iron coordinated to solvent, contaminating the FeMoco preparation).
- Interestingly, a long Fe-Fe interaction at 3.7 Å was also observed in literature
- Largely based on the Mo K-edge EXAFS results several proposals for the structure of the M center have been put forwarded (Fig).
- The MoFe proteins from *Clostridium pasteurianum* has a crystal of space group $P2_1$, with two molecules per unit cell of dimensions 70 x 151 x 122 Å. A molecular two-fold axis presumably relates equivalent sites in the two $\alpha\beta$ dimers that make up the protein molecule.
- Preliminary refinement reveals that the two FeMoco units per protein are about 70 Å apart and the four P clusters are grouped in two pairs.
- Single crystal EXAFS studies provided useful structural information of the Mo site.
- For different crystal orientations (relative to polarized X-ray beam), the amplitude of the Mo-Fe EXAFS changes by a factor of 2.5 & that of Mo-S EXAFS changes slightly



Proposed models for FeMoco

- Analysis of an isotropy of the Mo-Fe EXAFS using the available crystallographic information is consistent with either a tetrahedral $MoFe_3$ geometry such as that found in thiocubane (Figure) or a square-based pyramidal $MoFe_4$ arrangement of metals.
- The observed orientation-dependence of the iron amplitudes is too small for clusters containing a linear or planar arrangement of iron and molybdenum (Figure B, C), and too large for arrangements that involve regular disposition of iron about molybdenum.
- Moreover, the lack of anisotropy of the sulfur EXAFS argues against an $MoS_3(O/N)_3$ model that has molybdenum coordinated by sulfur atoms that bridge only to Fe atoms disposed to one side of the molybdenum.
- Significant anisotropy for the Mo-S EXAFS (of opposite polarization, and smaller than that for Mo-Fe) would be expected for such an arrangement of sulfur atoms.
- However, the cubane model of Figure, which provides the best model of both geometric and electronic structure, remains viable if one of the nonbridging ligands to molybdenum is a sulfur atom (rather than oxygen or nitrogen) with a bond length similar to that of the bridging sulphides.

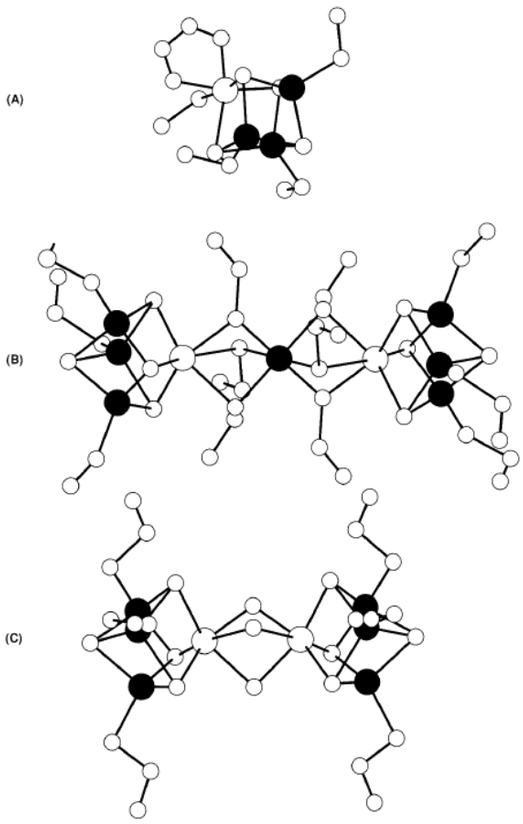


Figure 7.32
Structures of thiocubanes that display Mo-S and Mo-Fe distances similar to FeMoco: (A) $(Fe_3MoS_4)_2(SR)_9^{3-}$; (B) $(MoFe_3S_4)_2Fe(SR)_{12}^{3-/4-}$; (C) $MoFe_3S_4(SEt)_3(cat)CN^{3-}$.

Substrate reactions

- The two-component Mo-nitrogenase enzyme catalyzes the reduction of N_2 to $2NH_4+$ as its physiological reaction.
- Concomitant with the reduction of N_2, H_2 evolution occurs, with electrons supplied by the same reductants that reduce N_2
- The limiting stoichiometry appears to be
- If N_2 is omitted from the assay, all the electrons go to H_2 evolution.
- The rate of electron flow through nitrogenase is independent of whether the enzyme is producing only H_2, producing both NH_4+ and H_2, or reducing most of the alternative substrates.
- Many alternative substrates are known for this enzyme.
- The most important of these from a practical perspective is acetylene, C_2H_2, which is reduced by the Mo nitrogenase exclusively to ethylene, C_2H_4
- Acetylene can completely eliminate H_2 evolution by nitrogenase
- FeMo protein alone acts as an uptake hydrogenase.
- H_2 in the presence of [FeMo] causes the reduction of oxidizing dyes such as methylene blue or dichlorophenolindophenol in the absence of Fe protein (*only known catalytic reaction displayed by the FeMo protein alone*).
- The H_2 evolution and uptake by [FeMo] suggest that understanding H_2 interaction with transition-metal/sulfur centers may be crucial to understanding the mechanism of nitrogenase action.

Nitrogenase substrate reactions.

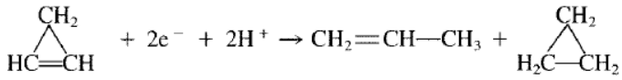
Two-electron reductions

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

$C_2H_2 + 2e^- + 2H^+ \rightarrow C_2H_4$

$N_3^- + 2e^- + 3H^+ \rightarrow NH_3 + N_2$

$N_2O + 2e^- + 2H^+ \rightarrow H_2O + N_2$



Four-electron reductions

$HCN + 4e^- + 4H^+ \rightarrow CH_3NH_2$

$RNC + 4e^- + 4H^+ \rightarrow RNHCH_3$

Six-electron reductions

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

$HCN + 6e^- + 6H^+ \rightarrow CH_4 + NH_3$

$HN_3 + 6e^- + 6H^+ \rightarrow NH_3 + N_2H_4$

$RNC + 6e^- + 6H^+ \rightarrow RNH_2 + CH_4$

$RCN + 6e^- + 6H^+ \rightarrow RCH_3 + NH_3$

$NCNH_2 + 6e^- + 6H^+ \rightarrow CH_3NH_2 + 2NH_3$

$NO_2^- + 6e^- + 6H^+ \rightarrow NH_3$

Multielectron reductions

$RNC \rightarrow (C_2H_6, C_3H_6, C_3H_8) + RNH_2$

$NCNH_2 + 8e^- + 8H^+ \rightarrow CH_4 + 2NH_3$

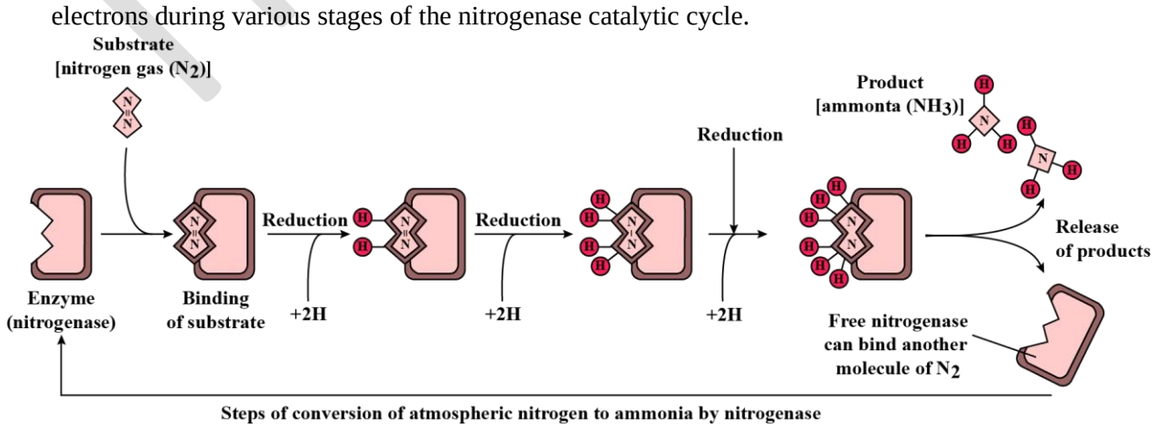
Role of ATP

- ATP hydrolysis appears to be mandatory, and occurs during electron transfer from [Fe] to [FeMo].
- Dissociation of [Fe] and [FeMo] following electron transfer is probably the rate-limiting step in the overall turnover of the enzyme.
- The thermodynamics of N_2 reduction to NH_3 by H_2 or by its redox counterpart flavodoxin or ferredoxin is favorable.
- The requirement for ATP hydrolysis must therefore arise from a kinetic necessity.
- This requirement is fundamentally different from the need for ATP in other biosynthetic or active transport processes, wherein the free energy of hydrolysis of ATP is needed to overcome a thermodynamic limitation.
- The reduction products of the N_2 molecule viz. N_2H_2 (diimide), N_2H_4 (hydrazine), and NH_3 (and its protonated form, NH_4^+) are isolable.
- In the presence of H_2, only the formation of ammonia is thermodynamically favored
- However, this does not mean that nitrogenase must form NH_3 directly without the formation of intermediates.

- It is possible for these reactive intermediates to be significantly stabilized by binding to a metal-sulfur center or centres.
- Detailed kinetic studies have suggested a scheme in which intermediates (diimide, hydrazine) with bound and probably reduced nitrogen are likely to be present.

Dinitrogen activation

- Dinitrogen activation refers to process of activating dinitrogen for use in chemical reactions
- The activation usually requires high temperature and pressure. Also presence of suitable catalyst is desirable. (Laboratory/industry)
- For free N_2, the N-N stretching frequency appears at 2331 cm^{-1}, while in the complexes with terminal N_2, the frequency shifts to 2200-1850 cm^{-1}.
- The shift is primarily due to the π-acceptor properties of the complexed N_2 (comparable to that of nitrile and less than CO).
- N_2 is also a very poor σ-donor due to the σ-bonding nature of HOMO
- The π-acceptor property of N_2 largely depends on the chemical state of the metal center (π-donor properties of the metal)
- Already bound ligand also influence the π-donor properties of the metal.
- A strong σ-donor property of already bound ligand favors M→N_2 π-back bonding while π-acceptor property of the same opposes it.
- The π-acceptance of N_2 indicates that in the complex M-N-N (like Ru), the N_2 moiety gains an additional electron density
- This makes the metal bound N_2, a better base than free N_2
- Because of enhanced basicity of the bound N_2, it is possible to protonate it.
- The N-N bond (multiple) length elongation (1.13Å from 1.10Å) and red shifting of the stretching frequency ν(N-N) indicate the lowering of bond order in N_2 due to complexation
- Thus, enhanced basicity and lowering of bond order support in favor of activation of N_2 to some extent via complexation.
- In bacteria, the activation of dinitrogen occurs readily in presence of nitrogenase.
- The process of nitrogen activation involves several intricate steps:
- **N_2-binding**: N_2 binds to FeMoco at the active site of nitrogenase, a challenging step in nitrogen fixation due to inertness of N≡N bond
- **Electron transfer**: Binding of N_2 induces the transfer of electrons to the FeMoco from electron releasing system such as ferredoxin or flavodoxin via Fe-protein
- **Reduction of N_2**: the electrons transferred to FeMoco are used to reduce N_2 to NH_3 via a series of complex reactions (exact mechanism is yet to be known)
- **Protonation and release**: H+ generated during the reduction is essential requirement for conversion of N_2 to NH_3. On completion of the reduction, NH_3 is released from the enzyme
- **ATP utilization**: the overall process requires energy in the form of ATP, used as driving force in various steps of N_2 reduction
- **Involvement of other metal centers**: Other than FeMoco, electron transport pathways involve complex series of reaction catalyzed by metalloenzymes. One of the key metal center involved is Fe-S cluster. It serves as electron carrier in the electron transfer pathways. It undergoes redox reaction shuttling the electrons during various stages of the nitrogenase catalytic cycle.



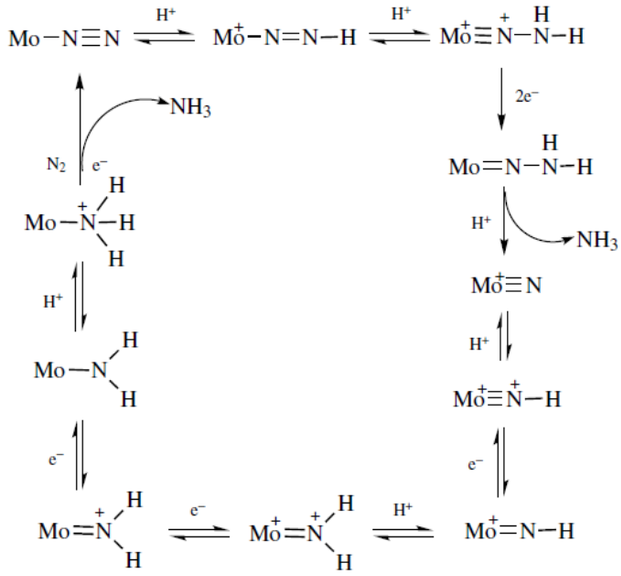
Steps of conversion of atmospheric nitrogen to ammonia by nitrogenase enzyme complex found found in nitrogen - fixing bacteria

Possible reaction pathways for reduction of N_2 to NH_3

Binding N_2 in η^1-mode in the FeMoco (asymmetric, Chatt-Schrock type mechanism)

- The two N-centres is coordinated to the Mo in low oxidation state in η^1-N_2 fashion

- Mo-N≡N complex experiences stepwise reduction and proton addition (proton coupled reduction)
- First, the distal-N (noncoordinating-N) is reduced in 3e/H+ additions and released as NH_3 with the cleavage of N-N bond producing the nitrido complex
- Then the metal bound-N (present as nitride) is reduced in 3e/H+ additions producing the second NH_3 molecule.
- This pathway of successive reduction of the two nitrogen centres of N_2 is commonly known as Chatt-Schrock mechanism or Chatt mechanism
- The cycle operates between molybdenum oxidation states Mo(0) and Mo(IV)
- The asymmetric N_2 attachment mode appears to involve the molybdenum atom of the M center but not the iron atoms except as possible electron providers.

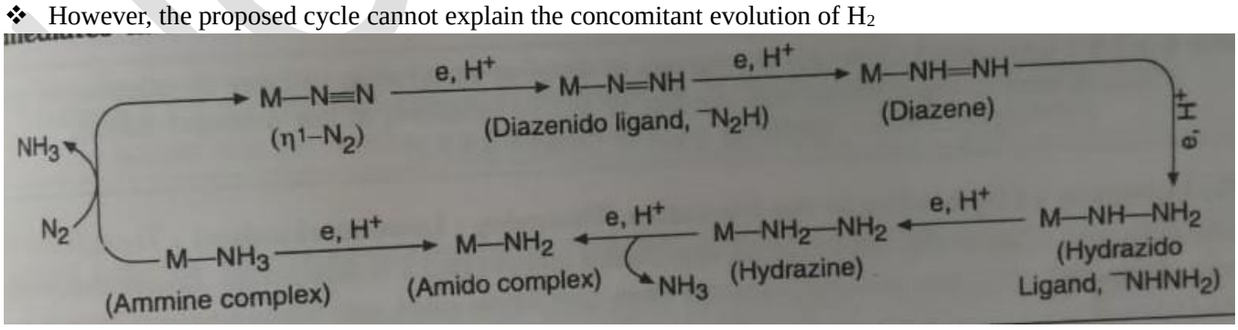


Proposed nitrogenase catalytic cycle

- Distal pathway of reduction cannot explain the formation of N_2H_2 and N_2H_4 as the product
- This pathway is valid in vitro while alternating pathway of reduction is possibly valid in vivo

Binding N_2 in η^1-mode in the FeMoco (symmetric, alternating)

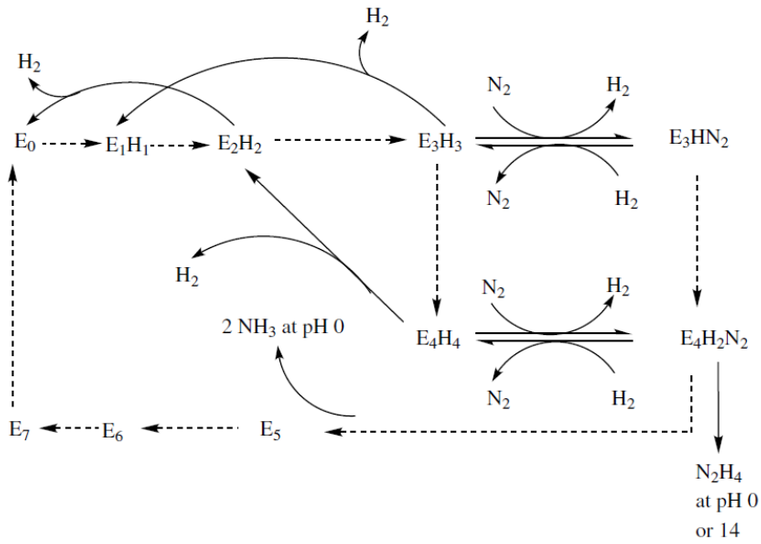
- The active site for dinitrogen binding can occur either to Fe or Mo, most probably involves Fe atom.
- The coordinated η^1-N_2 (M-N≡N, M=Fe or Mo) will experience the proton coupled electron transfer (PCET) reaction in an alternating or a symmetric pathway.
- The two N-atoms are reduced alternatively with the cleavage of the N-N multiple bond.
- The electron rich coordinated N_2 can experience the electrophilic attack by H^+.
- The process is proceed via the formation of symmetrical intermediate like diazene and hydrazine
- However, the proposed cycle cannot explain the concomitant evolution of H_2



Possibility of N_2 binding at Mo center (Thorneley-Lowe mechanism)

- No crystal structure evidence (no vacant site around Mo to accommodate N_2)
- Some groups have suggested that carboxylate group of homocitrate is dislodged from the Mo-center after 3e reduction of the enzyme
- The 3e reduction is followed by opening of the Mo bound carboxylate group of homocitrate allows dinitrogen to bind with the Mo-center
- Dinitrogen des not bind with the resting enzyme (bound to partially reduced enzyme)
- The bound N_2 is reduced after the release of H_2 (H_2 evolution cannot be eliminated during N_2 reduction)
- The scheme considers stepwise reduction through the intermediacy of a large number of possible states of the enzyme.

- The scheme can explain the H_2 evolution during N_2 reduction and inhibition of N_2 reduction by H_2



MoFe–protein cycle for reduction and protonation of N_2.

V-Nitrogenase – An alternative nitrogenase

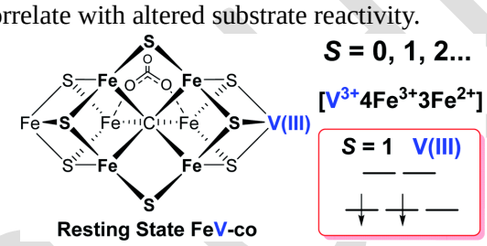
- Mo-nitrogenases (first by Bortels in 1930) are the most exclusively studied nitrogenases, more than a dozen distinct Mo enzymes are known.
- V-nitrogenases are found in members of the bacterial genus Azotobacter as well as the species *Rhodopseudomonas palustris* and *Anabaena variabilis*.
- Bortels further reported V-stimulated nitrogen fixation in 1935 (then could not gather considerable attention)
- Starting in the 1970s, attempts were made to isolate a V-nitrogenase
- Two groups isolated a V-containing nitrogenase (1971) from *A. vinelandii*
- Earlier, it was thought that V might substitute for Mo in nitrogenase, not that there was a separate system.
- The isolated enzyme was reported to be similar to the Mo enzyme, but had a lower activity and an altered substrate specificity.
- Presence of small amounts of Mo, presumed to be sufficient to account for the low activity
- The V was suggested to play a stabilizing role for [FeMo], allowing the small amount of active Mo-containing protein to be effectively isolated.
- It was not considered as a truly alternative nitrogenase system with protein and metal centers both differing from that of the Mo nitrogenase.
- Until 1980, Mo-was thought to be essential for nitrogen fixation
- The observation of nitrogen fixation in *A. vinelandii* when it was starved for Mo changed the whole concept.
- Alternative nitrogenase component proteins from different species of *Azotobacter* are isolated (1986), & suggested the presence of V in one component and absence of Mo in any component.
- One of the two components of the V-nitrogenase is similar to the Fe protein of nitrogenase.
- The similarity is evident in the isolated proteins from *A. vinelandii* and in the genetic homology between *nif* H (the gene coding for the subunit of the Fe protein in the Mo-nitrogenase system) and *nif* H* (the corresponding gene in the V-based system).

Comparison of alternative nitrogenase proteins[a]

Property	*Av*1[47]	*Av*1*[47]	*Ac*1*[50]
Molecular weight	240,000	200,000	210,000
Molybdenum[b]	2	<0.05	<0.06
Vanadium[b]	—	0.7	2
Iron[b]	30–32	9.3	23
Activity[c]			
H^+	2200	1400	1350
C_2H_2	2000	220	608
N_2	520	330	350
EPR *g* values	4.3	5.31	5.6
	3.7	4.34	4.35
	2.01	2.04	3.77
		1.93	1.93

[a] *Av*1 is the FeMo protein of *Azotobacter vinelandii*, *Av*1* is the FeV protein of *A. vinelandii* and *Ac*1* is the FeV protein of *A. chroococcum*.

- Both Fe proteins have an (α_2 subunit structure, and contain a single Fe_4S_4 cluster that is EPR-active in its reduced state.
- FeV proteins (from *A. vinelandii* and *A. chroococcum)* have an $\alpha_2\beta_2\delta_2$ subunit structure.
- Metal composition and spectroscopic comparisons between the FeMo and FeV proteins
- Although there is the major difference involving the presence of V instead of Mo in the FeV protein and in the probable presence of the small δ subunits (13 kDa), the two nitrogenase systems are otherwise quite similar.
- In each, a system of two highly oxygen-sensitive proteins carries out an ATP-dependent N_2 reduction with concomitant H_2 evolution.
- The Fe proteins have the same subunit structure and cluster content, and are spectroscopically very similar.
- The V versions of the larger protein have somewhat lower molecular weights than their Mo analogues, and by MCD spectroscopy seem to contain P-like clusters.
- The FeV site still may be an S = 3/2 center (by EPR, although its EPR differs significantly from that of the FeMo center).
- The V-S and V-Fe distances as measured by EXAFS are similar to those in thiocubane VFe_3S_4 clusters and to Mo-S and Mo-Fe distances like those in [FeMo], which are in turn similar to those in $MoFe_3S_4$ thiocubanes.
- Likewise, XANES indicates VS_3O_3 type coordination in [FeV] nitrogenase similar to the MoS_3O_3 coordination suggested by XANES for FeMoco.
- The "FeV cofactor" is extractable into NMF, and can reconstitute the *nif* B-, FeMoco-deficient mutant of the Mo system.
- On substitution of V for Mo, the proteins and their respective M-Fe-S sites do not differ drastically, their compositional changes correlate with altered substrate reactivity.



- The V and Mo enzymes differs considerably in substrate specificity and product formation
- The FeV nitrogenase has a much lower reactivity toward acetylene than does the Mo system. The FeV system yields significant amounts of the four-electron reduction product, ethane while the FeMo system gives ethylene from acetylene.
- The detection of ethane in the acetylene assay may prove a powerful technique for detecting the presence of the V nitrogenase in natural systems.
- This reactivity pattern is found in the *nif* B- mutant reconstituted with FeVco, indicating that the pattern is characteristic of the cofactor and not the protein.
- Reactivity varies from Mo to V in otherwise similar protein systems clearly adds weight to the implication of the M-Fe-S center (M = V or Mo) in substrate reduction

Nitrogenase Model systems

- Three types of model systems for nitrogenase may be considered.
- First, there are transition-metal sulfide clusters that resemble the FeMoco or FeVco centers of the active proteins (yet no definite model systems)
- A second approach uses the reactions of N_2 and related substrates or intermediates with metal centers in order to gain insights into the way in which transition-metal systems bind N_2 and activate it toward reduction (bears little direct chemical resemblance to the nitrogenase active sites yet carry out nitrogen fixation from which one can predict how to activate N_2)
- Finally, there are other inorganic systems that display some of the structural and possibly some of the reactivity characteristics of the nitrogenase active sites without binding or reducing N_2 or precisely mimicking the active center.
- These model systems are helpful in learning effectively about nitrogenase reactivity.

1. Transition-Metal Sulfide Models for Nitrogenase Sites

- Although there has been great activity in synthetic Fe-S cluster chemistry, there is to date no example of a spectroscopic model for the P-cluster sites in nitrogenase.

- If the P-clusters are indeed asymmetrically bound high-spin Fe_4S_4 clusters, then the recent work on high-spin versions of Fe_4S_4 clusters and site-selectively derivatized Fe_4S_4 centers may hint that appropriate model systems are forthcoming.

2. Fe-Mo-S Cluster Models for FeMoco

- Despite the importance of P-clusters, the modeling of the FeMoco center has properly received the most attention.
- The significant structural parameters that any model must duplicate are the Mo-S and Mo-Fe distances determined by EXAFS.
- Spectroscopically, the S = 3/2 EPR signal provides a stringent feature that model systems should aspire to mimic.
- Many FeMoS clusters have been prepared in the quest to duplicate the FeMoco center, but *none* of the chemically synthesized clusters can reactivate the (UW45 or *Nif* B -) cofactor-less mutants, perhaps because of their lack of homocitrate, which only recently has been discovered as a key component of FeMoco.
- Undoubtedly, new FeMoS clusters containing homocitrate will be prepared, and perhaps these will activate the mutant proteins, thereby revealing a close or full identity with FeMoco.
- Despite the absence of homocitrate, some interesting model systems have been investigated.
- It is beyond the scope of this chapter to give a comprehensive account of FeMoS chemistry.
- We concentrate on the so-called "thiocubane“ model systems.
- Heterothiocubane models were first synthesized using self-assembly approaches analogous to those used for the simpler Fe-S model systems.
- The reaction uses tetrathiomolybdate, MoS_4^{2-} ,as the source of Mo, and leads to the double cubane structures (Figure 7.32A, B)

$$MoS_4^{2-} + Fe^{3+} + SR^- \longrightarrow (MoFe_3S_4)_2(SR)_9^{3-} \quad \text{and} \quad (MoFe_3S_4)_2Fe(SR)_{12}^{3-,4-}$$

- The Fe_7Mo_2Ss structure proved particularly interesting, since it was possible to complex the central ferric iron atom with substituted catecholate ligands and eventually isolate a single thiocubane unit (Figure 7.32C).
- Significantly, the single unit has S = 3/2 and Mo-S and Mo-Fe distances that match precisely those found by EXAFS for the M center of nitrogenase.
- Single cubes with VMO_0S_4 cores have also been prepared.
- Although the single thiocubanes display spectroscopic similarity and distance identity with FeMoco, they are not complete models.
- They are stoichiometrically Fe and S deficient, lack homocitrate, and most importantly, fail to activate the UW-45 and *Nif* B - mutants.
- Other interesting FeMoS (and FeWS) clusters with structurally distinct properties are shown in Figure 7.33.
- These include the "linear" $(MoS_4)_2Fe^{3-}$ ion, the linear $(WS_4)_2Fe[HCON(CH_3)_2]_2^{2-}$ ion, the linear $Cl_2FeS_2MS_2Fe_2Cl_2^{2-}$ (M = Mo, W), the "linear" $(MoS_4)_2Fe_2S_2^{4-}$ ion, the trigonal $(WS_4)_3Fe_3S_2$4-, the capped thioprismane $Fe_6S_6X_6[M(CO)_3]_2^{3-}$ (X = Cl, Br, I; M = Mo, W), and the organometallic clusters $MoFe_6S_6(CO)_{16}^{2-}$, $MoFe_3S_6(CO)_6(PEt_3)_3$, and $MoFe_3S_6(CO)_6^{2-}$. Structures suggested for FeMoco based on these and other chemically synthesized transition metal sulfides and on spectroscopic studies of the enzyme are shown in Figure 7.31

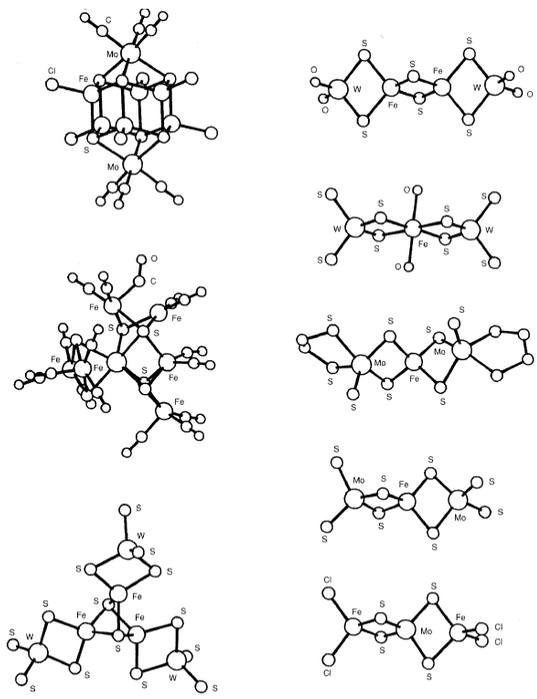
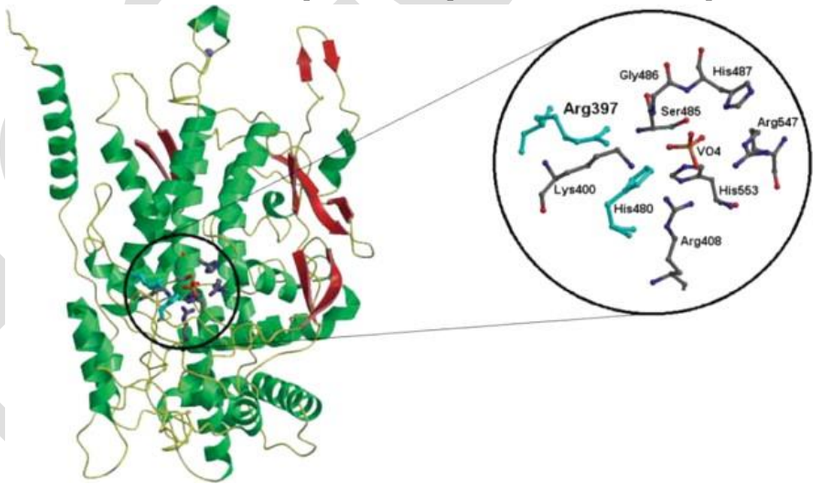


Figure 7.33
FeMoS and FeWS structures of potential interest with respect to nitrogenase.

V-Peroxidase

- Peroxidases are used as catalyst in the removal of peroxide
  $H_2O_2 + SubH_2 \rightarrow 2H_2O + Sub$
- Vanadium is essential for several organisms.
- Haloperoxidase represents the first, and best-characterized class of vanadium enzymes,
- Haloperoxidase is capable of catalyzing the two-electron oxidation of a halide by H_2O_2
- Chloroperoxidase (found in algae, seaweed, lichens and fungi) can oxidize both Cl^- and Br^- whereas bromoperoxidase (found in marine extracts) can only oxidize Br^-
- X-ray structures of several vanadate-dependent Haloperoxidase have been reported

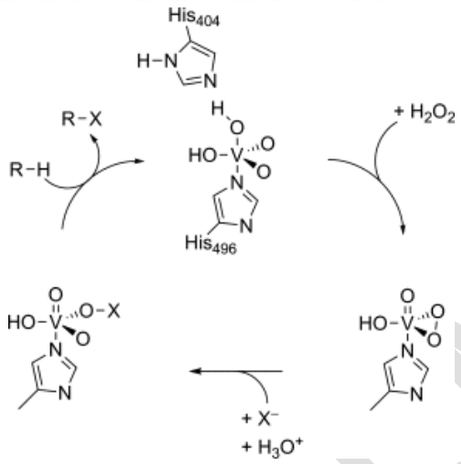


Structure and active site of the bromoperoxidase subunit from C. pilulifera.

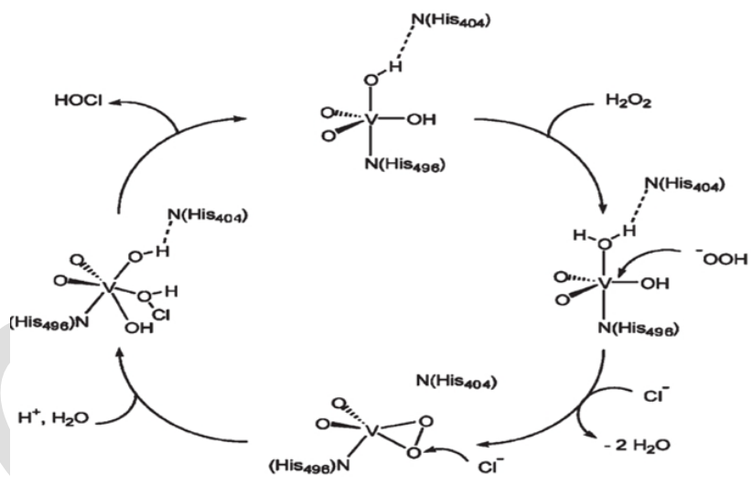
Vanadium bromoperoxidase

- It (V-BrPO) has been isolated from marine brown red algae
- It catalyzes the oxidation of Br^- by H_2O_2 and the concurrent formation of C-X bonds
- In the absence of an organic substrate, it also catalyses the generation of singlet dioxygen from the bromide assisted disproportionation of H_2O_2
- In contrast to corresponding heme iron Haloperoxidase, the production of dioxygen only occurs in the presence of Br^- or I^- anions.
- Apart from Br^- or I^- ions, V-BrPO can also catalyze the oxidation of Cl_2 by H_2O_2 leading to the chlorination of selected organic substates
- EPR studies have shown that the metal is present as vanadium (V); when the metal is reduced to vanadium (IV) state the enzyme looses its brominating activity.

- EXAFS studies suggest that it is in a distorted octahedral environment with two histidine nitrogen atoms and one oxo group bound to the metal
- The remaining three sites are probably occupied by three oxygen atoms from undetermined amino acids.
- A scheme for the reaction carried out has been advanced (Fig)
- However, the precise nature of the intermediate is not established and the exact role of the metal is not yet known
- The vanadium could act as an electron-transfer catalyst or a Lewis acid catalyst; it is also possible that it is the peroxide and not the metal that is involved in the reaction.



- On the basis of spectroscopic evidence, it is now thought that the oxidation state of the vanadium remains at V throughout catalysis
- The mechanism for both type of V-haloperoxidases are the same (Fig)



Proposed mechanism for the vanadium chloroperoxidase oxidation of chloride by H_2O_2

- The reaction proceeds by initial binding of H_2O_2 followed by protonation of bound peroxide and addition of the halide.
- NMR spectroscopy confirms the presence of VO_2–O_2 and there is no evidence for direct binding of halide to the vanadium ion.
- The rate limiting step in the catalysis is the nucleophilic attack of the halide on the protonated protein-peroxide complex, generating an 'X^+' species, which reacts directly with organic substrates (RH) to halogenate them (RX).
- In the absence of RH this step will generate singlet oxygen.

Hydrogenase

- Molecular hydrogen, H_2, is evolved by certain organisms and taken up by others.
- Hydrogenase is the enzyme responsible for the uptake or evolution of H_2
- Hydrogenases catalyze the conversion of molecular hydrogen to protons and electrons by a heterolytic splitting mechanism. $H_2 \rightarrow 2H^+ + 2e^-$
- In the activity of hydrogenase, the electron source the electron sink are well separated and different electron carriers make the link between them.
- In this electron transport chain, NAD^+ is converted to NADH at one end, but H_2 does not directly interact with NAD^+

- In fact, H_2 reduces the enzyme at one site and NAD^+ or other acceptors retrieve the electrons at other sites through the intermediacy of different electron carriers.
- The enzyme is found in many organisms
- It is found in several anaerobic bacteria, like the eubacterial *Clostridium pasteurianum* and *Acetobacterium woodii* and the archaebacterial *Methanosarcina barkerii.*
- Aerobic bacteria such as *Azotobacter vinelandii, Alcaligenes eutrophus,* and *Nocardia opaca,* and facultative anaerobes like *Escherichia coli* and various species of *Rhizobium* and *Bradyrhizobium* (leguminous plants), also contain hydrogenase
- Photosynthetic bacteria such as *Chromatium vinosum, Rhodobacter capsulatus,* and *Anabaena variabilis* (a filamentous cyanobacterium) also possess hydrogenase.
- Three types of hydrogenases are known:

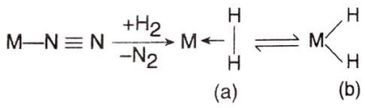
1) [NiFe] hydrogenase
2) [FeFe] hydrogenase
3) [Fe] hydrogenase

Properties of some representative hydrogenases.

Organism (designation)	MW (subunits)	Approximate composition
Clostridium pasteurianum (Hydrogenase I)	60,000 (1)	12Fe 22Fe
Clostridium pasteurianum (Hydrogenase II)	53,000 (1)	8Fe 17Fe
Acetobacterium woodii	15,000	Fe
Megasphaera elsdenii	50,000 (1)	12Fe
Desulfovibrio vulgaris (periplasmic)	56,000 (2)	12Fe
Desulfovibrio gigas	89,000 (2)	11Fe, 1Ni
Desulfovibrio africanus	92,000	11Fe, 1Ni
Methanobacterium thermoautotrophicum	200,000	Fe, 1Ni
Methanosarcina barkeri	60,000	8–10Fe, 1Ni
Methanococcus vanielli	340,000	Fe, Ni, 1Se, FAD
Desulfovibrio baculatus	85,000 (2)	12Fe, 1Ni, 1Se

Molecular properties of H_2

- It is formed by overlap of the two 1s orbitals of the two hydrogen atoms
- The H-H distance in H_2 is 0.74 A, and the bond dissociation energy is 103.7 kcal/mol.
- In molecular hydrogen, the existence of nuclear-spin energy levels is responsible for the distinction between *ortho (triplet, parallel)* and *para (singlet, antiparallel)* hydrogen
- Isotopic effect (protium, deuterium), radioactivity (tritium) and orientation of nuclear spin of hydrogen (ortho/para) is explored (NMR) as suitable mechanistic probe
- It is a reducing agent with half reaction $H_2 \rightarrow 2H^+ + 2e^-$, $E^o = -0.42$ V (at pH=7)
- The E^o value indicates that is one of the strongest biological reductants (can reduce O_2, NO_3^-, SO_4^{2-}, CO_2, fumerate etc.)
- The reverse reaction (proton reduction), producing H_2 can occur by using ferredox (Fd_{red})
- In the hydrogenase, the active site always contains the transition metal sulphides
- Dihydrogen can substitute the metal bound dinitrogen
- It may coordinate as a dihydrogen (a) or it may lead to an oxidative addition to form a dihydride complex (b)
- In molecular orbital terms, this overlap forms bonding σ (two e, act as donor) and antibonding σ* (vacant, act as acceptor) orbitals.
- The π-acceptance weakens the H-H bonding interaction.
- Sufficient build up of electron density in the σ*-MO via π-acceptance brings about the cleavage of H-H bond (formation of dihydride complex, oxidative addition)
- If $\nu_{(N-N)} \approx$ 2060-2160 cm-1, N_2 is replaced by H_2 to corresponding dihydrogen complex, and if $\nu_{(N-N)} <$ 2060 cm-1 (more back donation of electron cloud from the metal center to π* MO of N_2), the replacement leads to the formation of dihydride complex
- Thus, π-bonding makes a delicate balance between the dihydrogen and dihydride complex



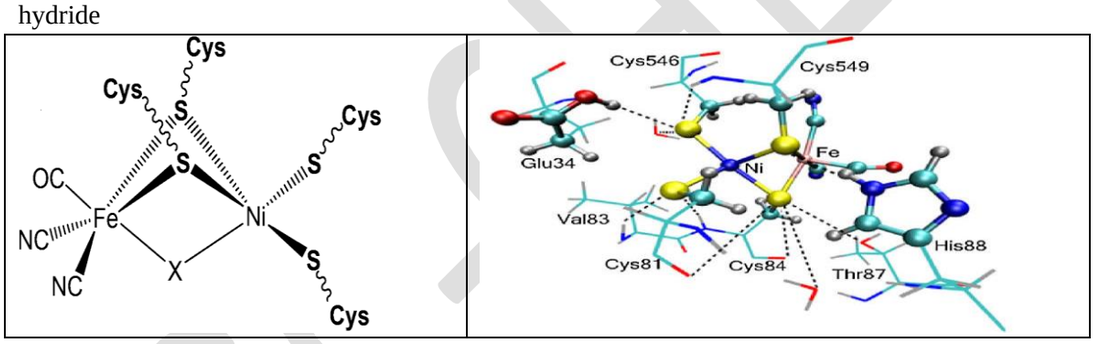
In vitro studies indicated that H_2 can react with the metal sulphides (in hydrogenase Fe-S and Ni-S sites are present) to generate thiol group

Nickel-iron [Ni-Fe] hydrogenases

- Presence of nickel in hydrogenases has been recognized relatively recently.
- In contrast to the Fe hydrogenases, the Ni enzymes possess a variety of compositions, molecular weights, activation behavior, and redox potentials.
- Some of the Ni hydrogenases contain Se (in the form of selenocysteine), some contain flavin (FMN or FAD), and all contain iron-sulfur centers (amounts ranging from 4-14 Fe atoms per Ni atom.
- Among the different Ni hydrogenases there is a common pattern of protein composition, to which many seem to conform (especially enzymes originating from purple eubacteria).
- There are two protein subunits, of approximate MW of 30 & 60 kDa, with the Ni-probably residing in the latter subunit.

Active site of [Ni-Fe] hydrogenases

- The active site is consists of a binuclear Ni-Fe active site present in the large subunit.
- Fe ion is coordinated by non-protein ligands (1CO and 2 CN).
- Two thiolates of cysteine residues in a terminal fashion.
- A third bridging ligand is found between Ni and Fe that varies (OH, OOH etc.)
- The low-spin Fe(II) ion is redox inactive throughout catalysis, whereas the Ni changes its oxidation state: [Ni(III), Ni(II) or Ni(I)].
- Once the enzyme is activated by hydrogen, the bridging position is supposed to be vacant or carrying a hydride



- The hydrogenase of the sulfate-reducing bacterium *Desulfovibrio gigas* is among the best investigated Ni-hydrogenase
- *D. gigas* hydrogenase contains a single Ni, two Fe_4S_4 clusters, and one Fe_3S_4 cluster. Of primary interest is the Ni site, which is thought to be the site of H_2 activation.
- EPR signals attributable to mononuclear Ni [enrichment with ^{61}Ni *(*I = 3/2)] have been used in numerous investigations of the role of Ni in hydrogenases.
- Three major Ni-EPR signals are known, which are called Ni-A (*g* values of these signals 2.32, 2.24, and 2.01), Ni-B (*g* values of these signals 2.35, 2.16, and 2.01), and Ni-C (*g* values of these signals 2.19, 2.15, and 2.01).
- Ni-C is thought to be associated with the most active form of the enzyme (called active); the other two are thought to originate from less active enzyme forms.
- Oxidized states (inactive), requires activation by H_2 or other reductants.

Ni-A state

- Crystal structures of Ni-A state [Ni-Fe] hydrogenases have indicated the presence of OOH bridging ligand in *D. gigas* (Dg) and OH bridging ligand in *Allochromatium vinosum*

Ni-B state

- The distance between the Ni and Fe atoms is slightly shorter (2.69 Å) than that of the Ni-A state (2.80Å). The electron density peak of the third bridging ligand (x) is assigned to an monatomic oxygen species, most probably an OH^-

Ni-C state

- A hydride is ligated to the bridging position in the Ni-C state (from spectroscopy)
- The central catalytic state Ni-C is light sensitive and is converted to Ni-L state
- The Ni-L state is probably an intermediate state between Ni-C and Ni-SIa

- Protonation of a terminal cysteine residue in Ni-L has been suggested (DFT and Raman)

Catalytic Active states

In the catalytic cycle only the three NiFe species (Ni-SIa, Ni-R and Ni-C) take part.

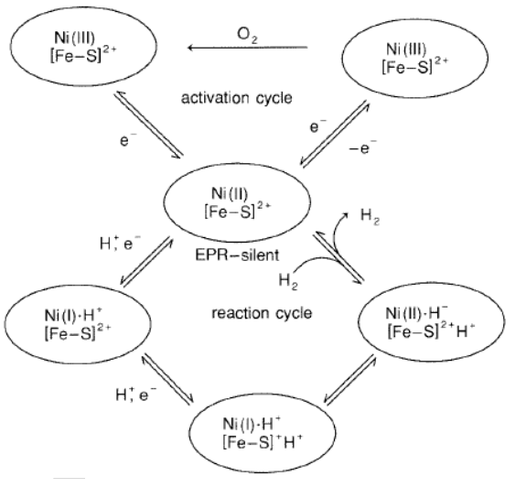
- Ni-SIa: It is the most oxidized active state [formal valance Ni(II), EPR-silent]
- Upon H_2 reduction, the third bridging ligand OH in the Ni-SIr state is released as a H_2O molecule from the active site. As a result, the Ni-SIa state carries a vacant bridge.

Ni-R state

- The Ni-R state is known as the most reduced active state.
- A recent ultra high-resolution crystal structure of the Ni-R state of *D. gisgas* [Ni-Fe] hydrogenase revealed the presence of a hydride bridge between the low-spin Ni(II) and Fe(II) and a protonated residue (cys 546).
- The proton which is attached to the cysteine, is expected to be transferred to the molecular surface through a hydrogen-bond network (proton transfer pathway).

Mechanism

- On hydrogen reduction the Ni-A EPR signal disappears, and the enzyme is converted into a higher-activity form (Ni-B arises from reoxidation of this form).
- Further progressive reduction of the enzyme gives rise to the Ni-C EPR signal, which also finally disappears. These redox properties show that Ni-C arises from an intermediate enzyme oxidation state.
- Although the Ni-A and Ni-B EPR signals almost certainly originate from low-spin Ni(III), the formal oxidation state of Ni-C is rather less certain.
- Both an Ni(I) site and an Ni(III) hydride have been suggested, with the former alternative currently favored because of the apparent absence of the strong proton hyperfine coupling expected for the latter.
- In the fully reduced enzyme, Ni-C is converted to an EPR-silent species. This has variously been suggested to be Ni(0), Ni(II), or an Ni(II) hydride. One possible reaction cycle is shown in figure.



Proposed activation/reactivity scheme for Ni hydrogenases

- Information on the coordination environment of the Ni has been obtained from both X-ray absorption spectroscopy and EPR spectroscopy.
- The Ni K-edge EXAFS of several different hydrogenases, and EPR spectroscopy of S enriched *Wolinella succinogenes* hydrogenase, clearly indicate the presence of sulfur coordination to nickel.
- A recent X-ray absorption spectroscopic investigation of the selenium-containing *D. baculatus* hydrogenase, using both Ni and Se EXAFS, suggests selenocysteine coordination to Ni.
- ESEEM (electron spin echo envelope modulation) spectroscopy of the Ni-A and Ni-C EPR signals indicate the presence of ^{14}N coupling, which probably arises from a histidine ligand to Ni.
- Interestingly, Ni-C, but not Ni-A, shows coupling to a proton that is exchangeable with solvent water. Although this coupling is too small to suggest a nickel hydride (consistent with conclusions drawn from EPR), the proton involved could be close enough to the Ni to play a mechanistic role.

Function of Ni-Fe hydrogenase

- The primary functions of hydrogenases are to provide energy for the organisms by oxidation of molecular hydrogen and to balance the redox potential of the cell.
- They can remove reducing equivalents by production of molecular hydrogen or provide electrons by splitting hydrogen. Depending on the location in the cell, hydrogenases may either be tuned for hydrogen evolution or hydrogen uptake.
- They may also be involved in establishing transmembrane proton gradients.

❖ Consulted Books

1. Bioinorganic Chemistry by Valentine, Gray, Lippard and Bertini
2. Bioinorganic Chemistry by Das and Das
3. Inorganic Chemistry by Huheey, Keiter, Huheey and Medhi
4. Inorganic Chemistry by Atkins
5. Inorganic Chemistry by Missler and Tar
6. EPG-Pathshala
7. *Bioinorganic Chemistry by Fenton*
8. *Other Available sources*