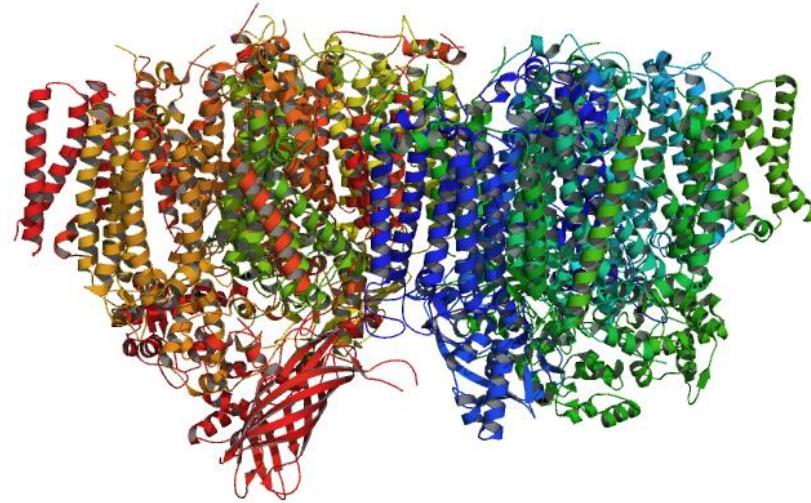
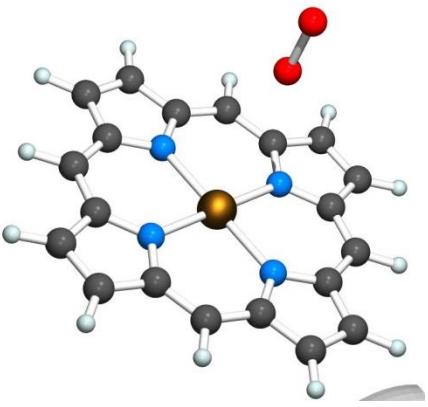
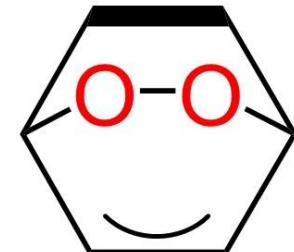


Bioinorganic Chemistry (BIC)

VIII. Metalloenzymes with Radical Intermediates



Dr. ($O_6S_4C_4Ar$) Lung Wa CHUNG(钟龙华)
(oscarchung@sustech.edu.cn)
Department of Chemistry



Quiz (27th May)

A simple quiz to **test the key and most representative systems, concepts or knowledge**, NOT test your memorization about **many detailed biological information**.

Contents (Parts I-VIII, only those we have learned in the classes)

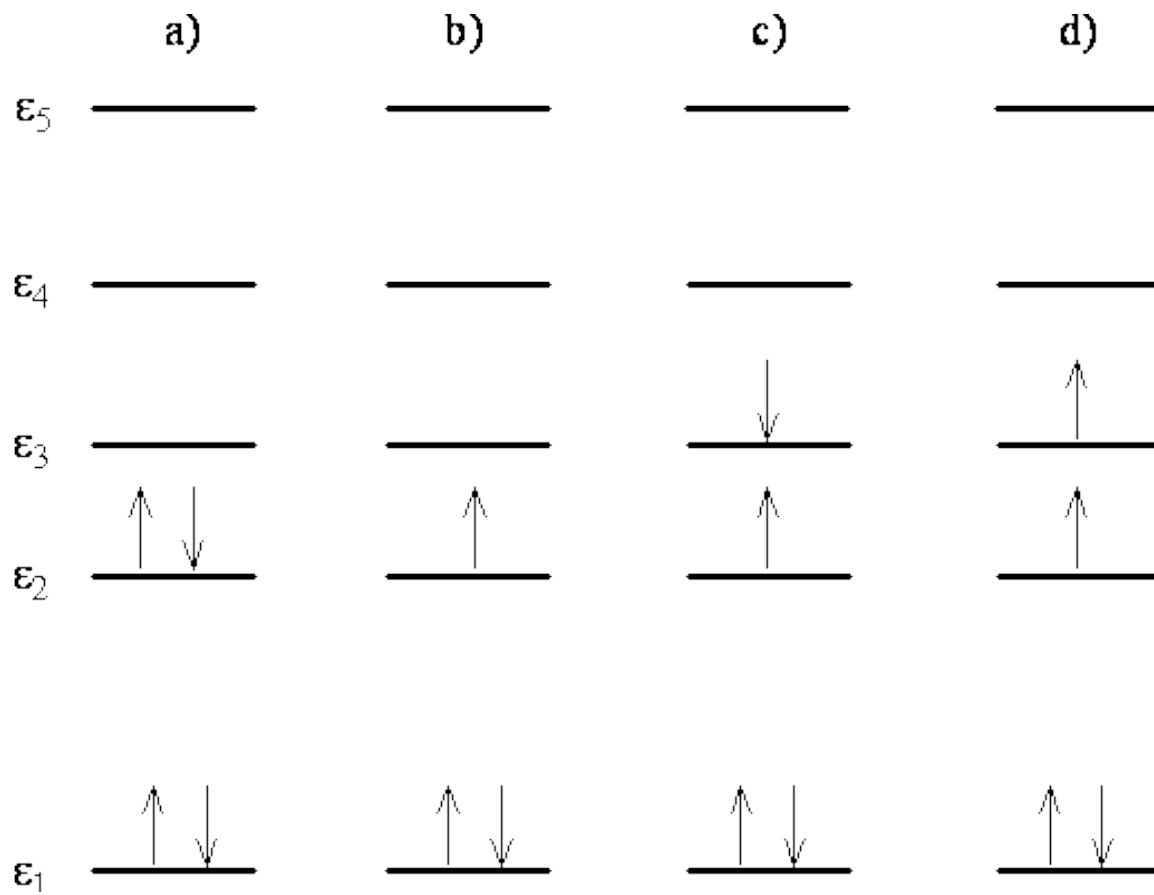
- 1. Multiple-choice (*32)**
- 2. Selected 2 short questions (mechanism-based (*~3) or metal cluster/content (*1)):**

1. Introduction to Radicals

Free Radicals

- A special type of molecules with **unpaired electron(s)**. Hydrogen atom is the simplest radical.
- Generally highly **reactive**: often chain process, hardly-controlled in solution or gas phase.
- **Good-controlled** radical reactions in enzymatic catalysis generally.
- Involved in atom abstraction (e.g. H atom abstraction), rearrangement & oxidation-reduction reactions.
- Contrast to polar (**heterolytic, 2e**) mechanisms, often follow radical (**homolytic, 1e**) mechanisms.

- Widely probed by EPR spectroscopy.
- Can serve as an organic equivalence of a 1st row transition metal: **open-shell electronic structures** (e.g. b).



Naming of Free Radicals

- use the suffix “-yl”.

e.g.

glycine → glycyl radical

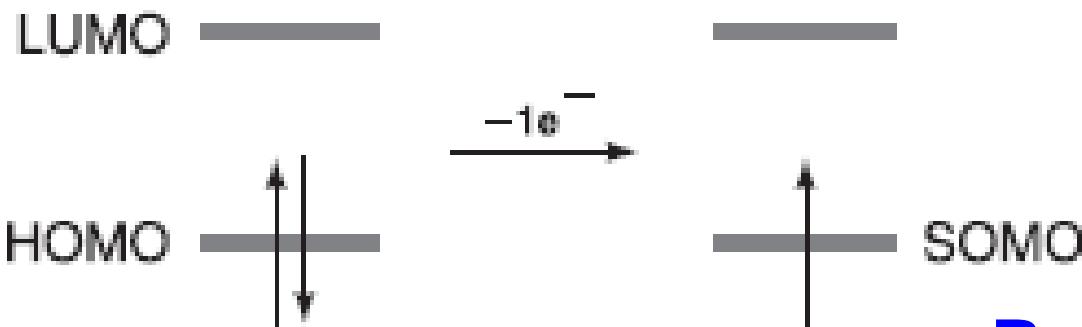
Thiol → thiyl radical

phenol (or phenoxide) → phenoxyyl radical

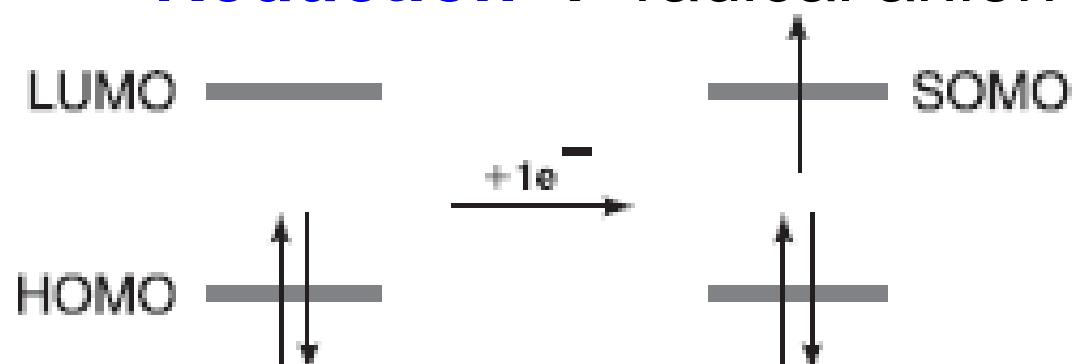
Formation of Radicals

1. Can often be obtained from **homolytic cleavage**.
2. May be formed from a closed-shell parent molecule either by **oxidation** (removing an electron from HOMO), or by **reduction** (accepting an electron to LUMO).
3. Could be formed by **light radiation** (photochemistry).

Oxidation → radical cation



Reduction → radical anion



Biological Radical Complexes

- Formation of biological radicals are often associated with/facilitated by specialized **redox sites** in enzymes.

1. The **side chain** of amino acids:

Tyr radical: Type I ribonucleotide reductase (RNR) & OEC of PSII.

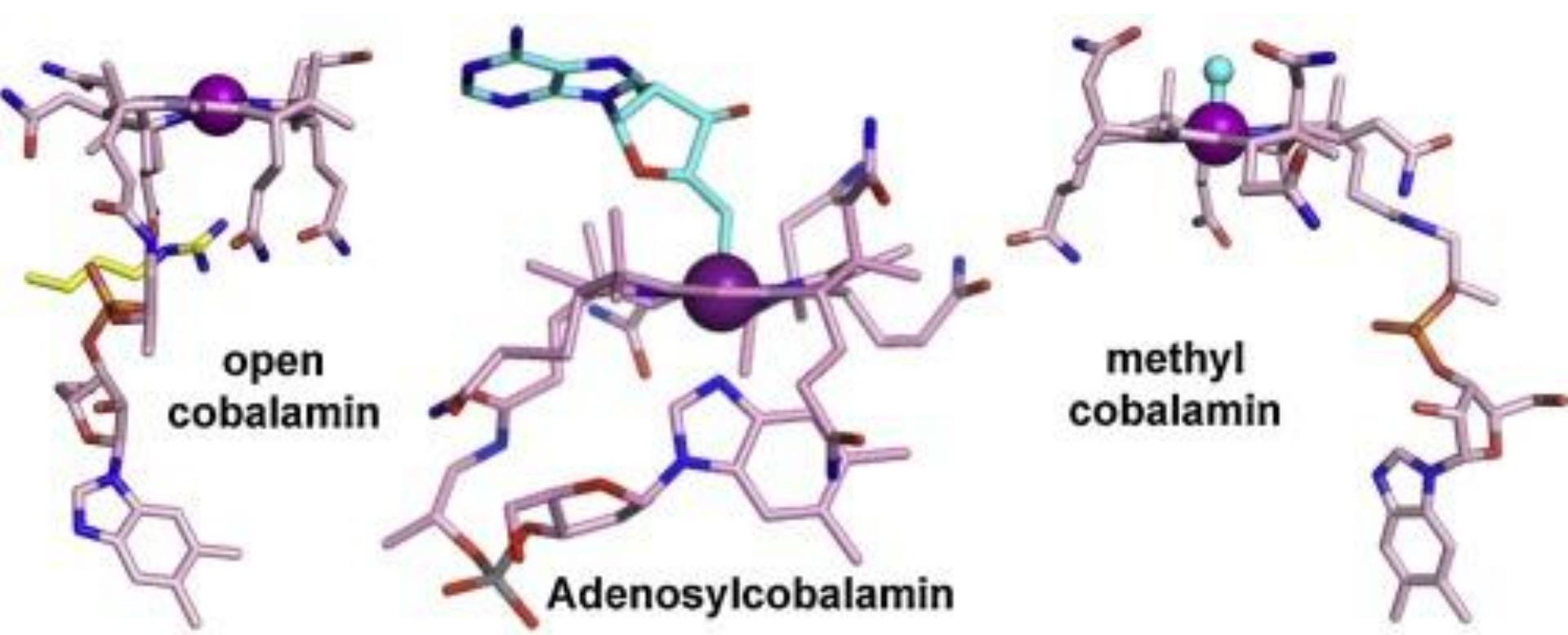
Trp radical: Cyt c peroxidase.

Thiyl radical: RNRs.

2. The **peptide backbone**: Type III RNR or pyruvate-formate lyase.

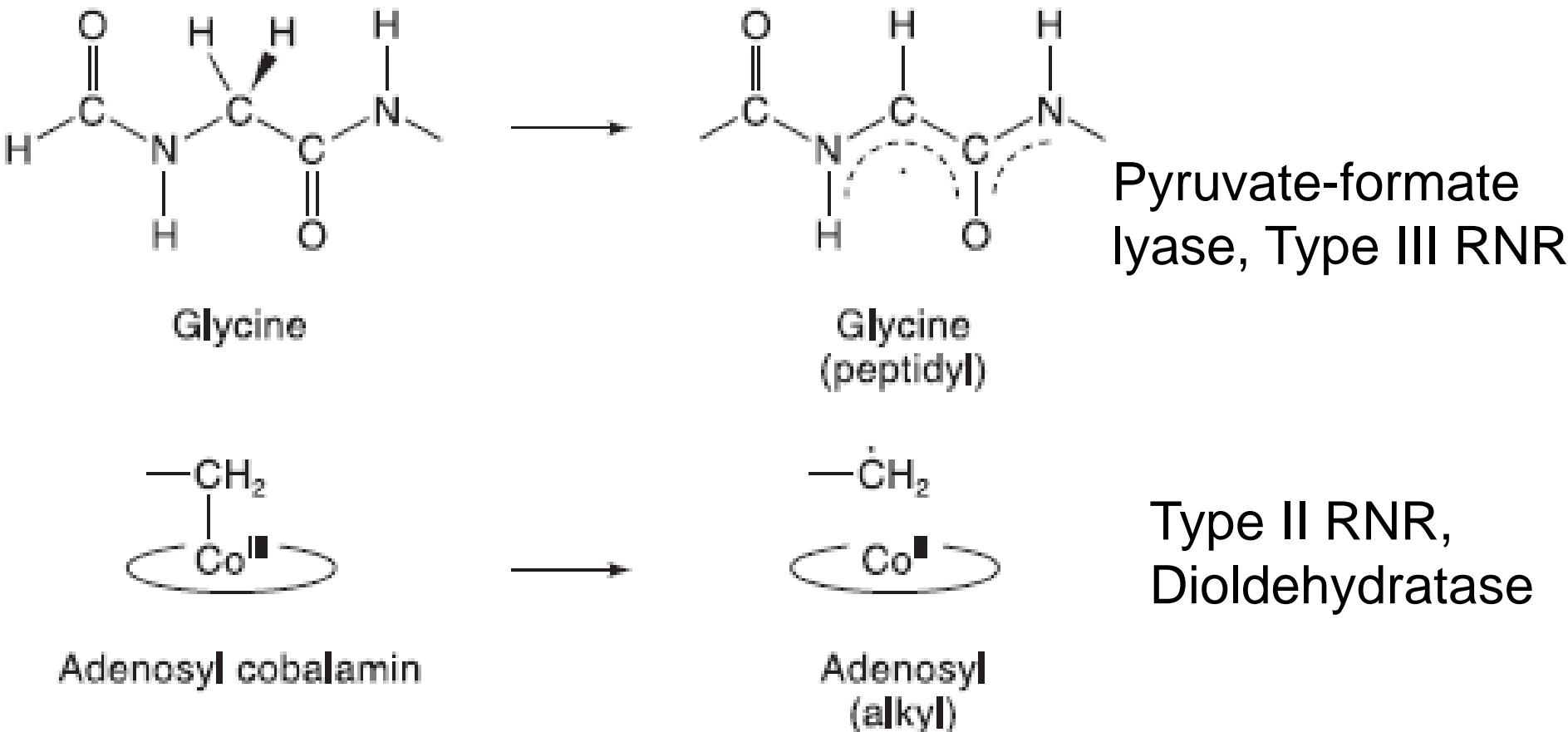
- Generally, these “radical” sites are deeply **buried** in the protein core & **protected** from the other reactions.

- Biological radicals could be generated by thermal or photochemical way. E.g.
- Carbon radical & Co(II) radical can be generated thermally or photochemically by homolysis of the Co-C bond in adenosylcobalamin (B_{12}).
- Photoionization of chlorophyll in photosynthesis.

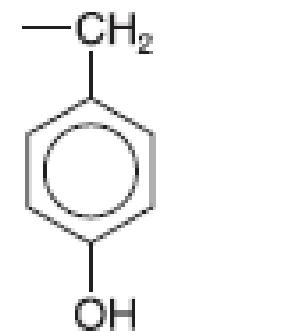


Examples of Enzymes involving Free Radicals

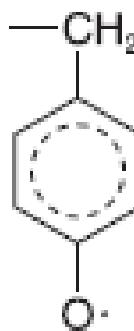
Carbon Radicals



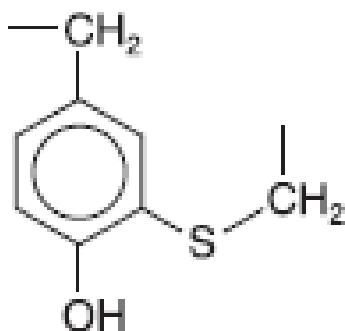
Oxygen Radicals



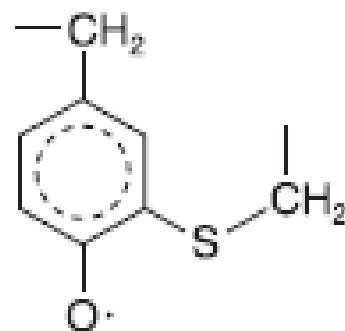
Tyrosine



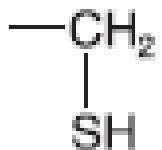
Type I RNR,
OEC of PSII,
Prostaglandin H synthase



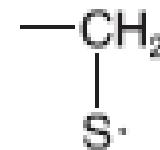
Cysteinyl-tyrosine



Cysteinyl-tyrosyl
(phenoxy)



Cysteine



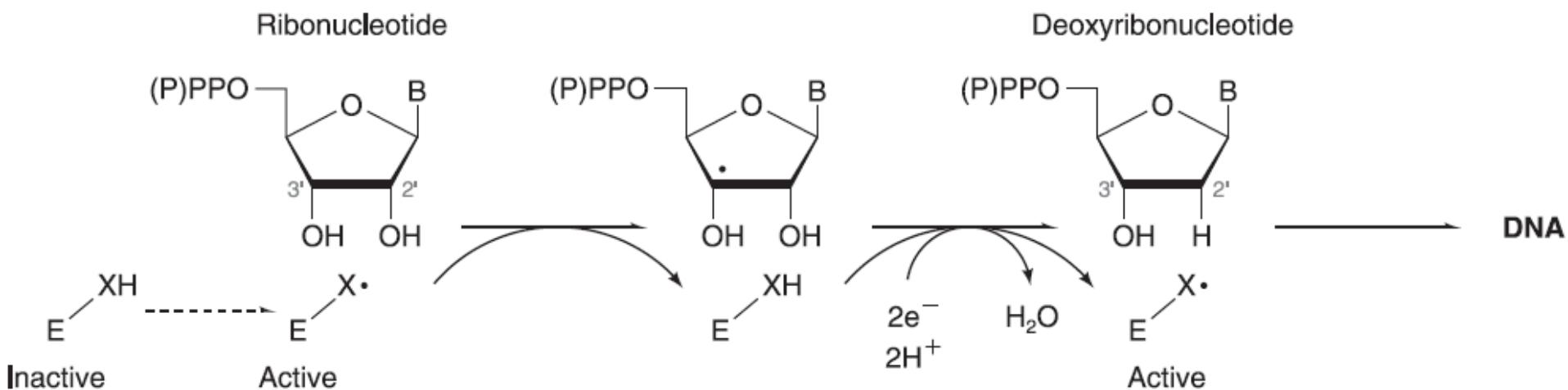
Cysteinyl
(thiyl)

Galactose oxidase,
Glyoxal oxidase

Sulfur Radical

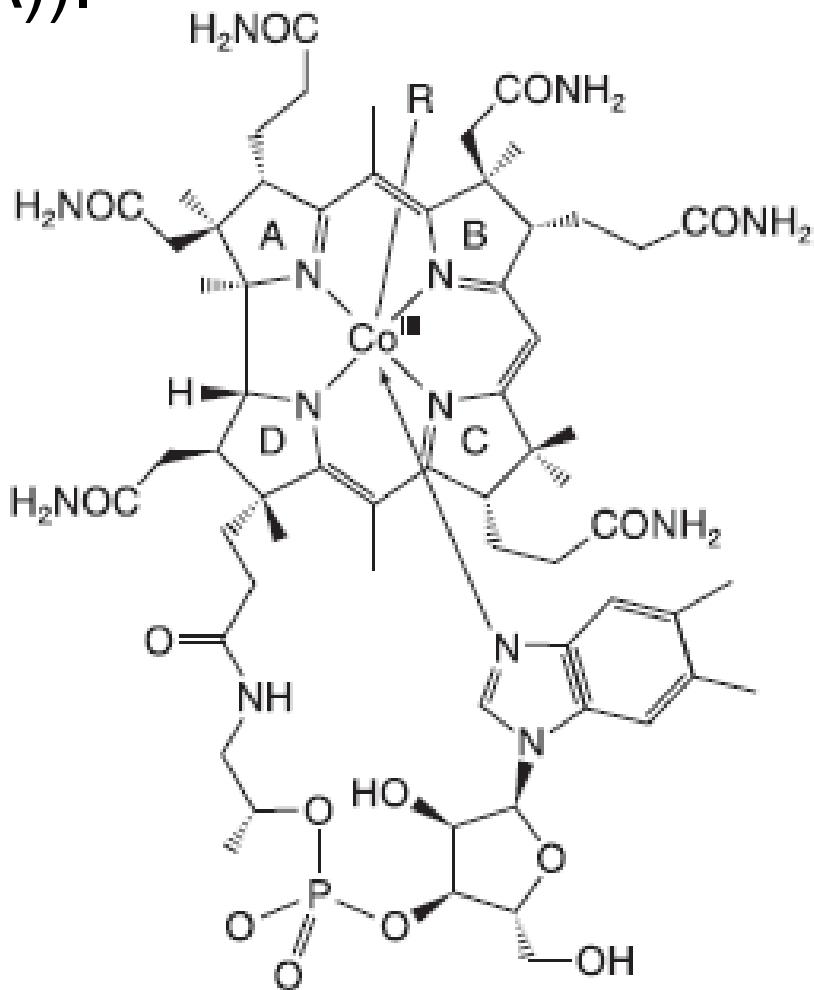
Pyruvate-formate lyase,
RNRs

- The **metal cofactors** can facilitate formation of protein radicals (e.g. **$1e^-$ redox** process).
- All types of **RNR** are catalyzed by **radicals**, which are generated from reactions of a metal cofactor: binuclear center (Type I), B_{12} (Type II), or FeS cluster (Type III).



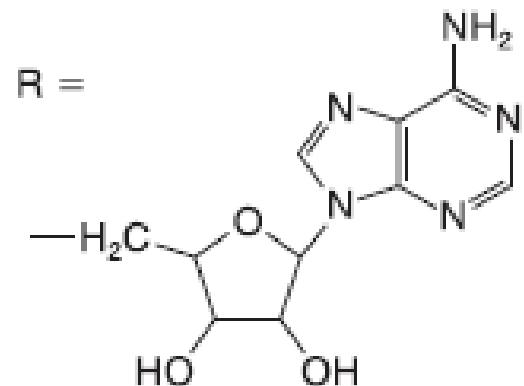
2. Cobalamins (B₁₂)

- **Cobalamins (Cbls):** one of the **most complex cofactors** in Nature.
- Different forms: methylcobalamin (**MeCbl**, R = Me) & adenosylcobalamin (**AdoCbl**, R = 5'-deoxyadenosine (5'-dA)).



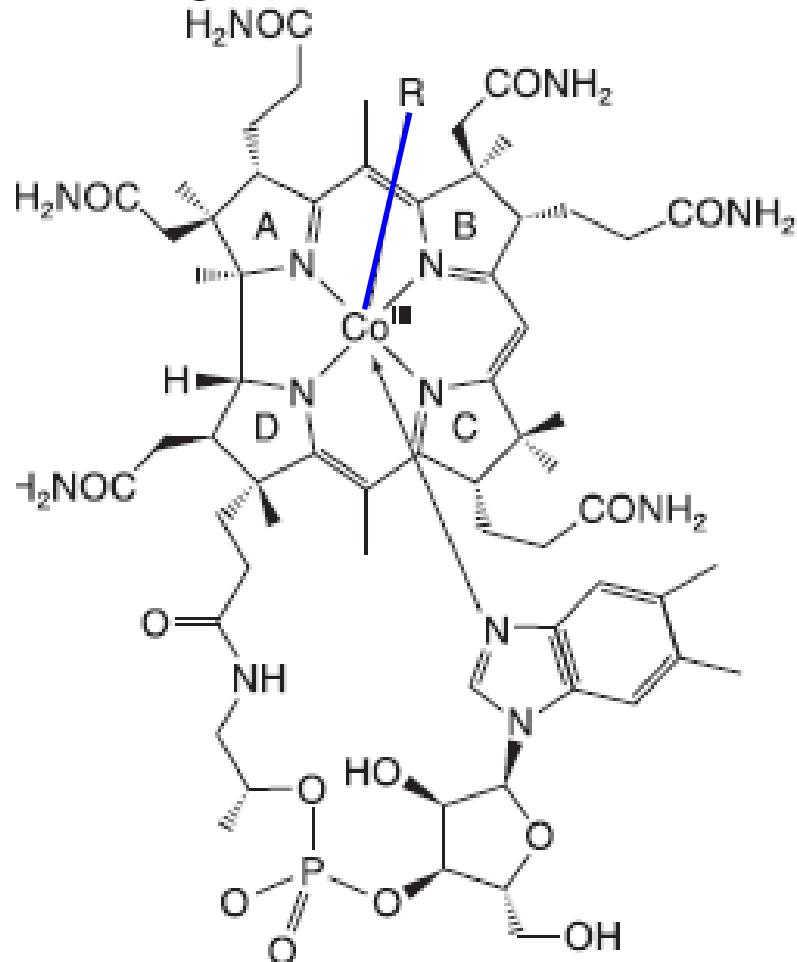
R = CN Vitamin B₁₂

R = CH₃ Methylcobalamin



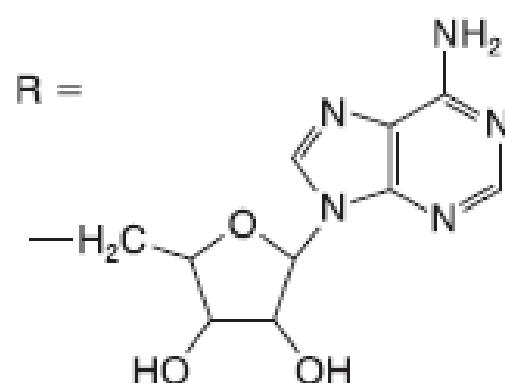
5'-dA-Adenosylcobalamin (coenzyme B₁₂)

- The Co-C bond in AdoCbl is relatively weak: (bond-dissociation enthalpy: ~31 kcal/mol in solution).
- After homolysis of AdoCbl, the radicals are within the solvent cage & the Co-C bond is reformed ($k \sim 1 \times 10^9 \text{ s}^{-1}$).



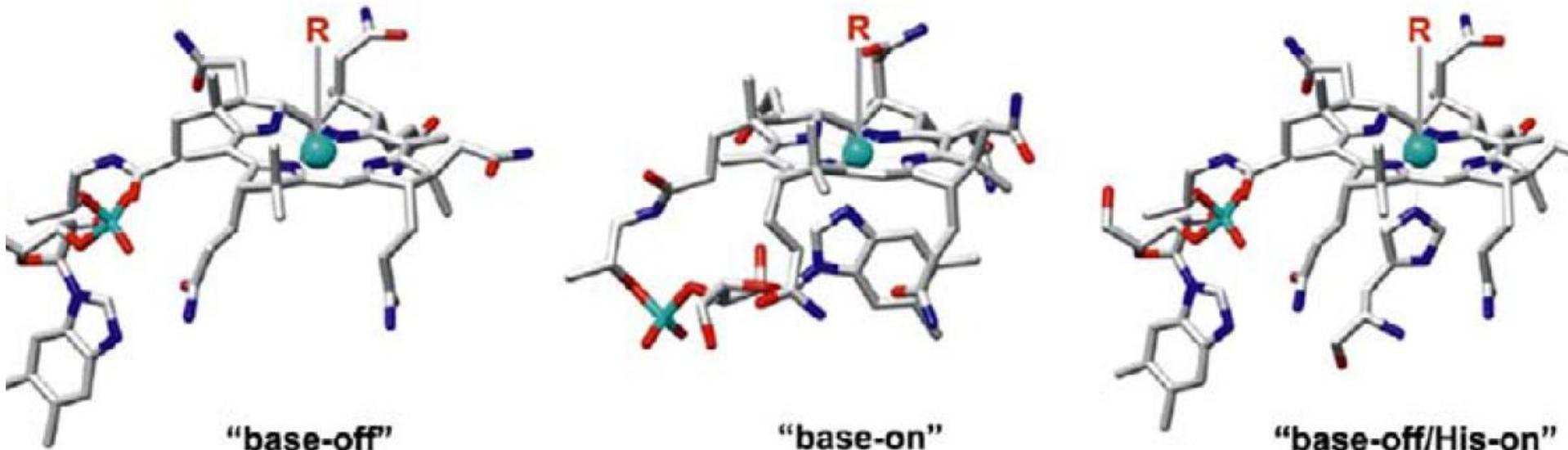
$R = \text{CN}$ Vitamin B₁₂

$R = \text{CH}_3$ Methylcobalamin



5' dA-Adenosylcobalamin (coenzyme B₁₂)

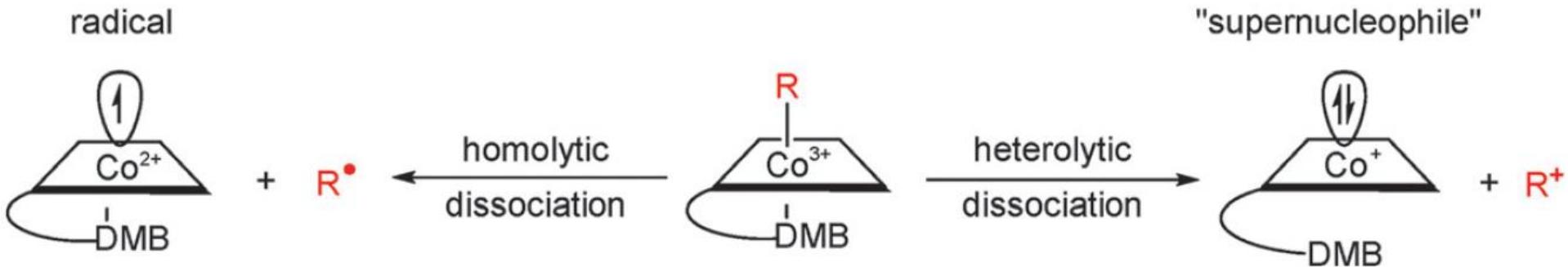
Coordination Modes & Oxidation States



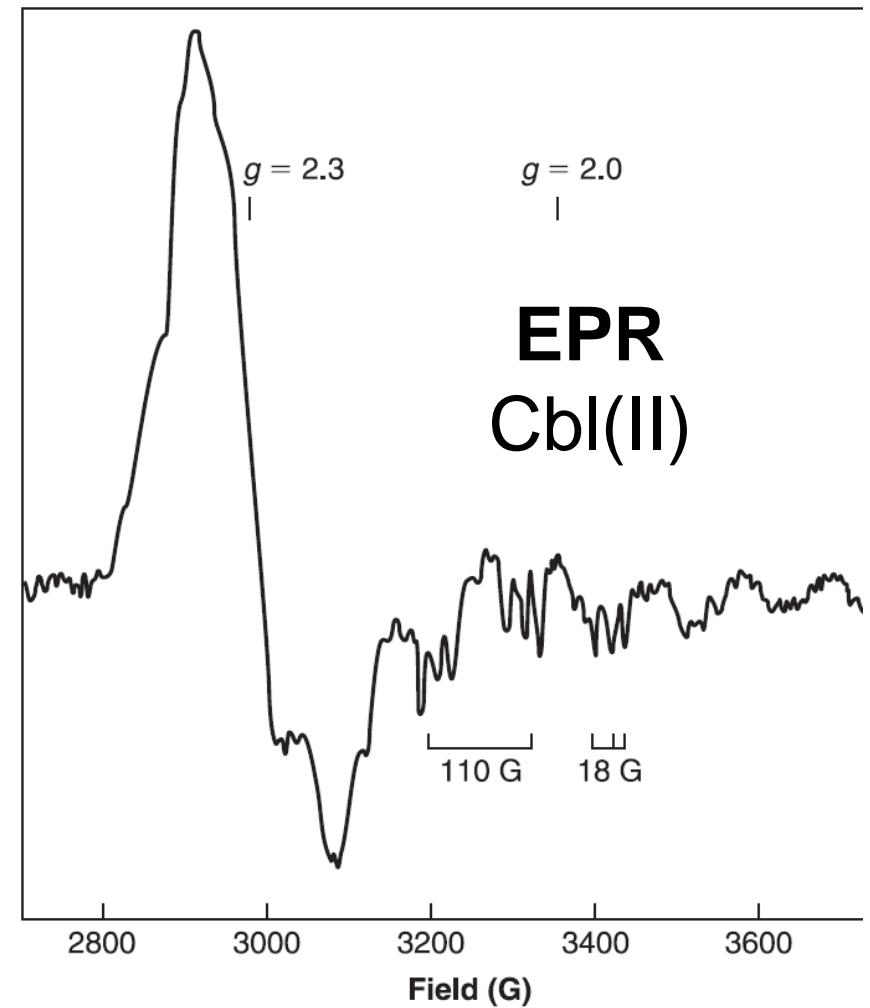
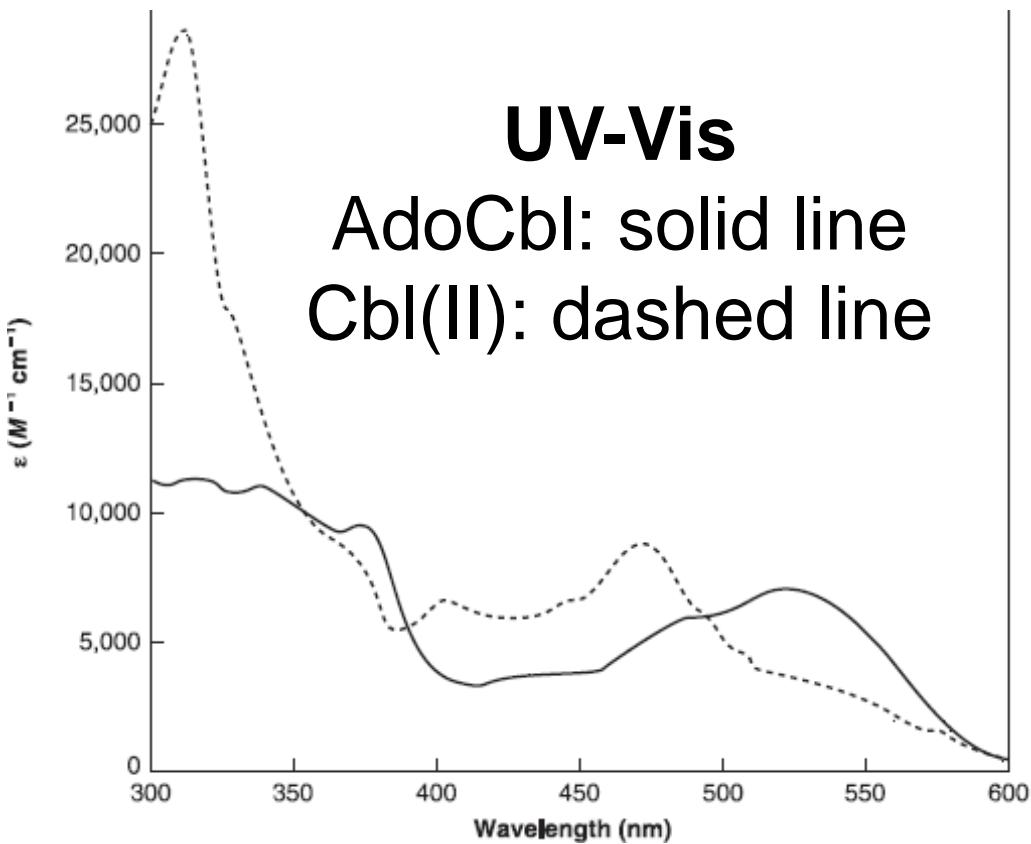
- The axial base group in some proteins can dissociate from the Co metal (**base-off**). While, a His of some proteins can coordinate to the Co metal (**His-on**).
- **3 possible oxidation states** of the Co under physiological conditions: **Co(I), Co(II) & Co(III)**.
- The resting state: low-spin Co(III).

- **AdoCbl** generally is the ultimate **radical chain initiator** (homolytic, **Co(II)** & **Co(III)**) to trigger the other **radical chemistry**.
- While, **MeCbl** follows **heterolytic** chemistry (**Co(I)** & **Co(III)**) to trigger the other heterolytic chemistry (e.g. methyltransferase).

Radical (1e) & Polar (2e) Mechanisms



- The UV-visible spectra & the paramagnetic nature of all three Co oxidation states have been well characterized.



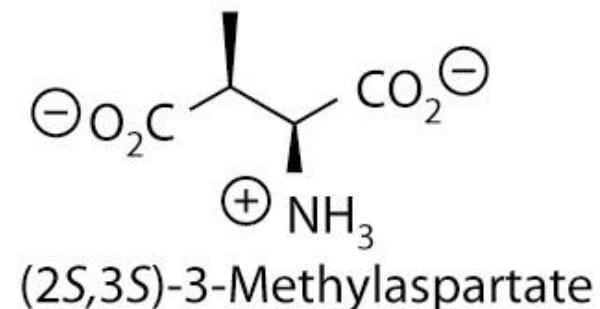
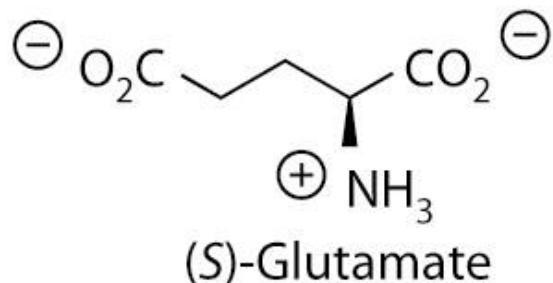
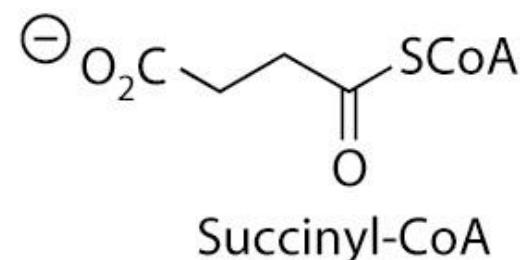
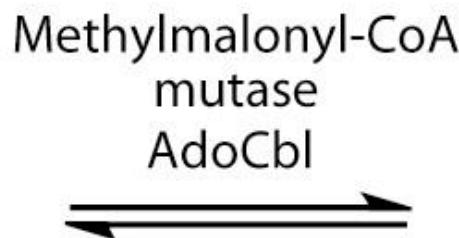
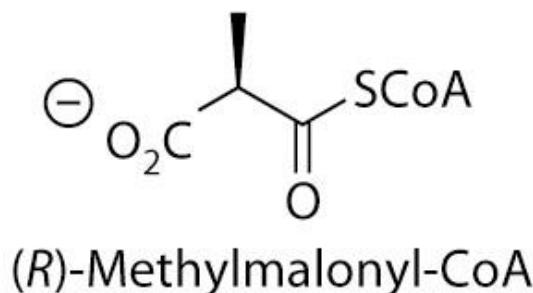
Enzymatic Systems Using AdoCbl

Enzyme	Substrate	Product	Migrating Group	Reversible / Irreversible
ADOCBL COFACTOR				
Glutamate mutase	L-Glutamate	L-threo-3-Methylaspartate	2-Glycyl	Reversible
2-Methyleneglutarate mutase	2-Methyleneglutarate	(R)-3-Methylitaconate	2-Acrylate	Reversible
Methylmalonyl-CoA mutase	Methylmalonyl-CoA	Succinyl-CoA	Formyl-CoA	Reversible
Isobutyryl-CoA mutase	Isobutyryl-CoA	n-Butyryl-CoA	Formyl-CoA	Reversible
β-Lysine-5,6-amino mutase	5-Aminolysine	6-Aminolysine	NH ₃ ⁺	Reversible
D-Ornithine-4,5-amino mutase	4-Aminoornithine	5-Aminoornithine	NH ₃ ⁺	Reversible
Diol dehydrase	1,2-Propanediol	Propionaldehyde	OH	Irreversible
Glycerol dehydrase	Glycerol	3-Hydroxypropionaldehyde	OH	Irreversible
Ethanolamine ammonia lyase	Ethanolamine	Acetaldehyde	NH ₃ ⁺	Irreversible
Ribonucleotide reductase (RNR)	Ribonucleotide	2'-Deoxyribonucleotide	OH	Irreversible
Toluene <i>cis</i> -dihydrodiol dehydrogenase	Toluene <i>cis</i> -2,3-dihydrodiol	Catechol	CH ₃	Irreversible
MECBL COFACTOR				
Methionine synthase	Homocysteine	Methionine	CH ₃	Irreversible

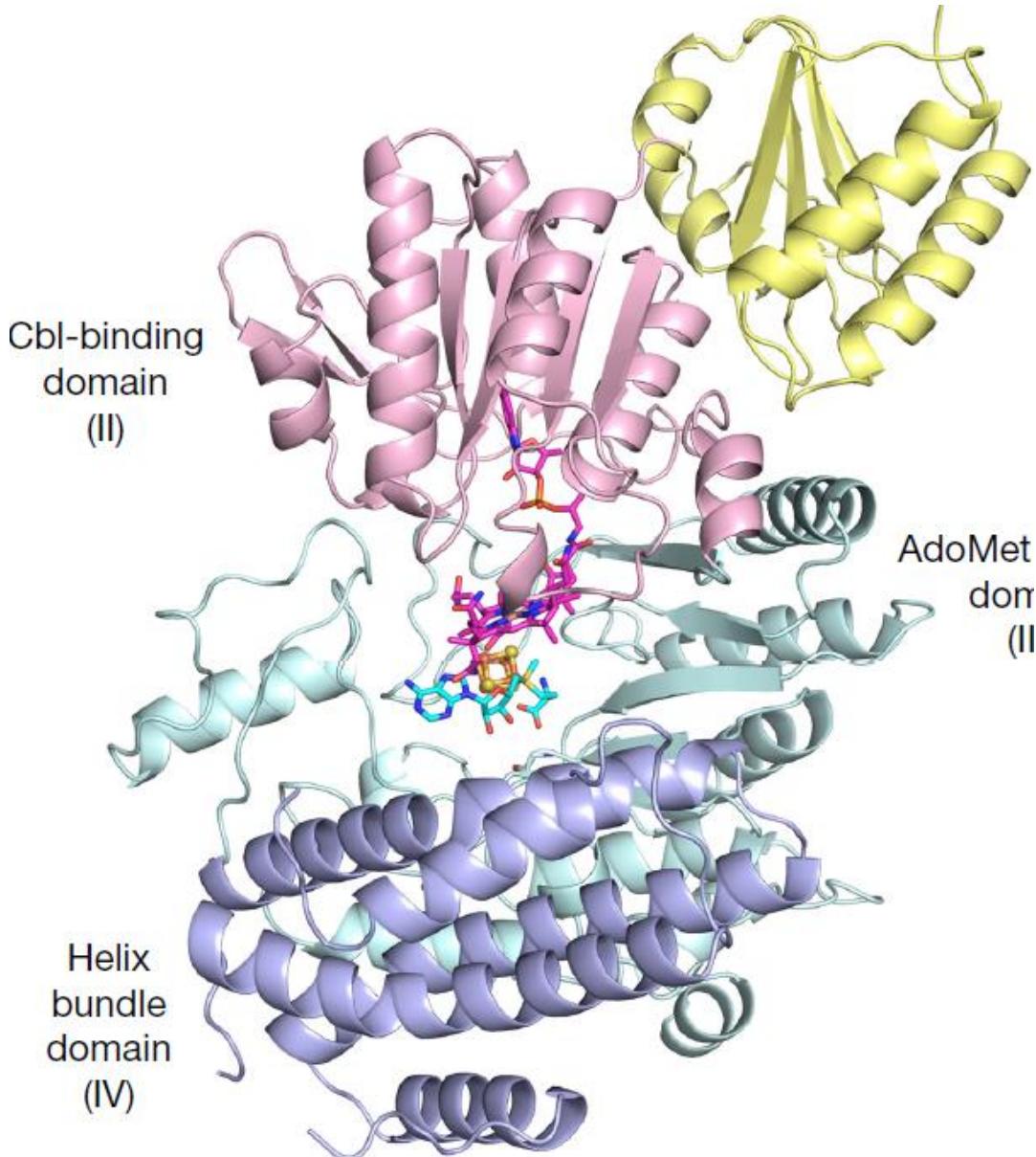
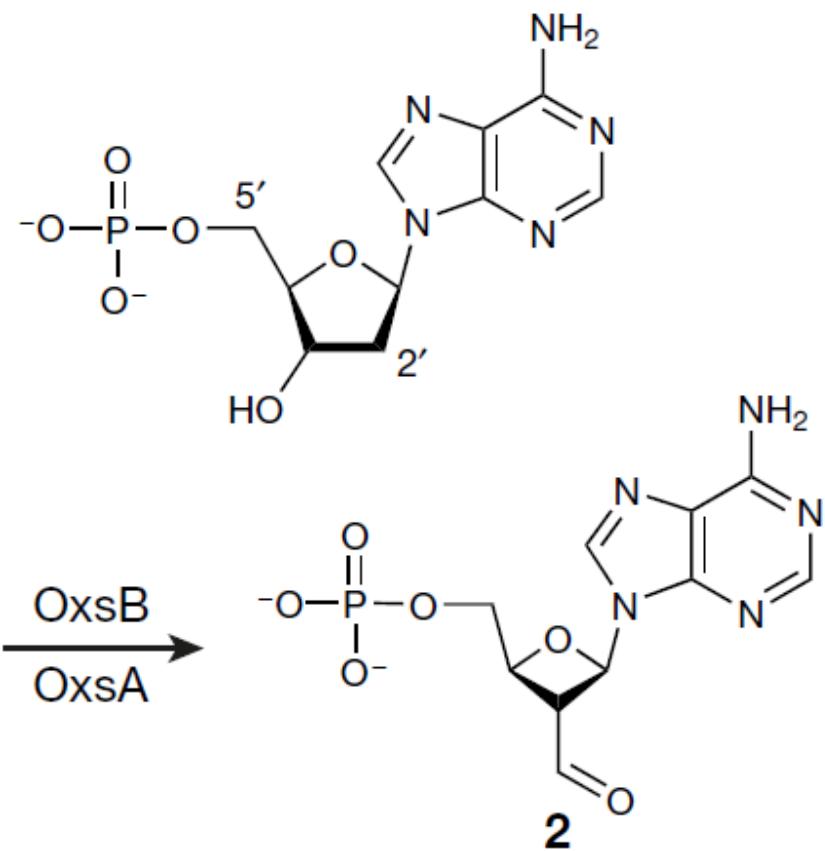
Diverse reactions involved.

Examples of Cobalamin Enzymes

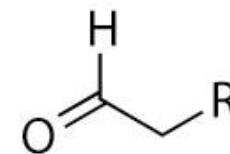
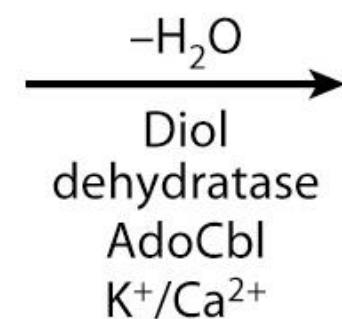
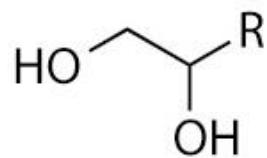
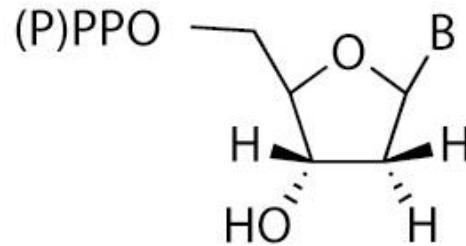
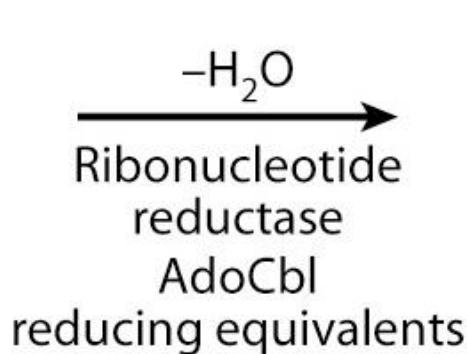
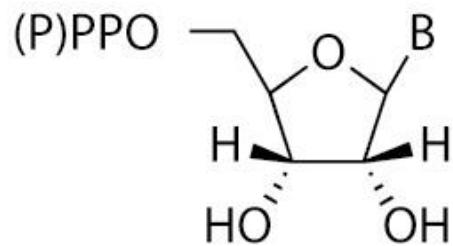
Class I: Carbon skeleton mutases



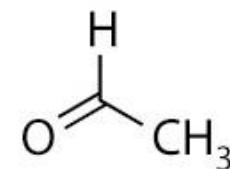
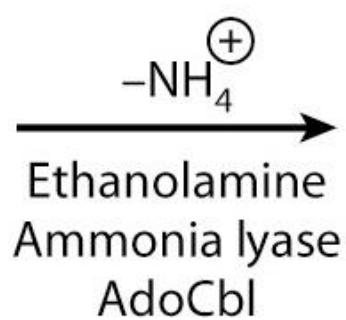
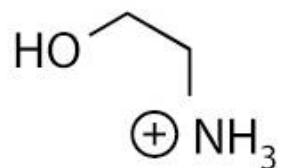
Recent New B₁₂-dependent AdoMet Radical Mutase



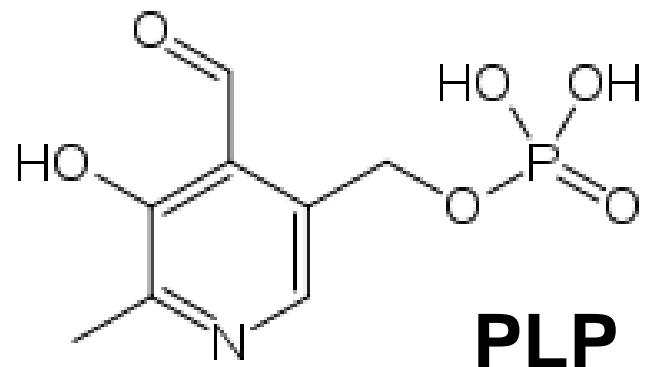
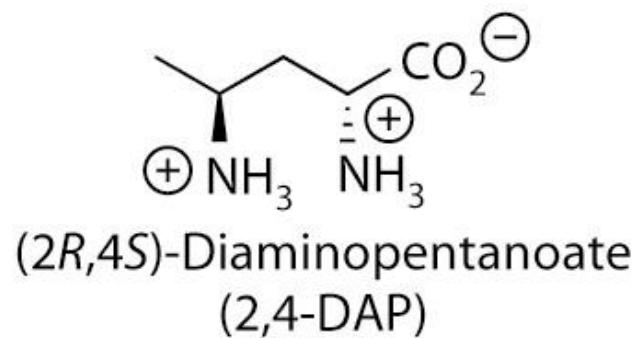
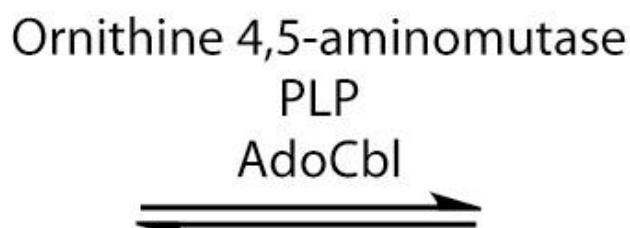
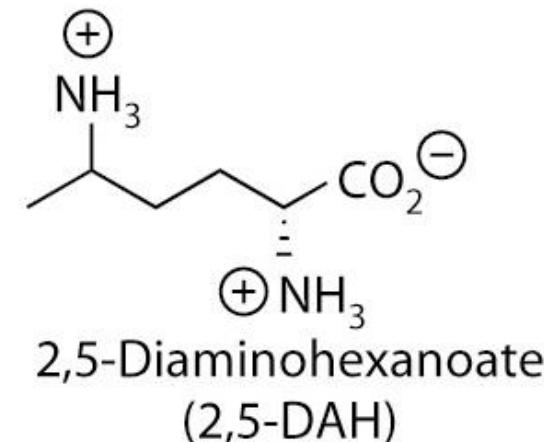
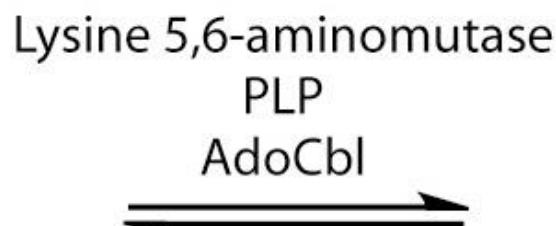
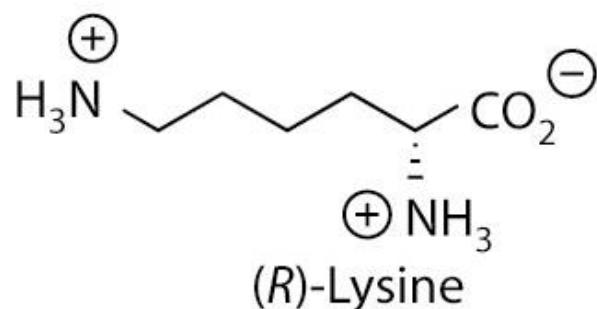
Class II: Eliminases



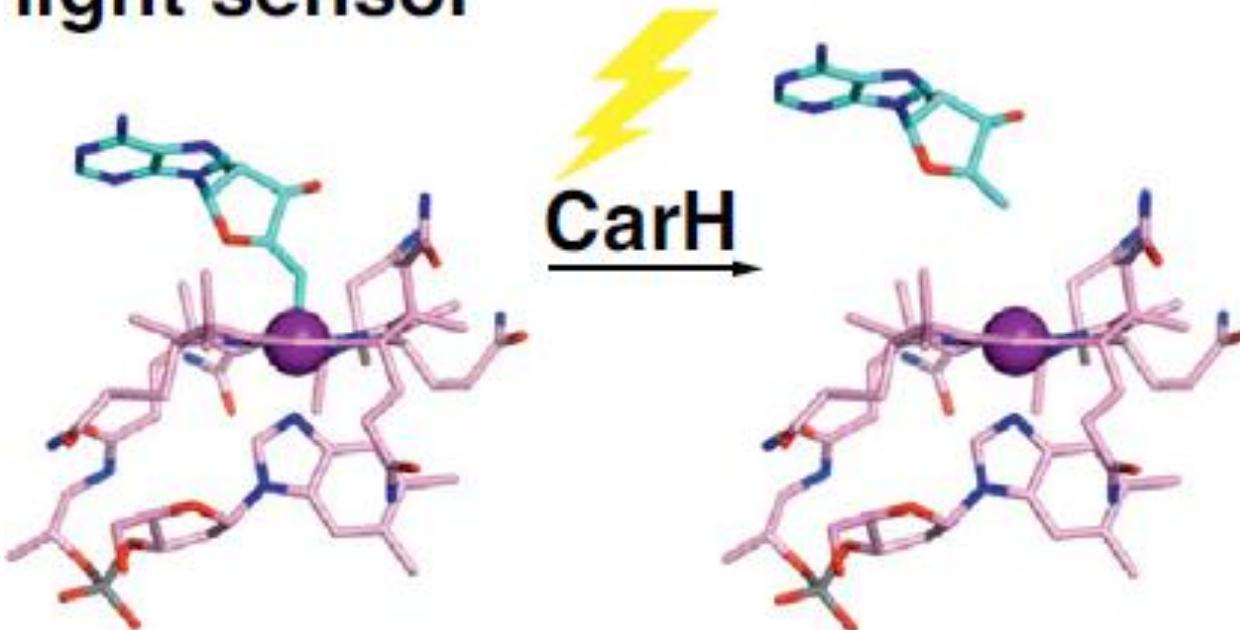
$\text{R} = \text{H}, \text{CH}_3$



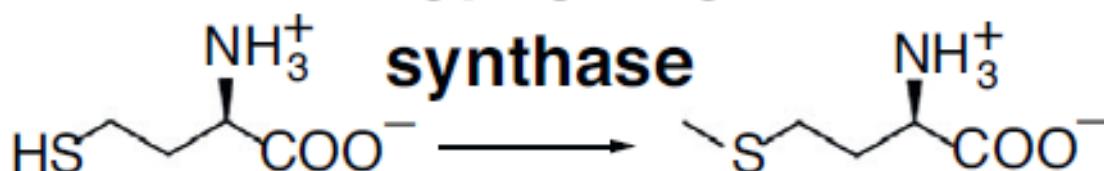
Class III: Aminomutases



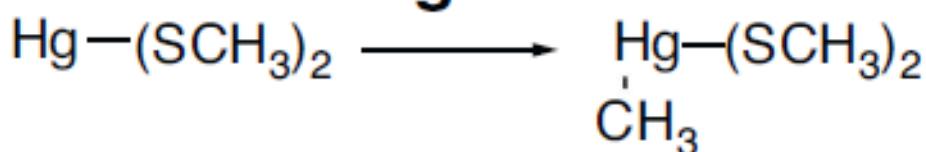
light sensor



methyltransferases methionine

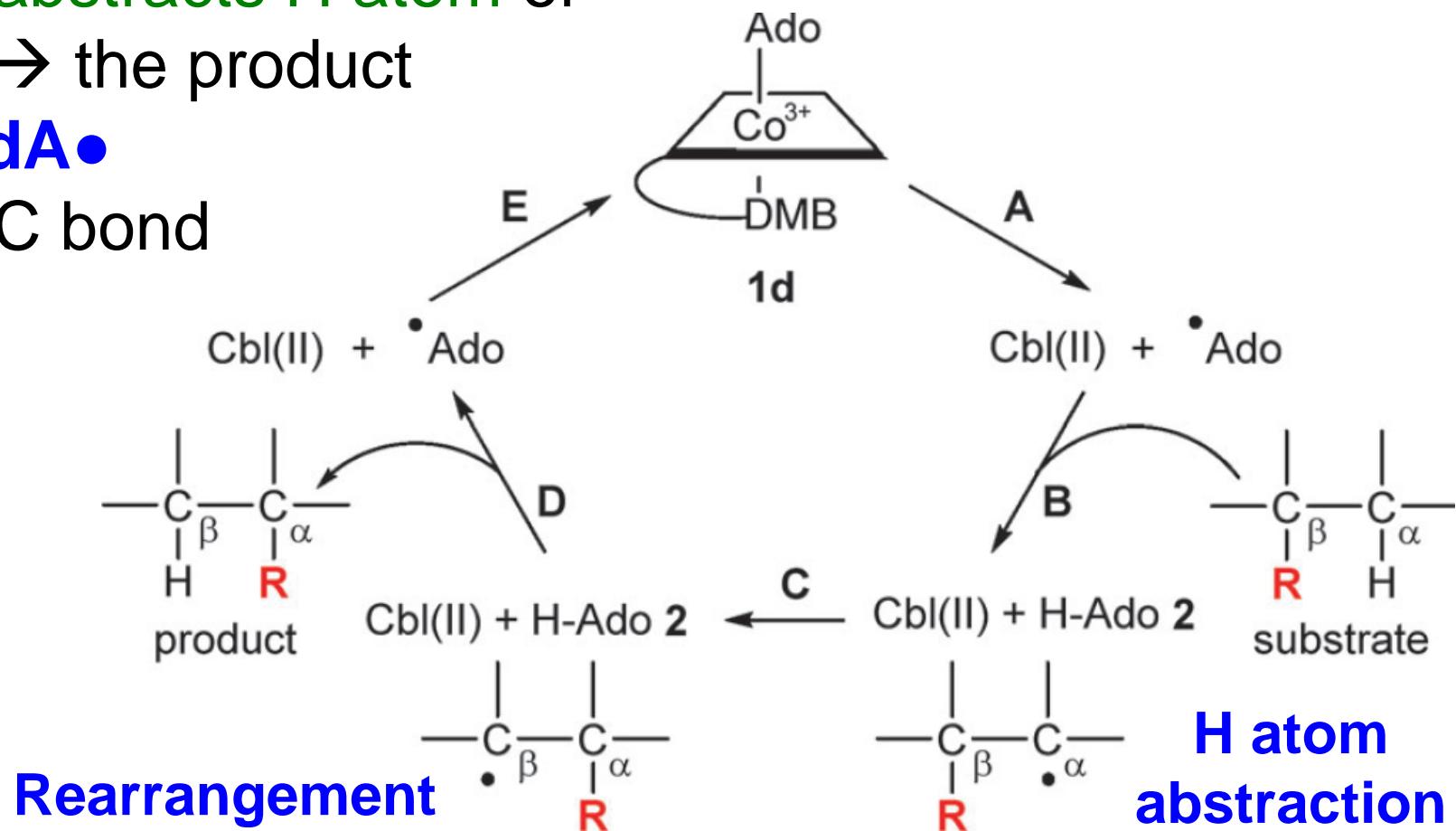


HgcA



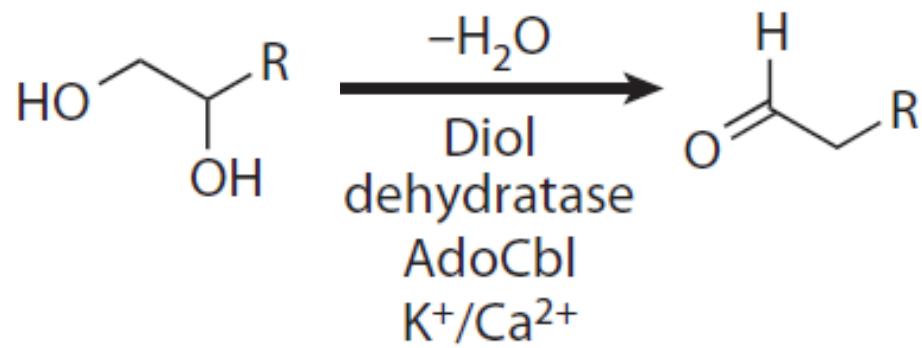
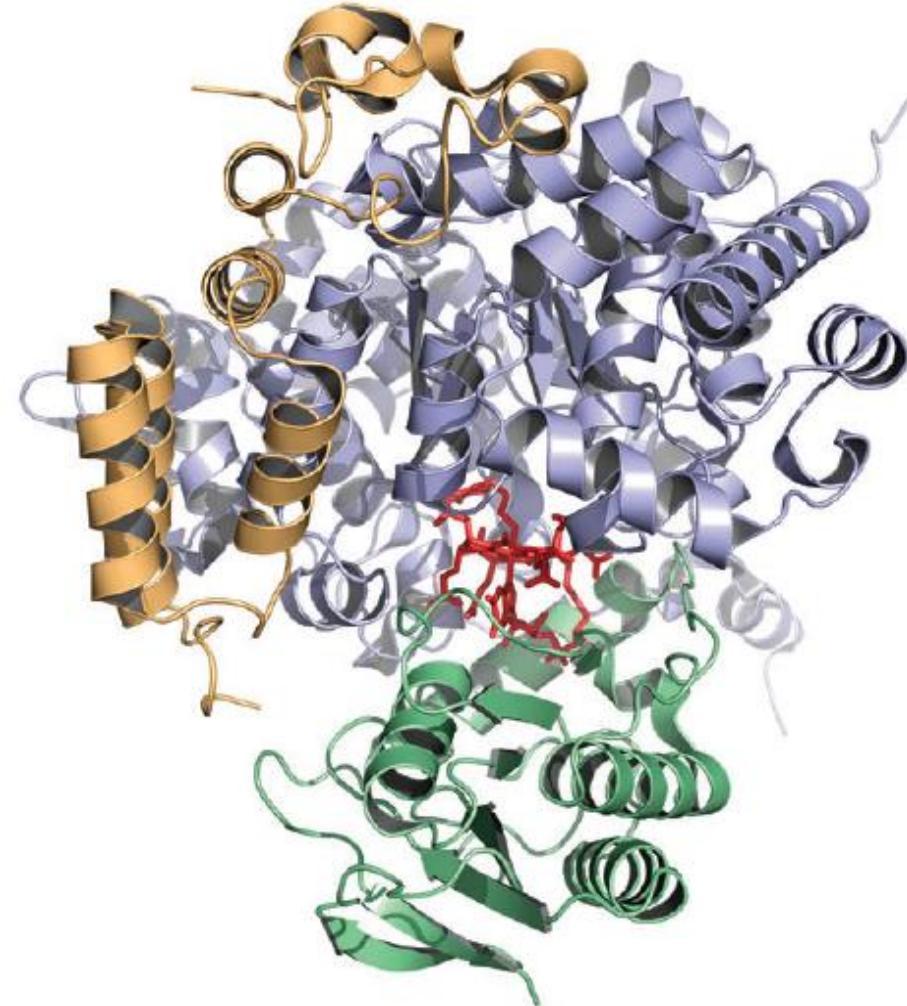
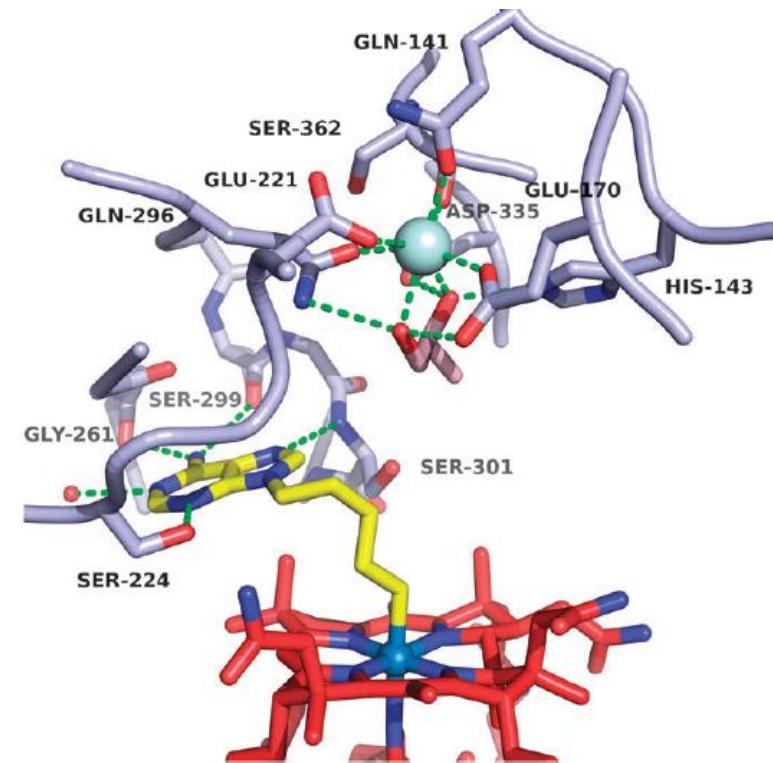
Proposed Generic Mechanism for Rearrangements

- (A) Homolytic bond cleavage \rightarrow 5'-dA \bullet (C \bullet)
- (B) 5'-dA \bullet abstracts a H atom of the substrate \rightarrow substrate radical (S \bullet)
- (C) Rearrangement of S \bullet \rightarrow the product radical (P \bullet)
- (D) P \bullet abstracts H atom of 5'-dA-H \rightarrow the product (P) & 5'-dA \bullet
- (E) Co-C bond reforms

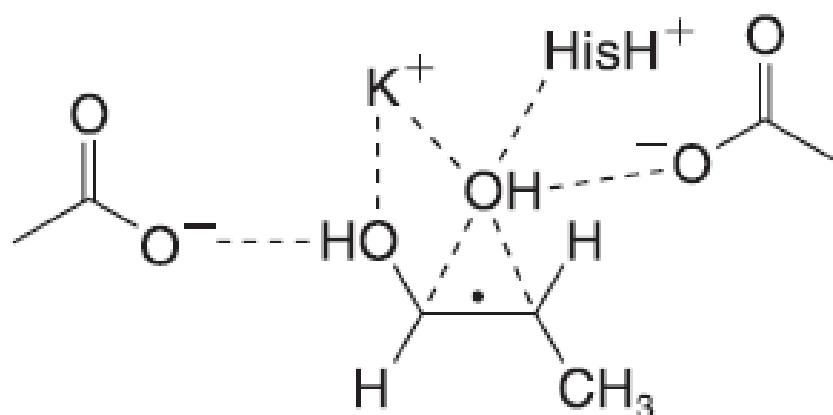
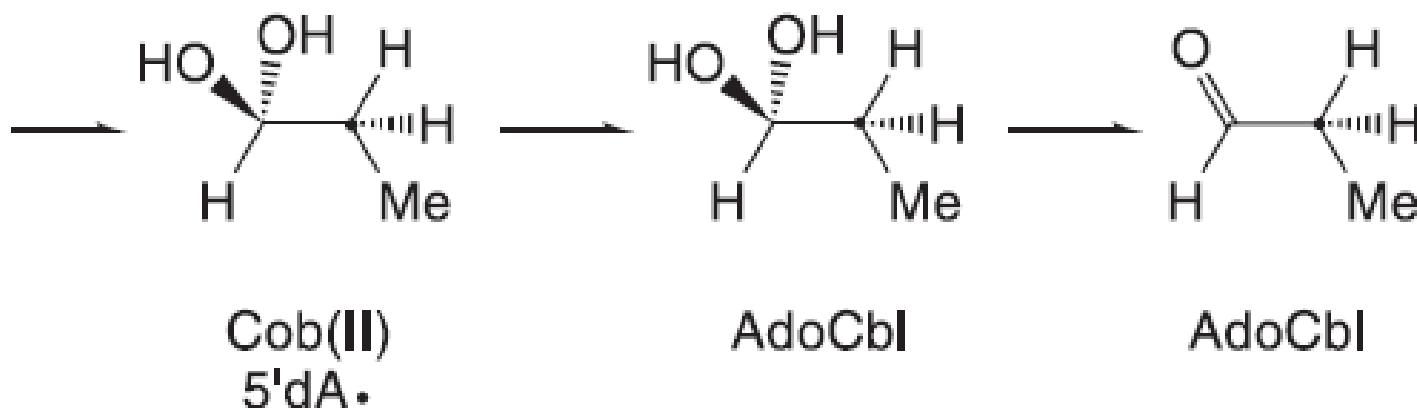
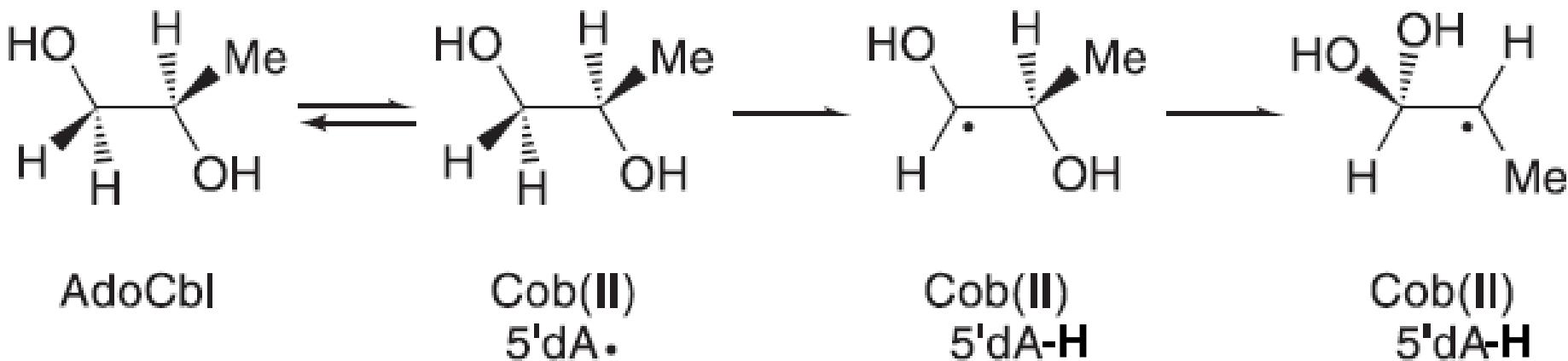


Diol Dehydrase (DD)

- Catalyzes dehydration of propanediol to give propanal.
- Model & structural studies showed that the **cob(II)-alamin** acts as a **spectator** in the **rearrangement** step.

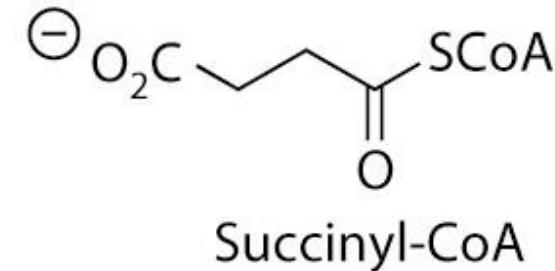
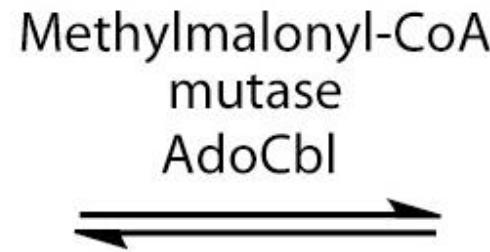
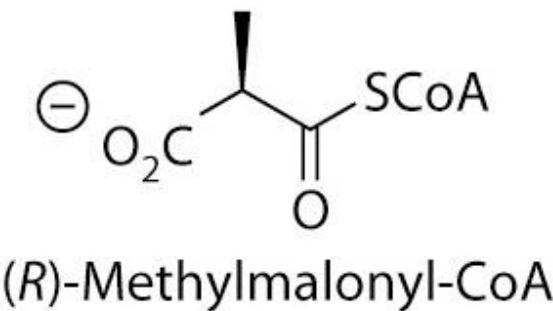
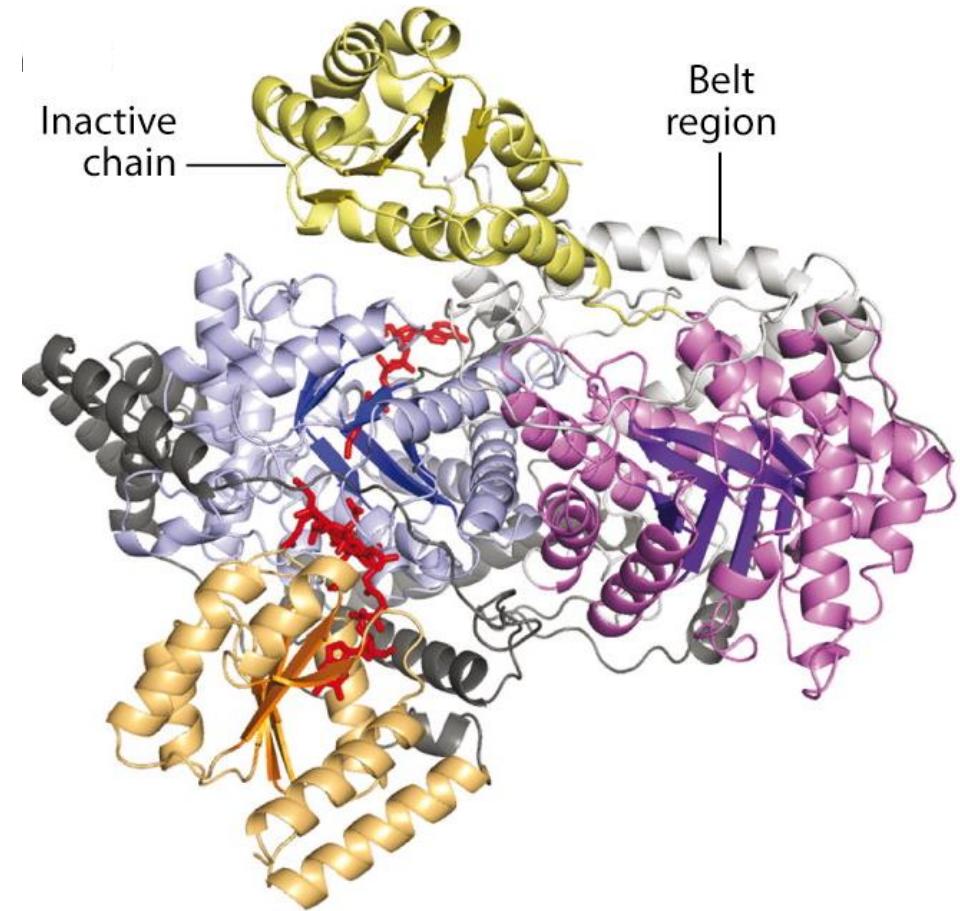


Proposed Mechanism



Methylmalonyl-CoA Mutase (MMCM)

- Catalyzes rearrangement between (*R*)-MMCoA & succinylCoA.
- Similar mechanism as in Diol Dehydrase.
- The role of the Cbl in the Co-C cleavage under debate (reactant destabilization, transition state stabilization; concert with H abstraction?).

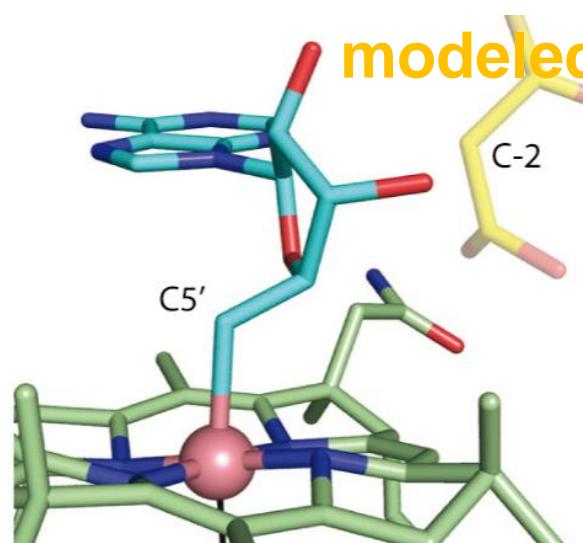
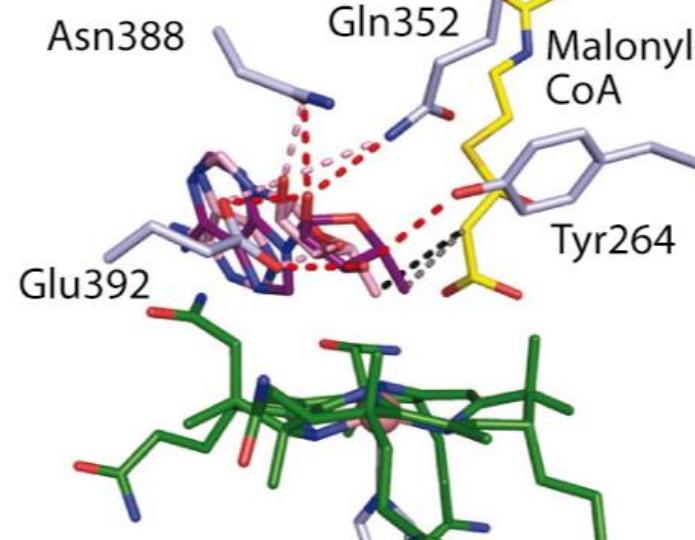
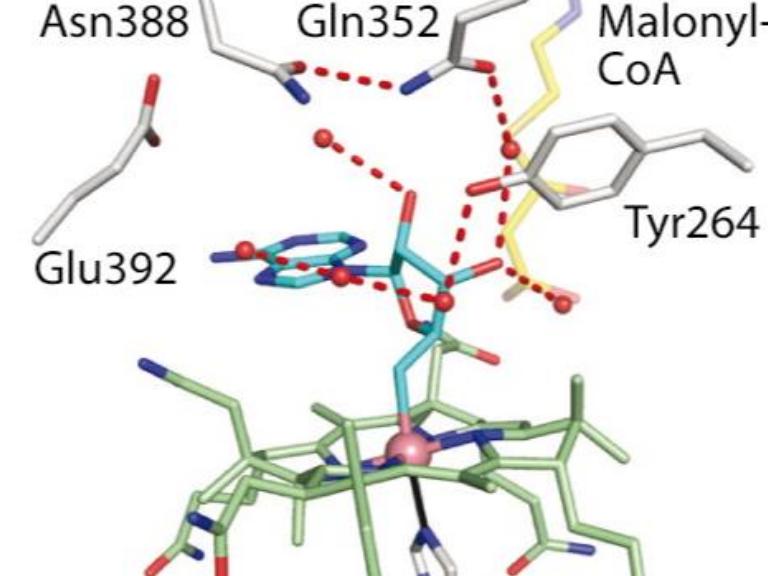


- The enzyme-catalyzed homolytic Co-C bond cleavage to form **5'-dA•** & cob(II)alamin can be **accelerated by** 10^{10} - 10^{11} in Cbls (compared to the non-enzymatic one).

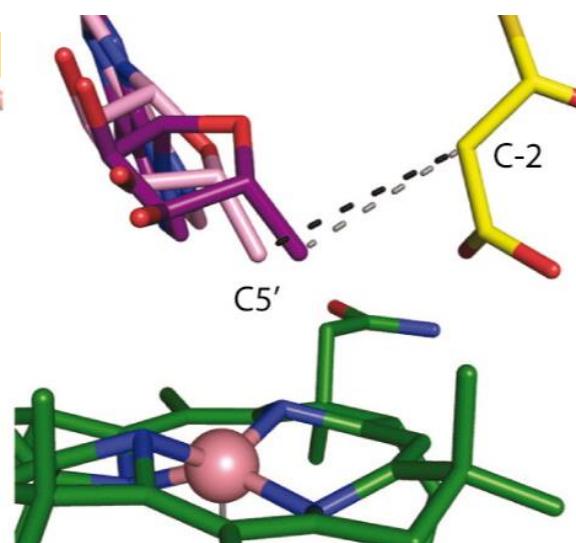
Facts (Co-C bond cleavage):

<u>In aqueous solution</u>	<u>In protein</u>
Rate constant $\approx 10^{-9} \text{ s}^{-1}$	$\kappa_{\text{cat}} \approx 2-300 \text{ s}^{-1}$
Activation barrier $\approx 31 \text{ kcal/mol}$	$\approx 17-16 \text{ kcal/mol}$
Reaction free energy $\approx 23 \text{ kcal/mol}$	$\approx 0 \text{ kcal/mol}$
Equilibrium constant $\approx 8 \times 10^{-18} \text{ M}$	≈ 1

Significant protein effect in Methylmalonyl-CoA Mutase



**the open form
(no substrate)**



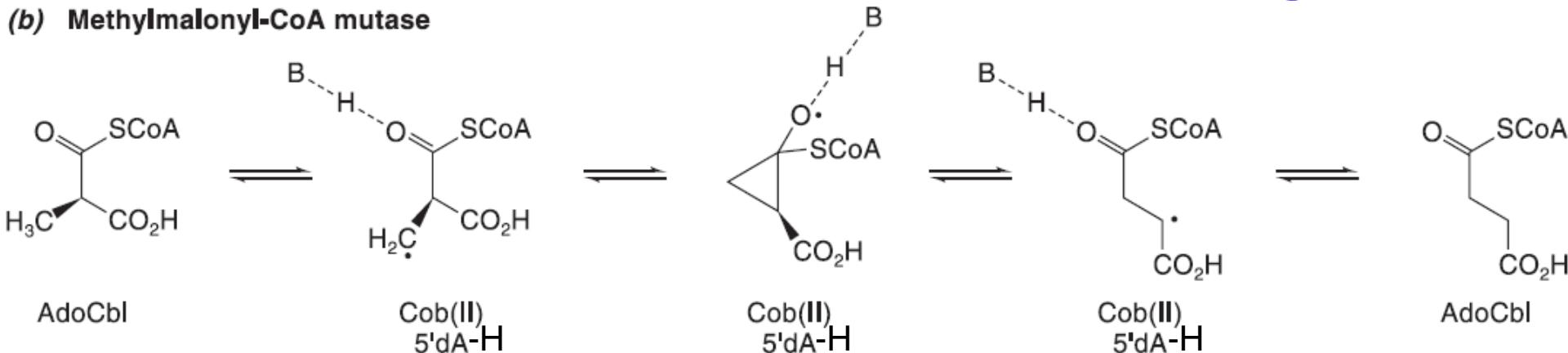
the closed form

Larger structural organization in the open & closed forms for substrate binding & protecting radical intermediates.

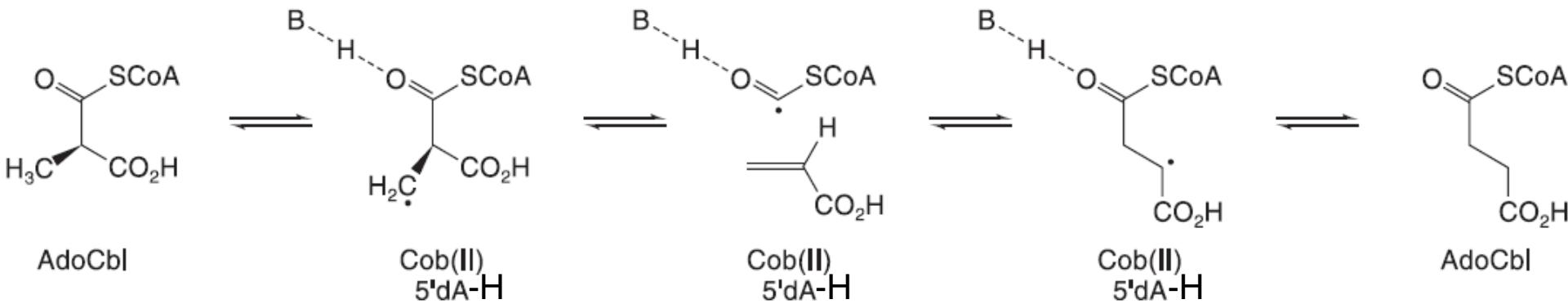
Different conformations of the ribose in Co(III) & (II).

Proposed Mechanism of the Rearrangement

(b) Methylmalonyl-CoA mutase

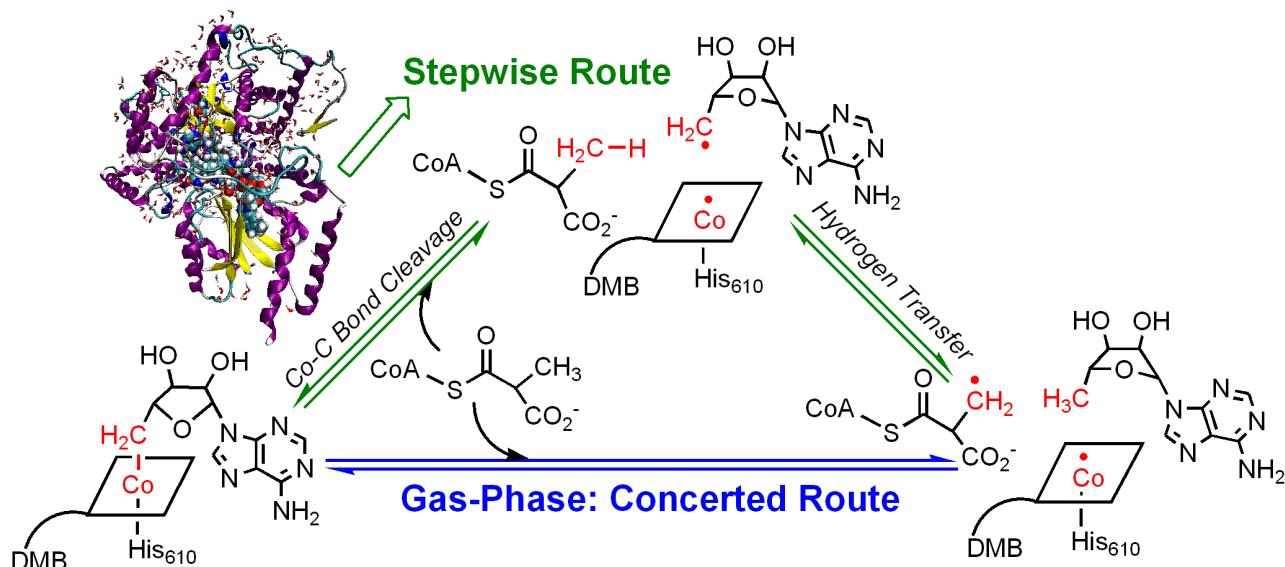
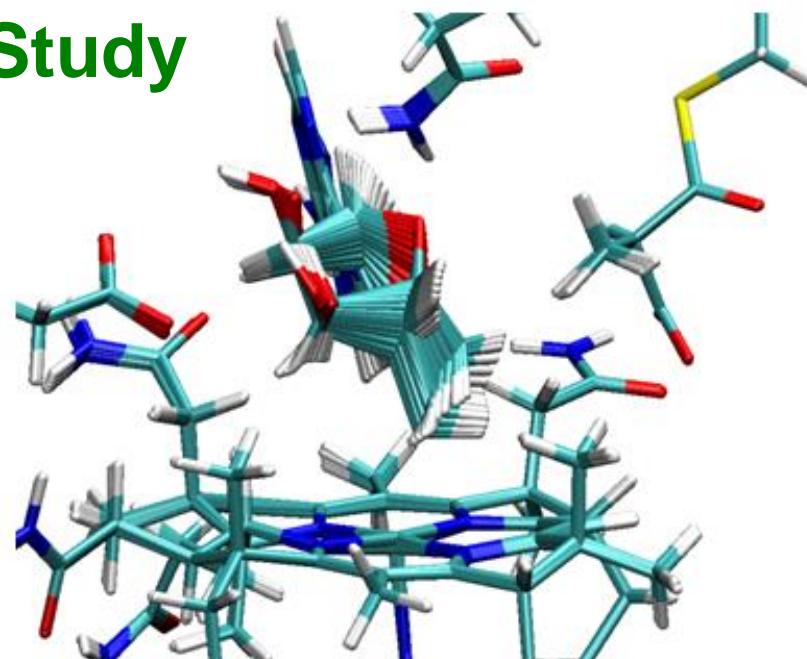
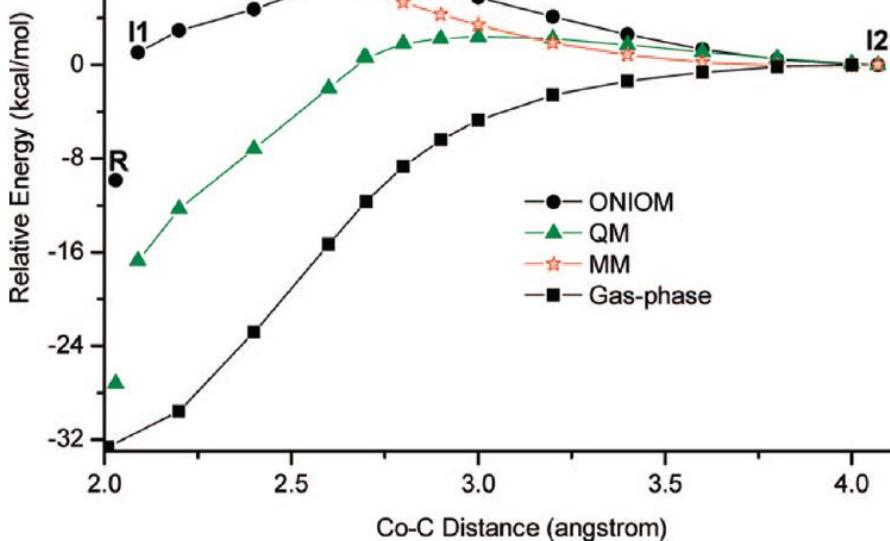


(c)



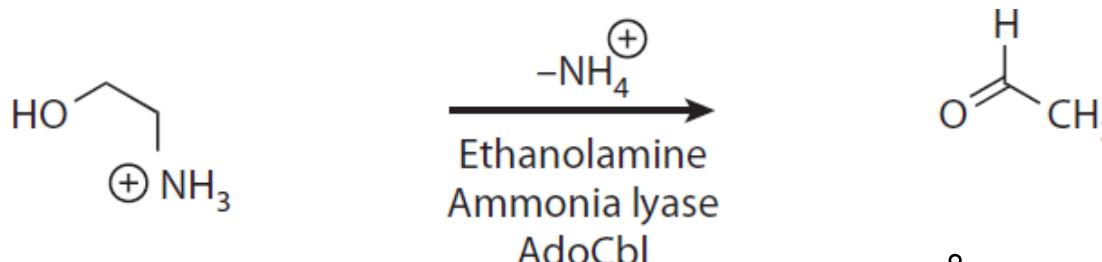
- A cyclopropyl oxy radical intermediate or an acrylate COCoA intermediate via a fragmentation-recombination mechanism.

Our QM/MM Study

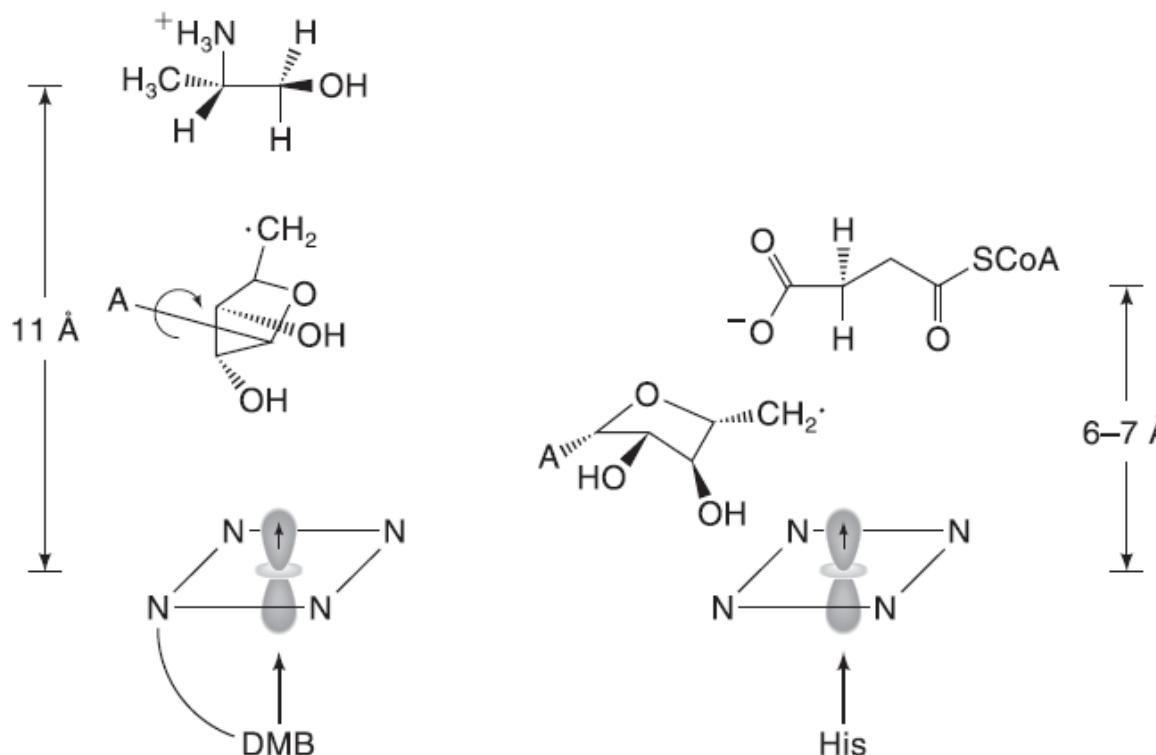


Ethanolamine Ammonia Lyase (EAL)

- Catalyzes the conversion of ethanolamine to MeCHO.

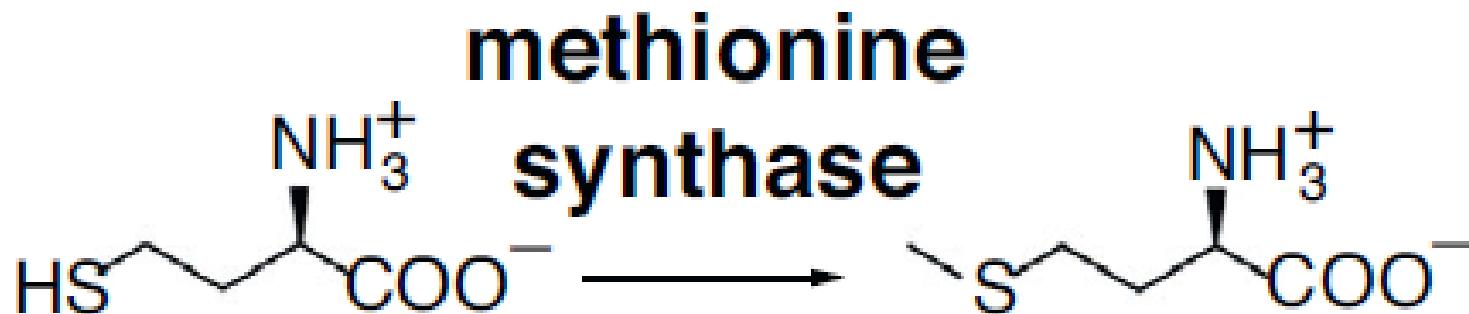


- EPR: the substrate radical is $\sim 11 \text{ \AA}$ from Co(II). After the homolysis, the 5'-dA was proposed to rotate near the substrate. While, that distance is $\sim 6-7 \text{ \AA}$ in MMCM.



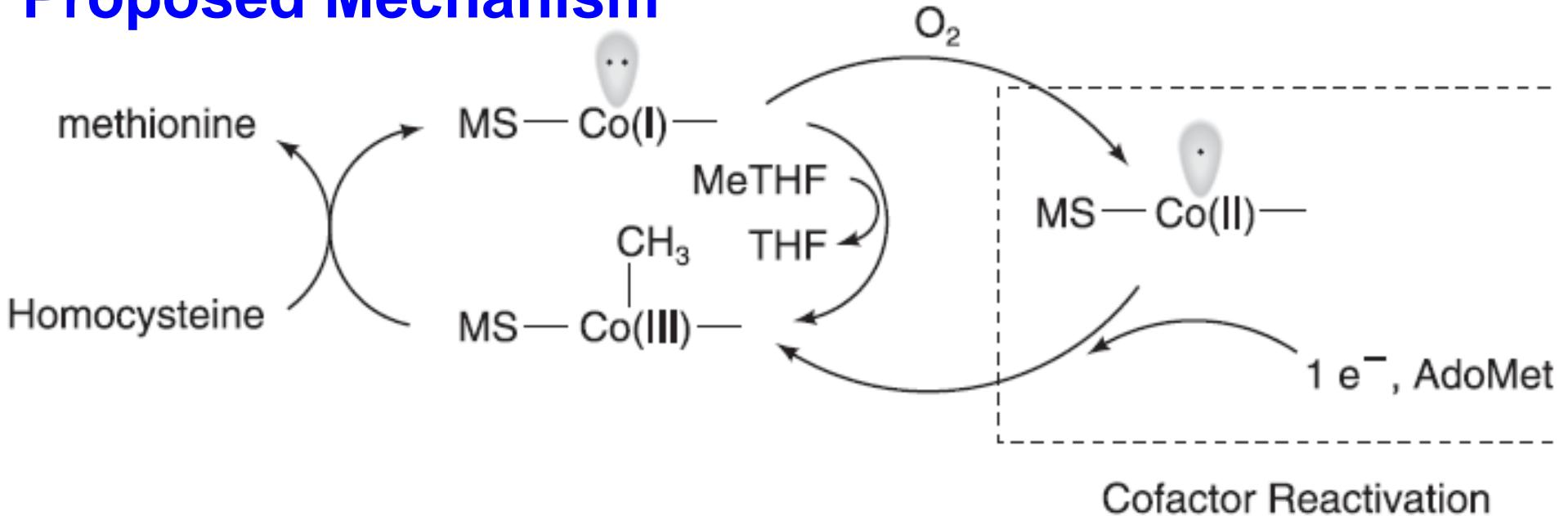
MeCbl-dependent Methionine Synthase (MS)

- A key role in one carbon metabolism.
- Convert homocysteine (activated by Zn^{2+}) into Met by using **3 organic cofactors**, **MeCbl**, N^5 -methyl tetrahydrofolate (**N^5 -MeTHF**), & S-adenosyl-methionine (**AdoMet**, or known as **SAM**).

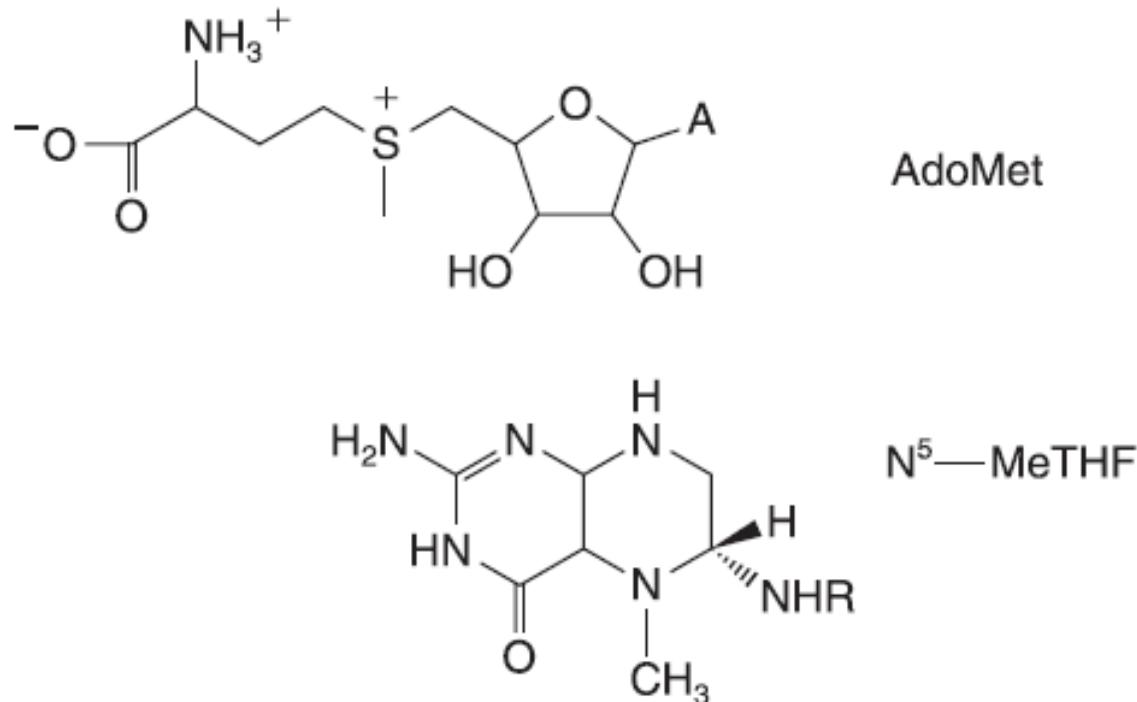


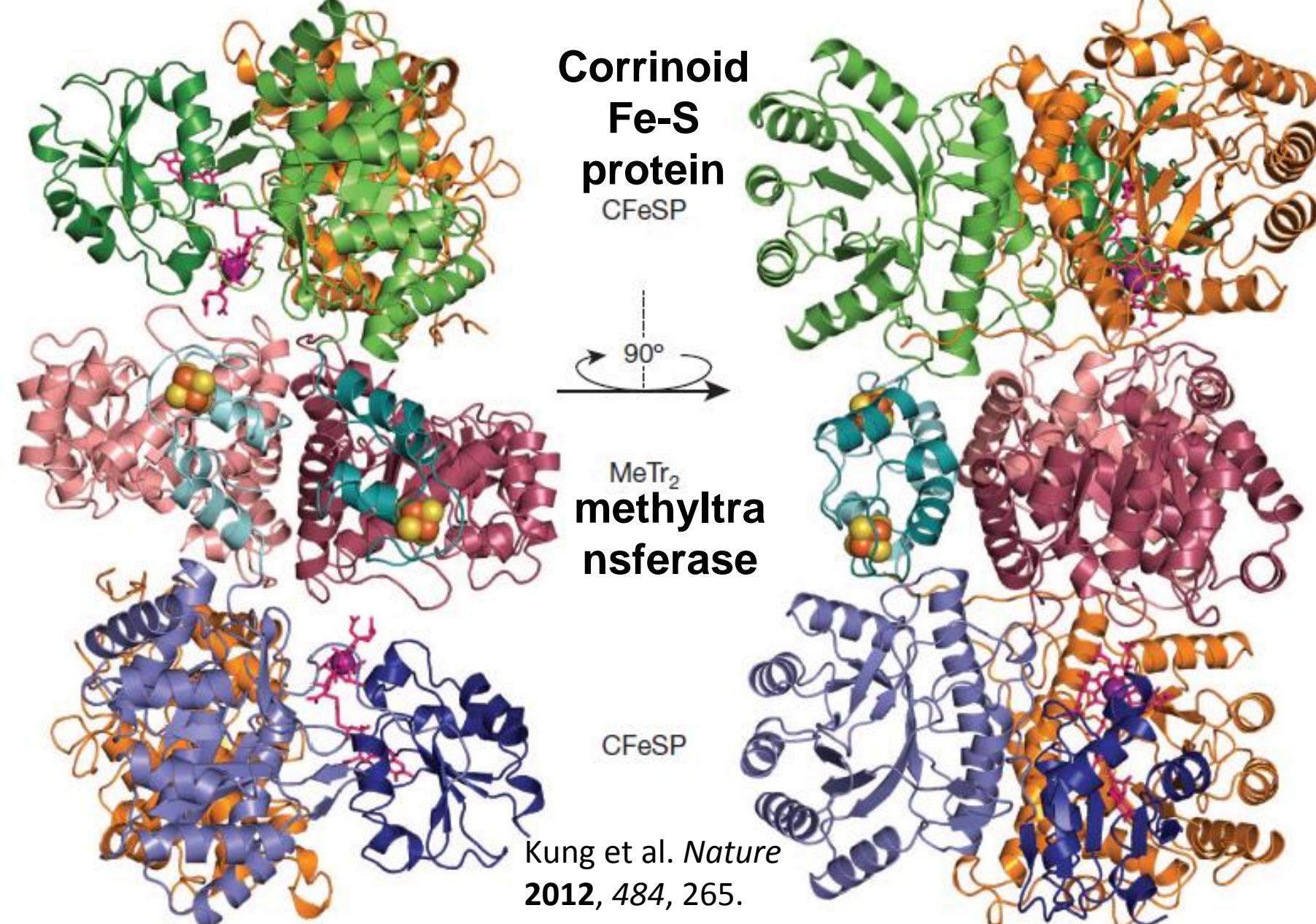
- A **large modular enzyme**: undergo reorganization to adopt multiple **conformations** for **activation**, **protection** & **catalysis** on the Cbl cofactor.

Proposed Mechanism

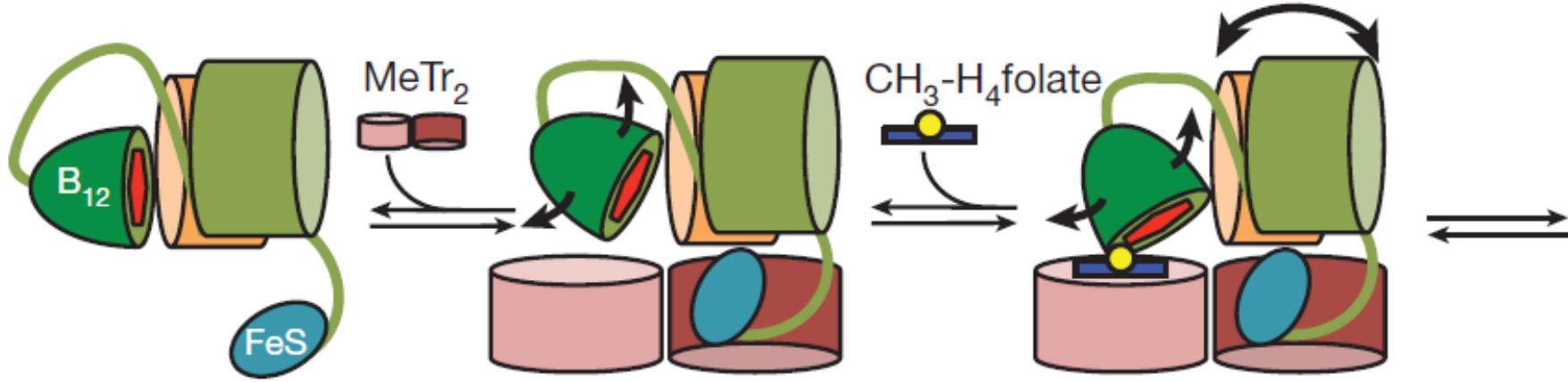


- Conversion with retention of configuration: likely two $S_{\text{N}2}$ reactions (not SET or oxidative addition).





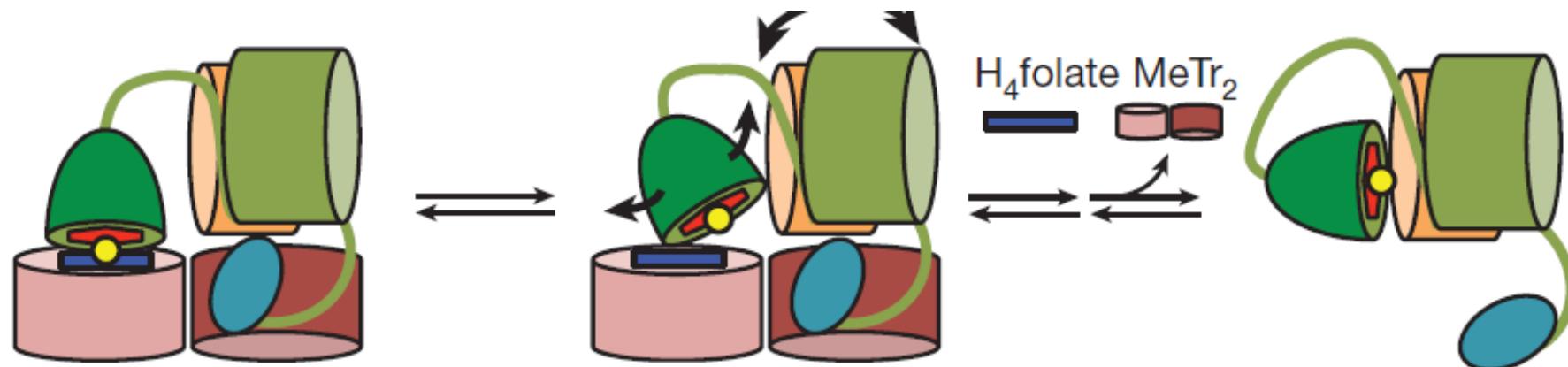
- *E. coli* MS has 4 modules connected by flexible linkers, one of the domains as a Cbl-binding domain.



“Resting” CFeSP:
Co(I) protected
(*Ch*CFeSP structure)

Folate-free complex:
 B_{12} “en route”
(this work)

$CH_3\text{-H}_4\text{folate}$ -bound complex:
 B_{12} equilibrium shifted
(this work)



‘Folate-on’ conformation:
Methyl transfer reaction
(transient)

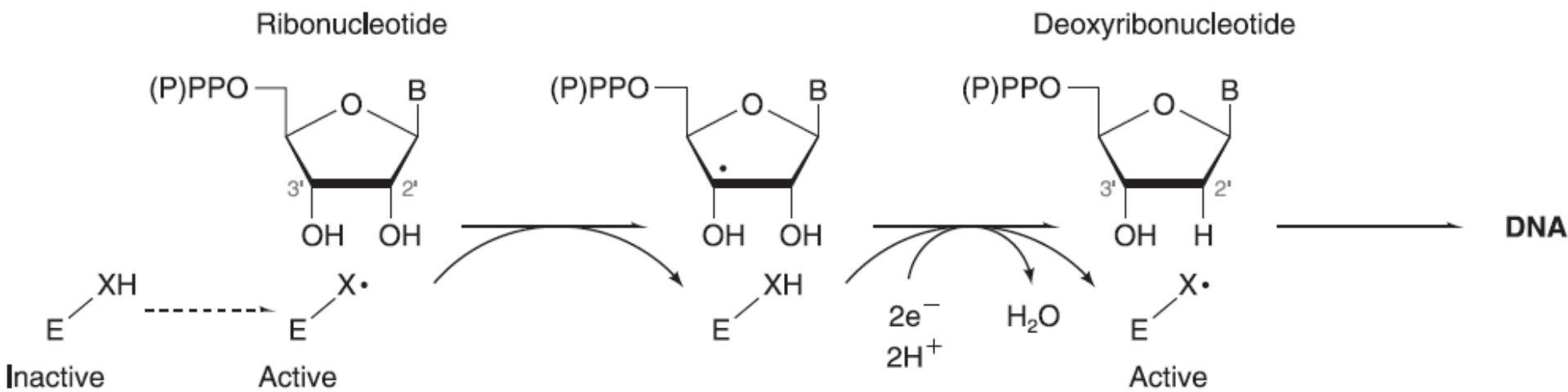
$H_4\text{folate}$ -bound complex:
 B_{12} equilibrium shifted
(this work)

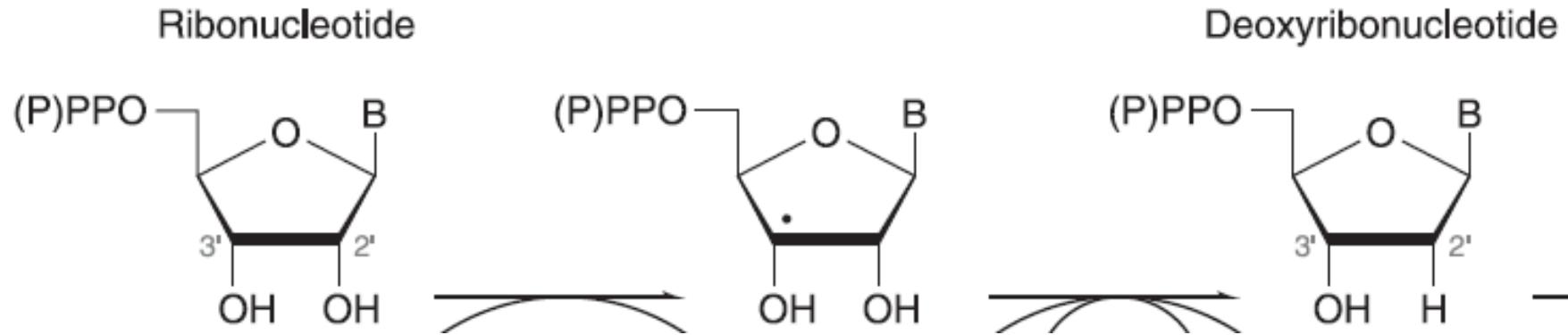
Product bound:
 $CH_3\text{-Co(III)}$ protected

3. Ribonucleotide Reductases (RNRs)

Ribonucleotide Reductases (NRs)

- Synthesis of deoxyribonucleic acids (DNAs) rely on 4 deoxyribonucleotides. The related ribonucleotides are **reduced** to form deoxyribonucleotides.
- Such reduction is catalyzed by allosterically regulated ribonucleotide reductases (**NRs**), which involves a **Cys radical** ($X = S$) to initiate the reaction (**H abstraction at the 3'** position of the substrate).





- Many deoxygenation of alcohols via ionic mechanisms are not (poly)functional group tolerant.
- S_N2 at C2' is blocked by steric & electronic factors. S_N1 at C2' is unfavorable, due to the adjacent electron-poor C.
- Deprotonation at C2' to form an anionic intermediate leads to elimination of the base (B) at the C1'.
- **Radical** reactions: the **only feasible way** for the reduction of the nucleosides.

- Three classes of RNR enzymes involve the formation of a stable radical precursor:

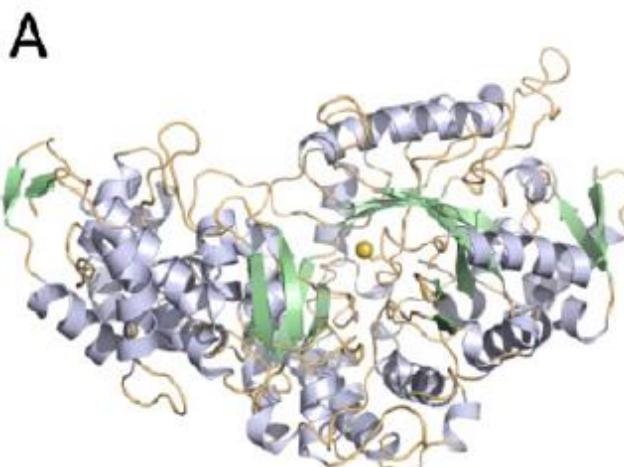
Class I: a tyrosyl radical

Class II: a Ado radical

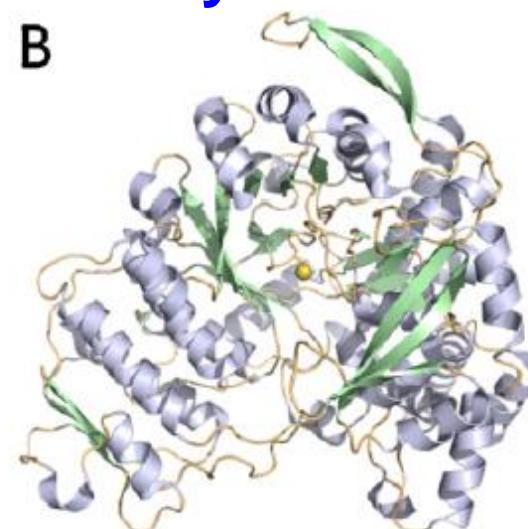
Class III: a glycyl radical

A redox-active metal center is required.

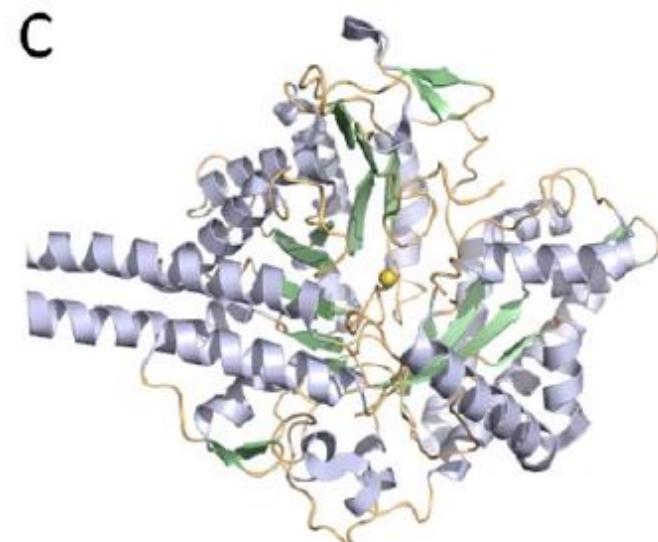
Catalytic subunit



Class I



Class II

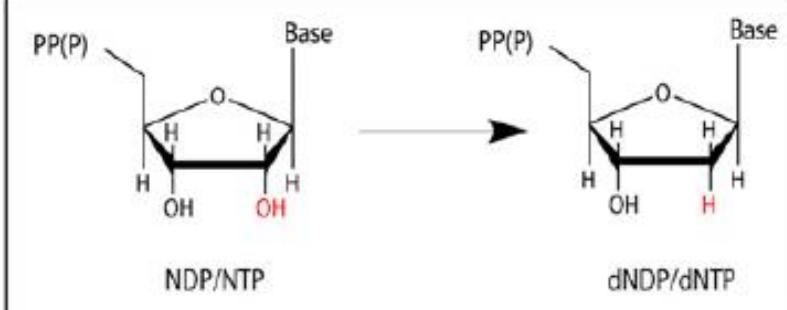


Class III

Electron donor

Thioredoxin
reductase

NADPH



Catalytic subunit

NrdA
(R1)

NDP

dNDP

Cys*

NrdE
(R1E)

NDP

dNDP

Cys*

NrdA
(R1)

NDP

dNDP

Cys*

NrdJ

NDP/NTP

dNDP/dNTP

Cys*

Formate

CO_2

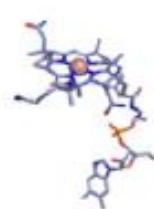
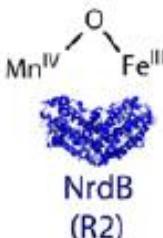
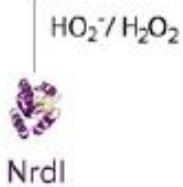
NrdD

NTP

dNTP

Cys*

Gly*



Adenosyl
cobalamin (B₁₂)



S-adenosyl
methionine



Radical initiator

Ia

Ib

Ic

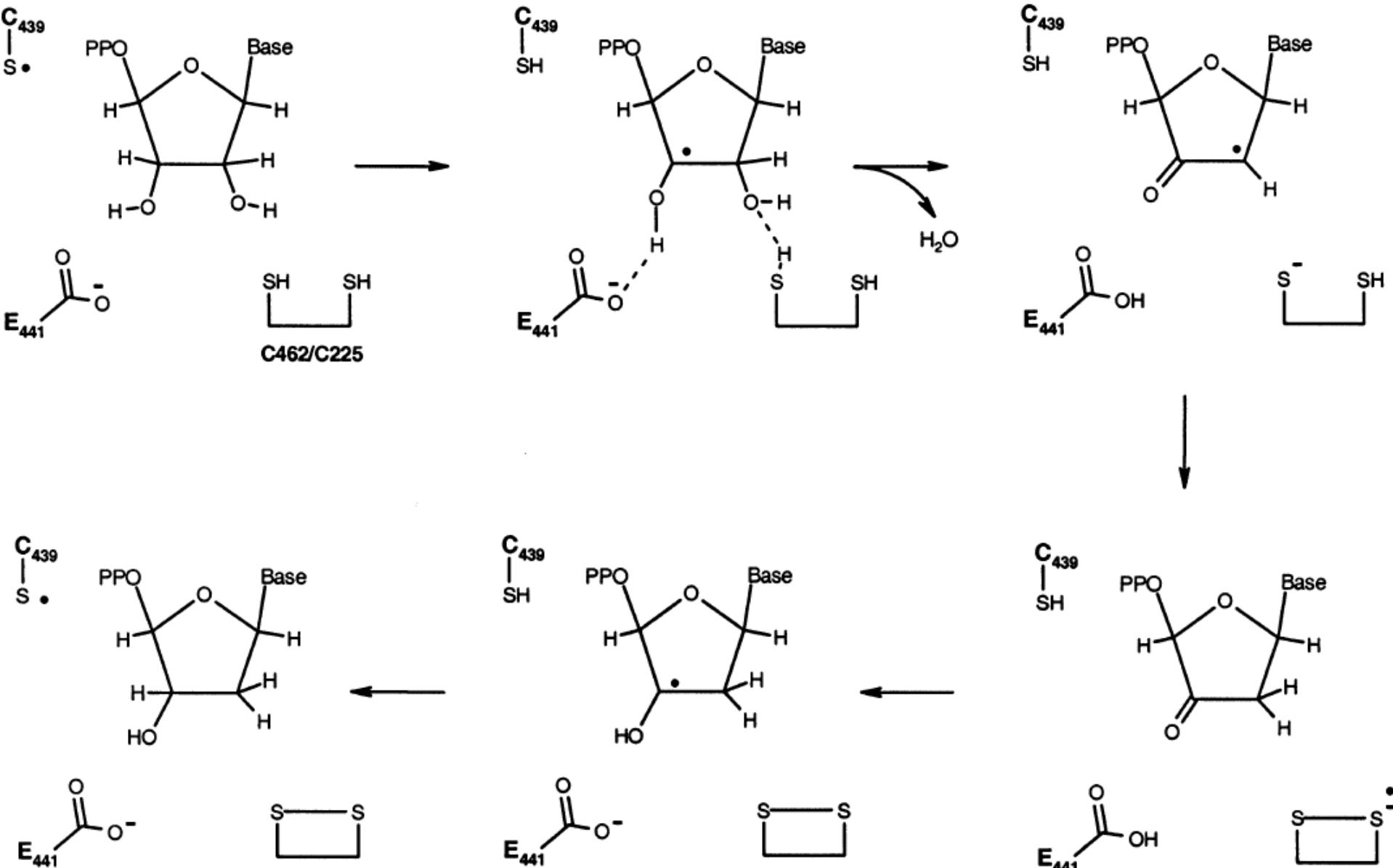
Class

II

III

Coord. Chem. Rev.
2013, 257, 3

Proposed General Mechanism



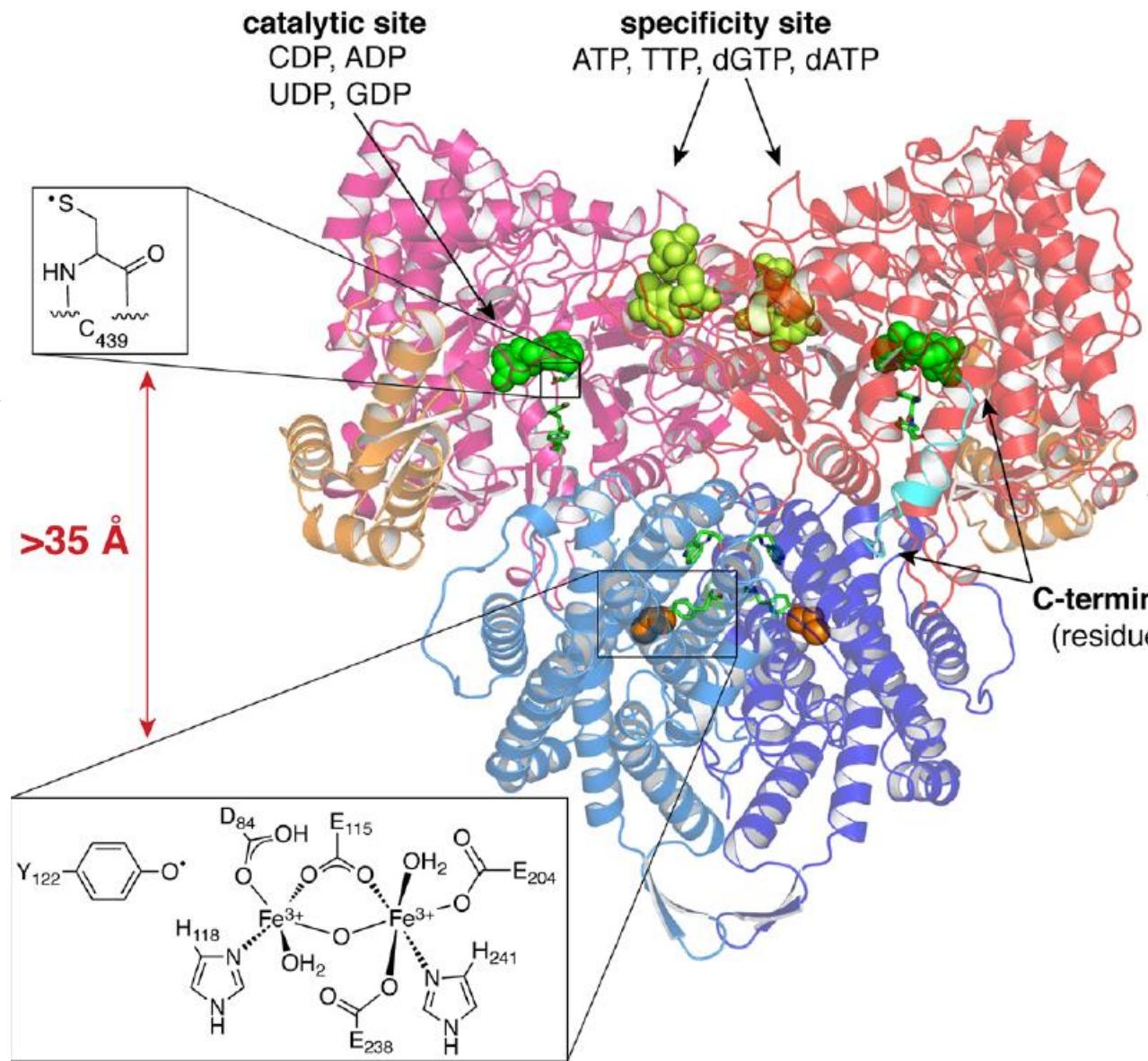
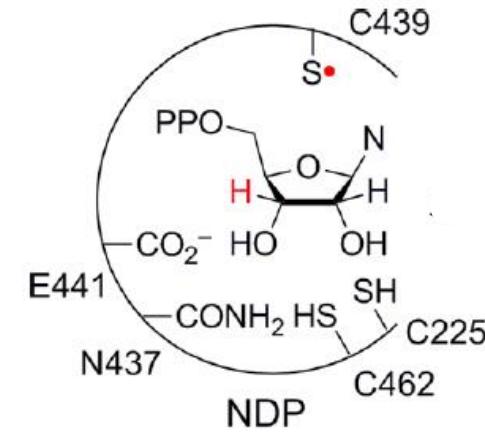
Class I RNR

- Strictly aerobic $\alpha_2\beta_2$ enzymes.

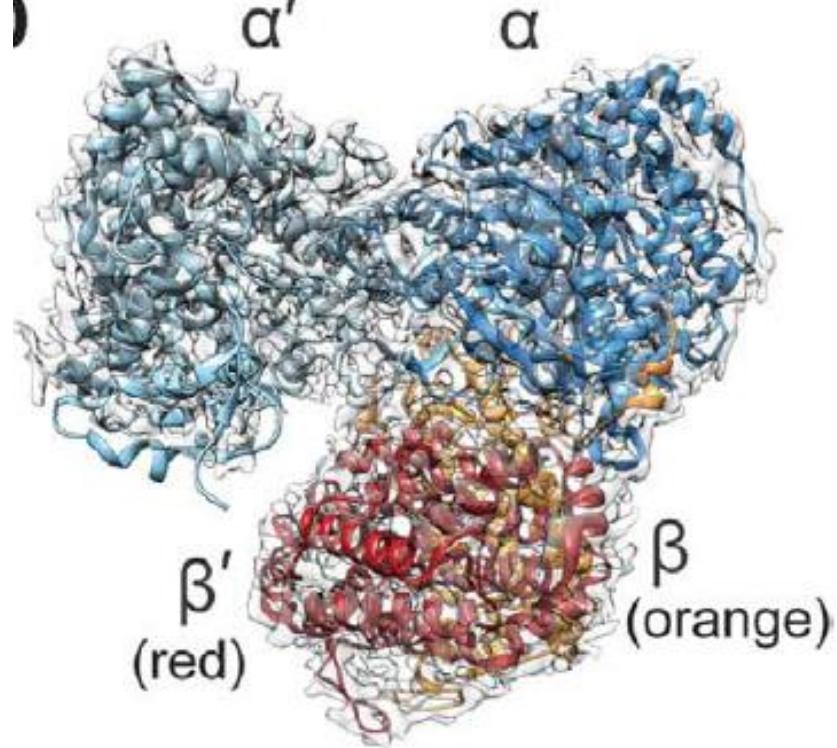
- Proteins **R1** (α_2) & **R2** (β_2).

- Protein **R1** has the binding sites for substrates & allosteric effectors.

The **substrate binding site** has **3 conserved Cys**.

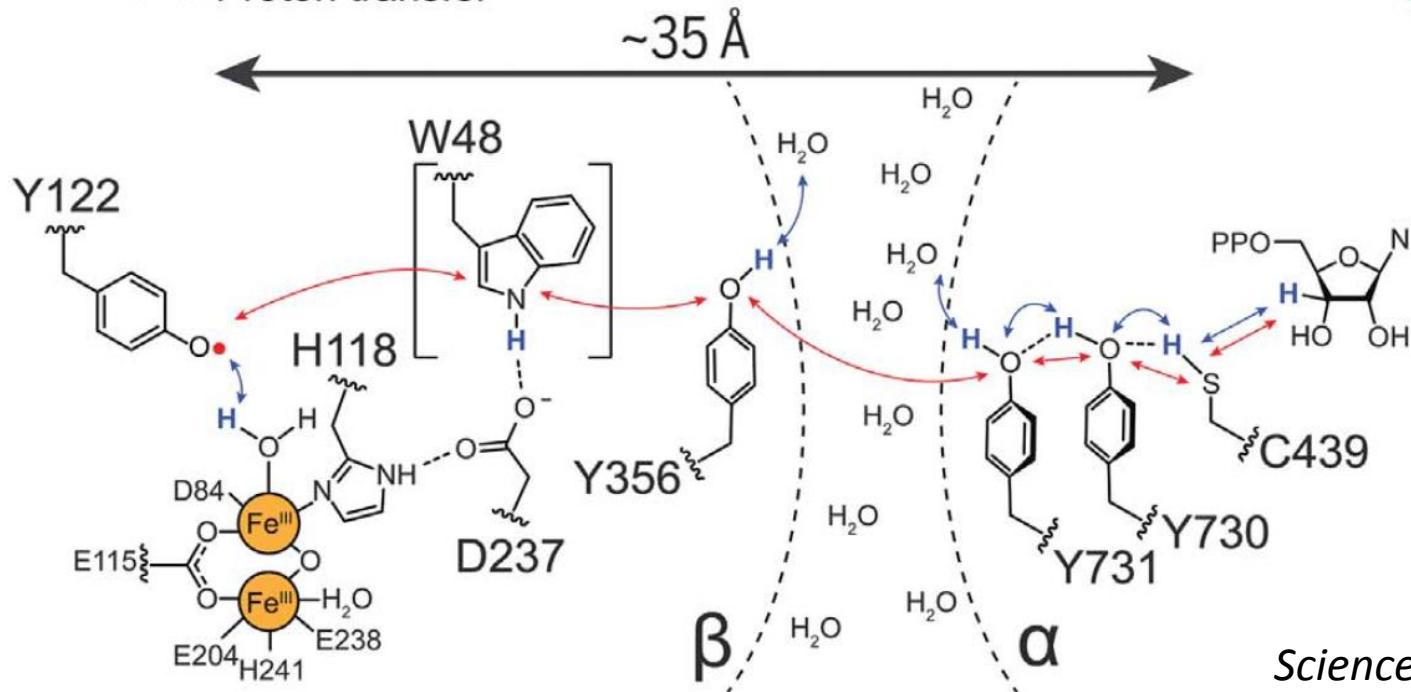


Structure of an active a2b2 RNR complex (cryo-EM)

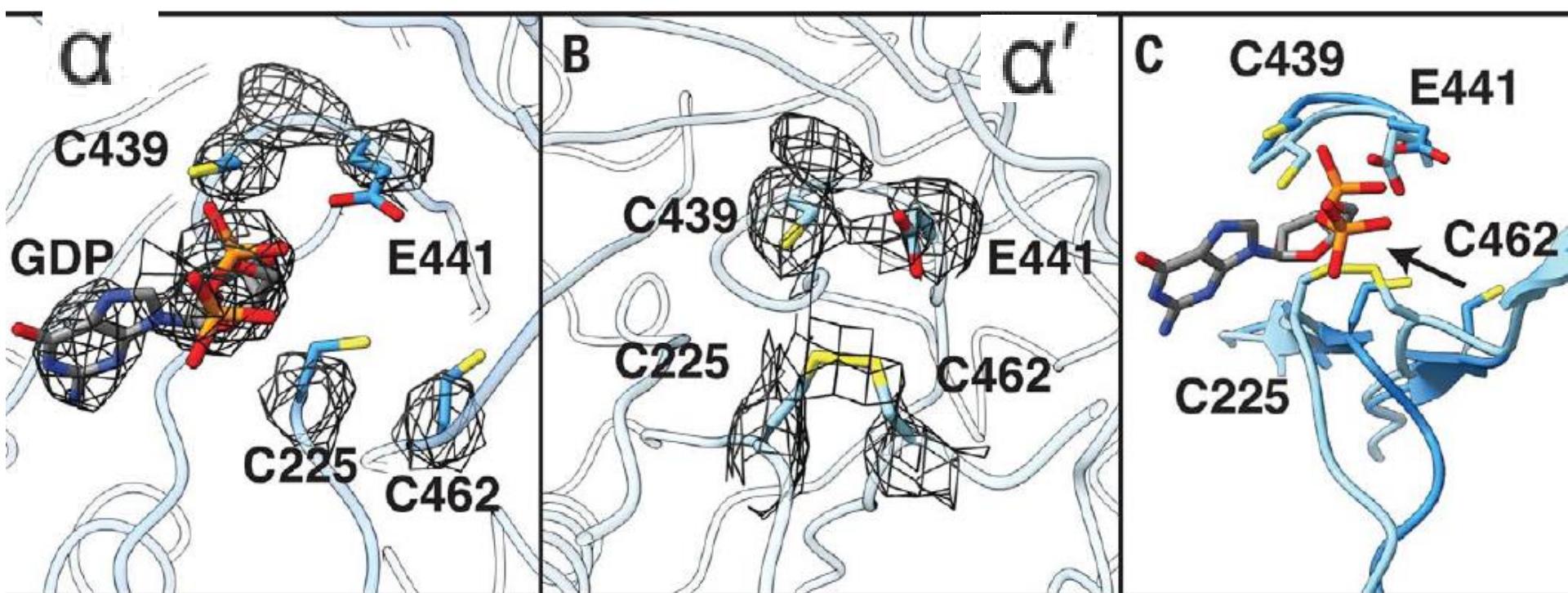


B

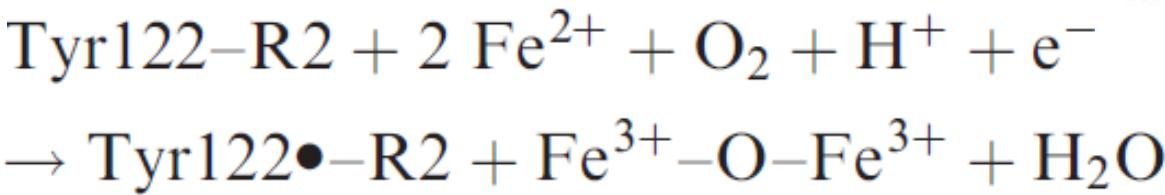
↔ Electron transfer
↔ Proton transfer



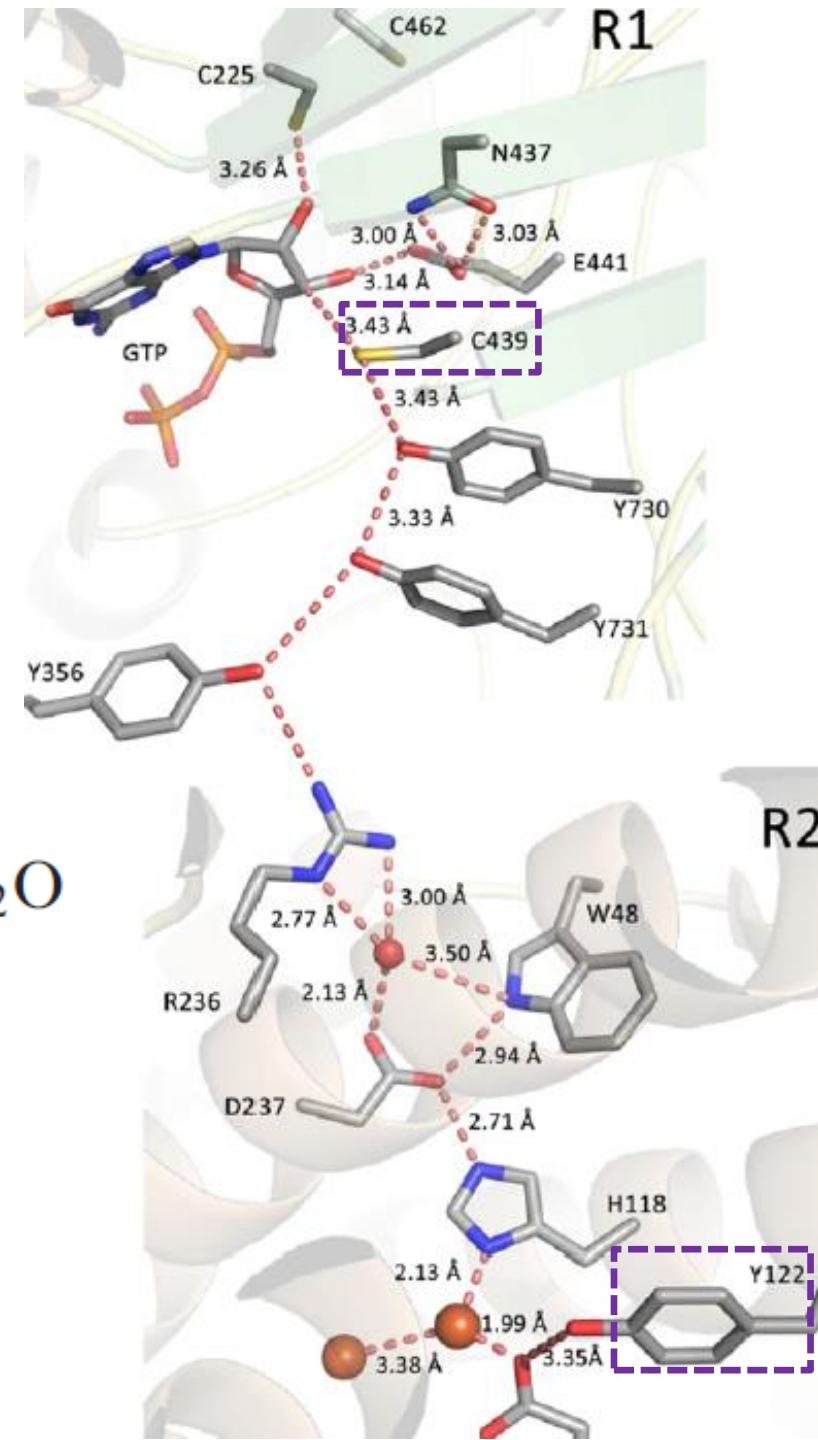
Structure of an active a2b2 RNR complex (cryo-EM)



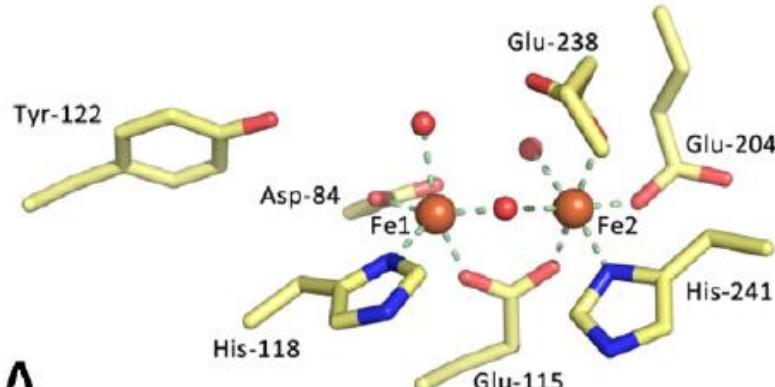
- Protein **R2**: a **non-heme diiron center** (ferric ions (Mn ions in some cases) with an oxo & a Glu bridging ligands), & a **Tyr radical**. The Tyr radical is formed from the metal ions with O_2 .



- This Tyr radical (R2) generates a transient **Cys radical** on protein **R1**, which **activates the substrate**.



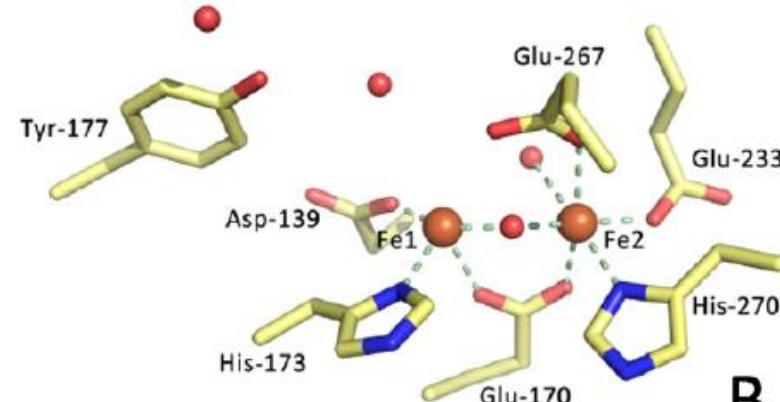
Escherichia coli



A

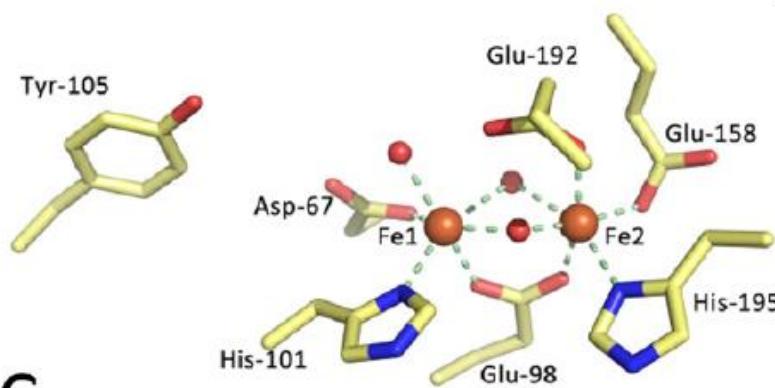
class Ia

Mus musculus



B

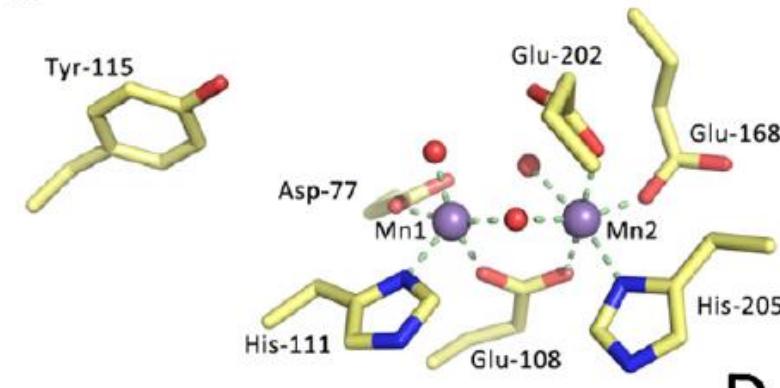
Salmonella typhimurium



C

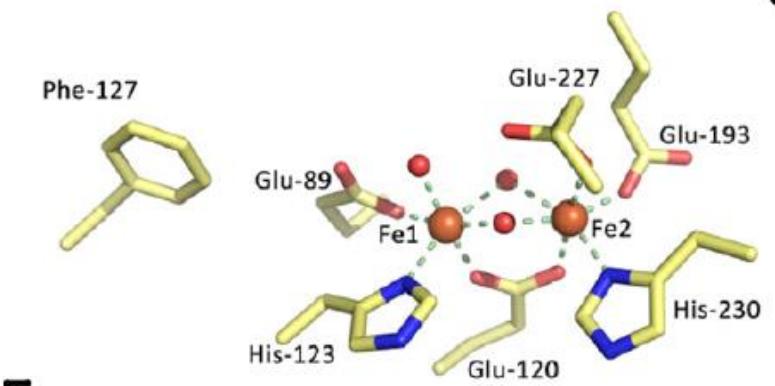
class Ib

Corynebacterium ammoniagenes

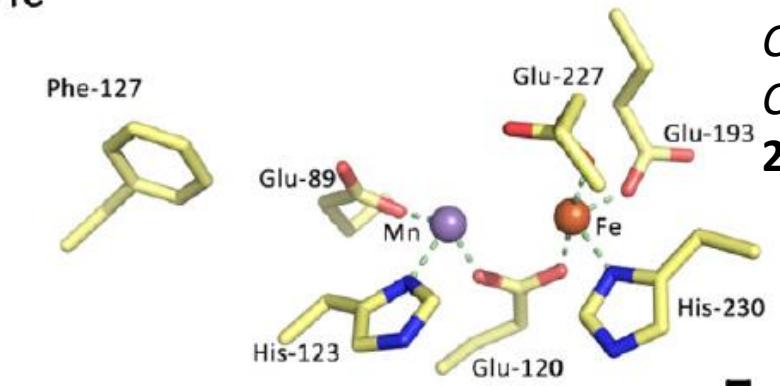


D

Chlamydia trachomatis

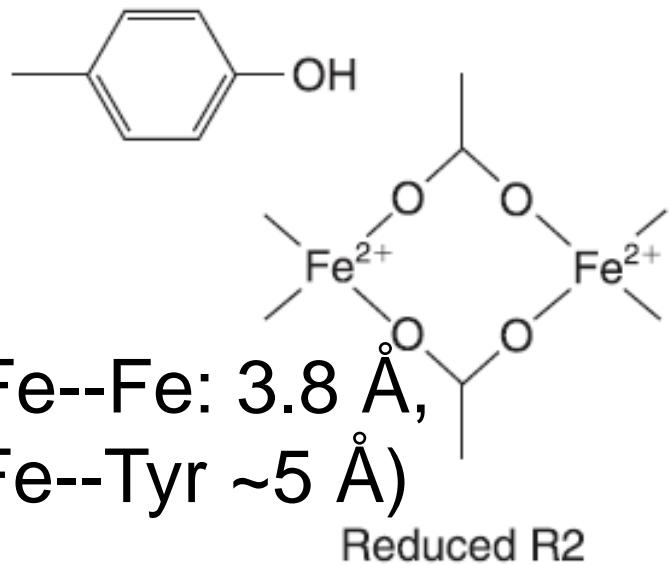


class Ic



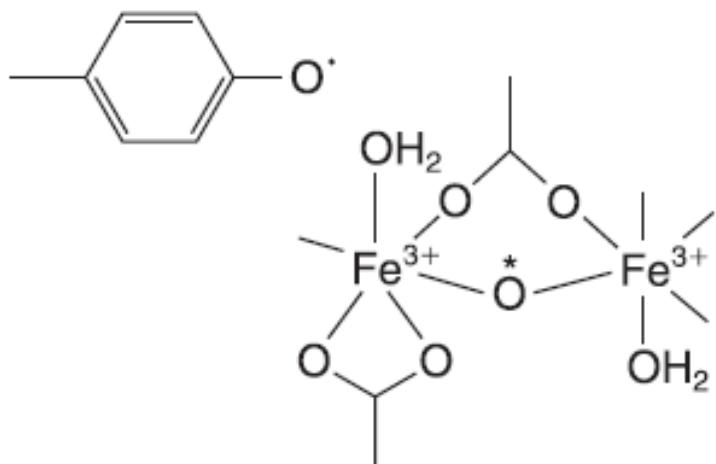
Coord.
Chem. Rev.
2013, 257, 3

Proposed Mechanism

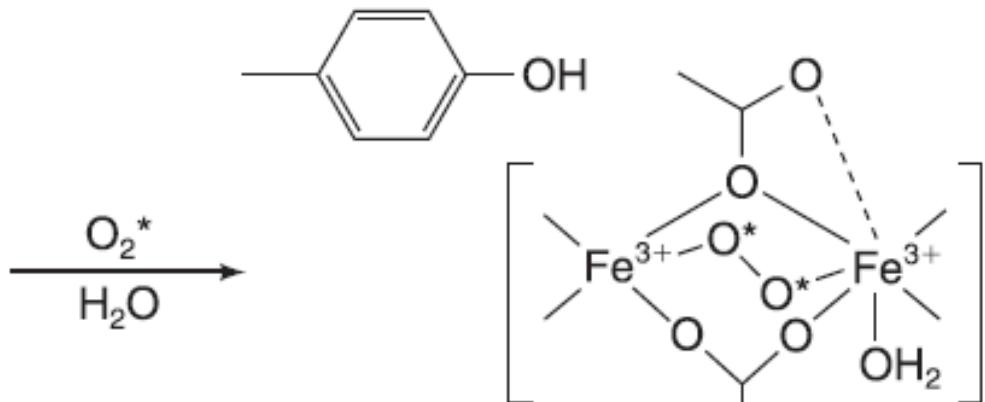


(Fe--Fe: 3.8 Å,
Fe--Tyr ~5 Å)
Reduced R2

ligand reorganization



Active R2

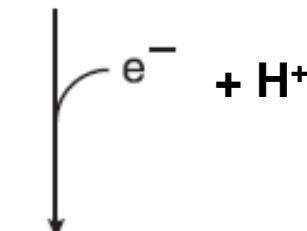
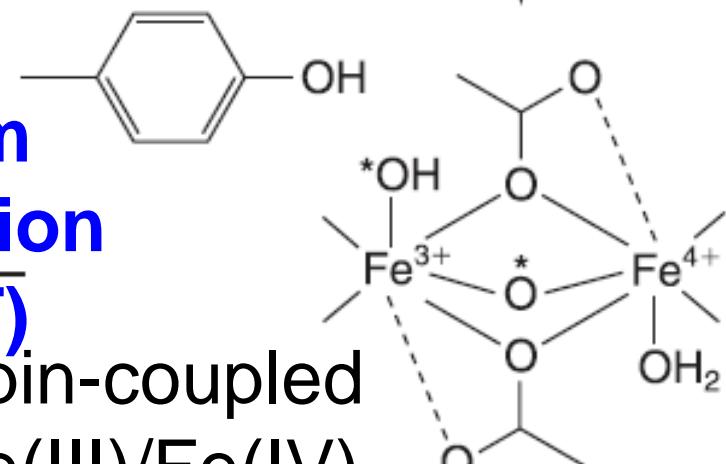


short-lived μ -1,2-peroxo diferric

**O-O bond
cleavage**

**H atom
abstraction
(PCET)**

Spin-coupled
 Fe(III)/Fe(IV)
(Fe--Fe: 2.5 Å) Compound X

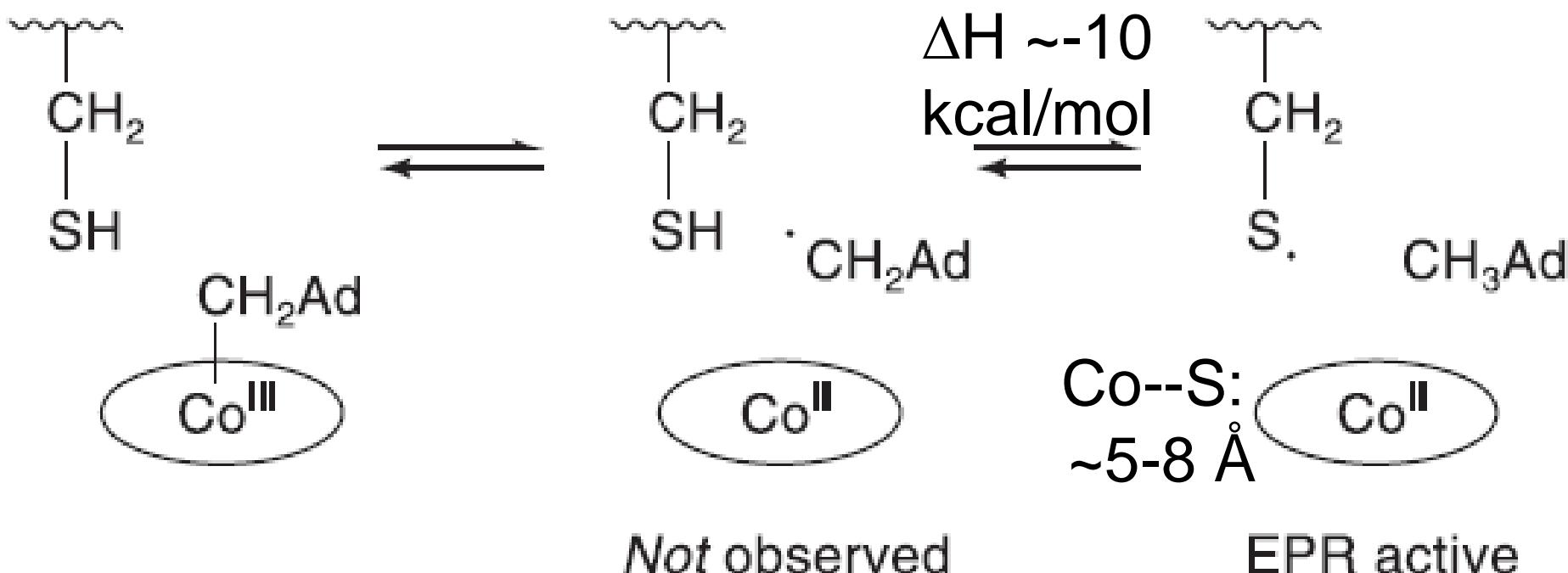


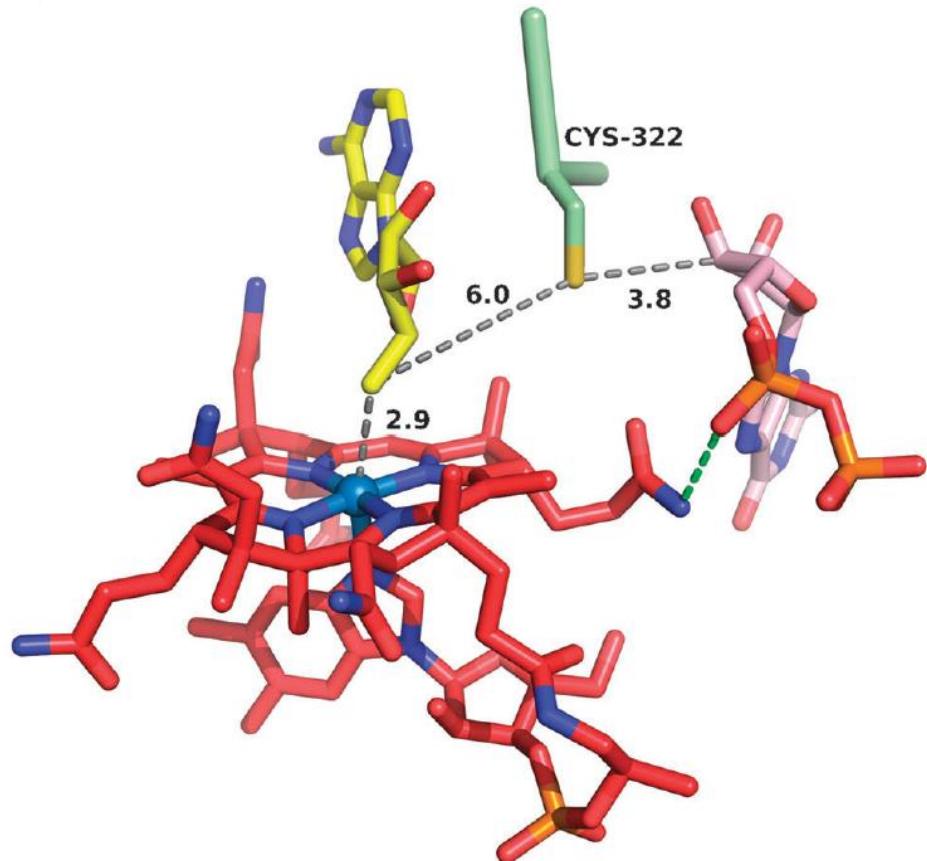
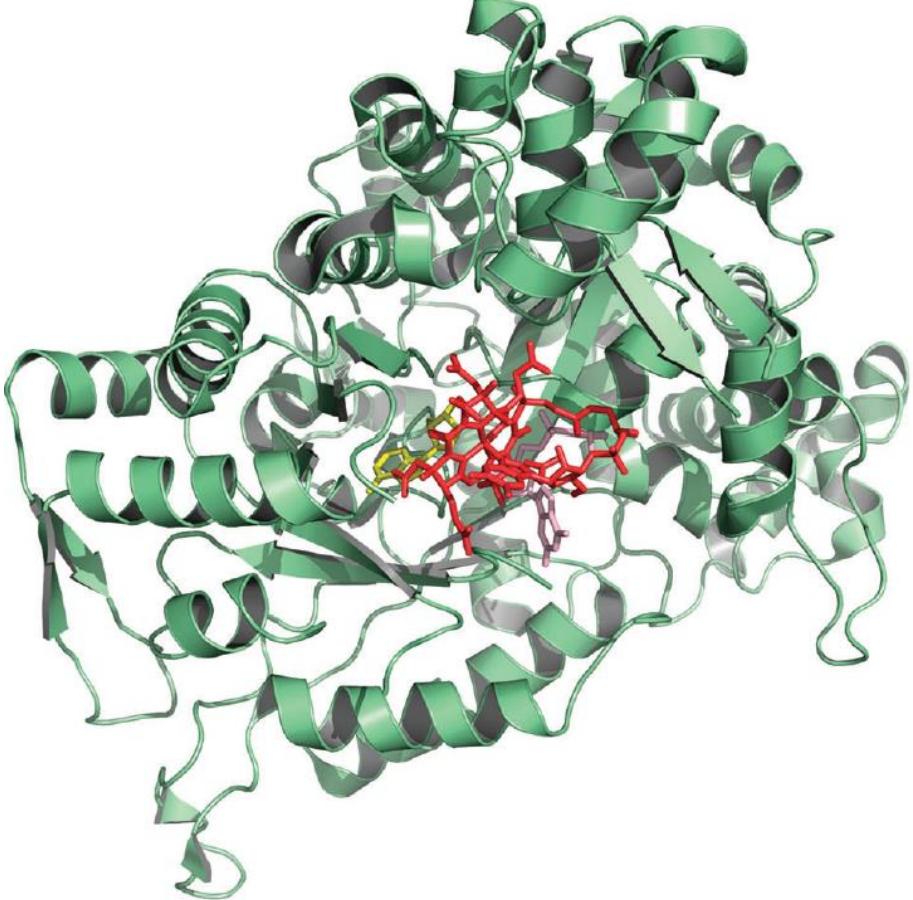
Class II RNR

- Found in bacteria & archaea.
- **AdoCbl** is required to act as a radical chain initiator.
- **3 key redox-active Cys** are also present & **1 transient Cys radical** is formed to catalyze the reduction.



Proposed Mechanism

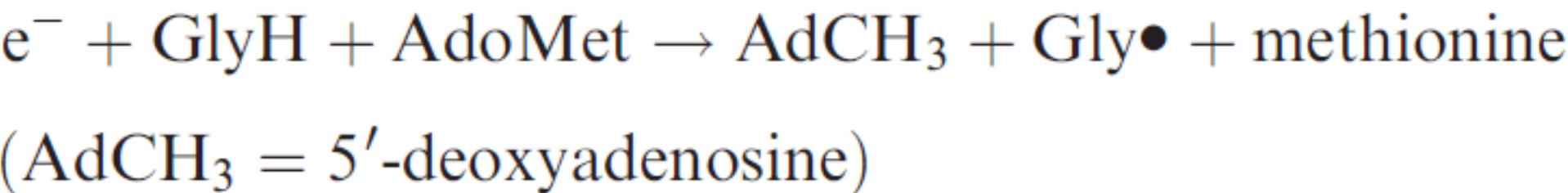




- The **Ado radical** is presumably formed (homolytic Co-C cleavage) to generate the **Cys radical**, which **abstracts H (C3') position of the substrate**.
- The Co-C bond cleavage can occur in the absence of the substrate.
- The base-on form in Cbl.

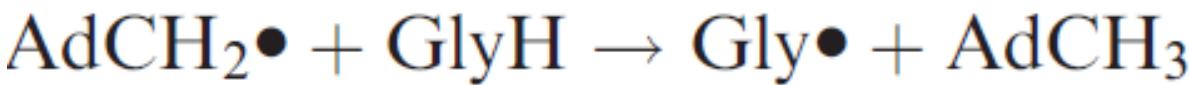
Class III RNR

- Oxygen-sensitive enzymes found in some anaerobes & methanogens.
- A **Gly radical** (via **1e⁻ oxidation**) is required for the reduction.

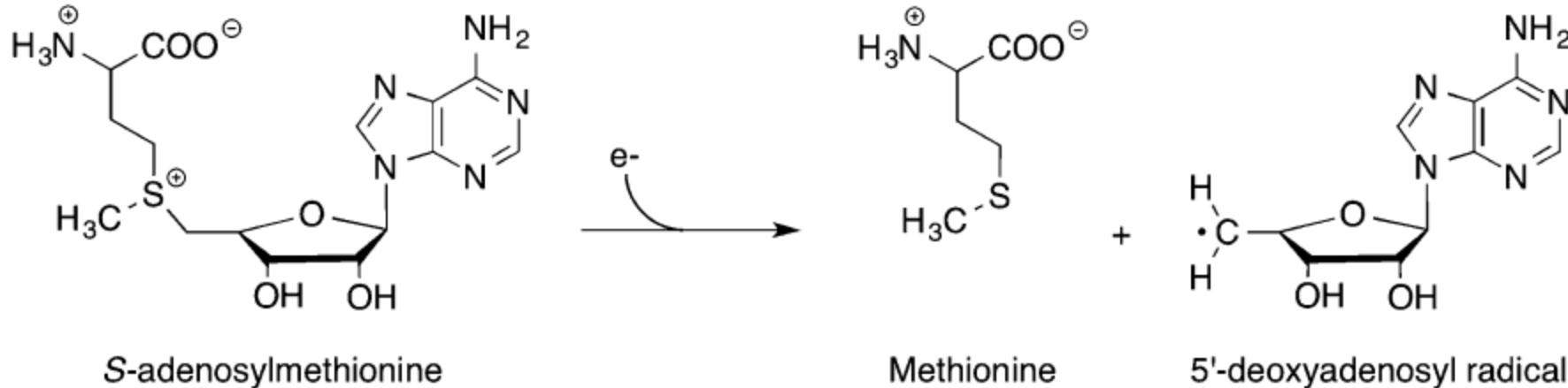


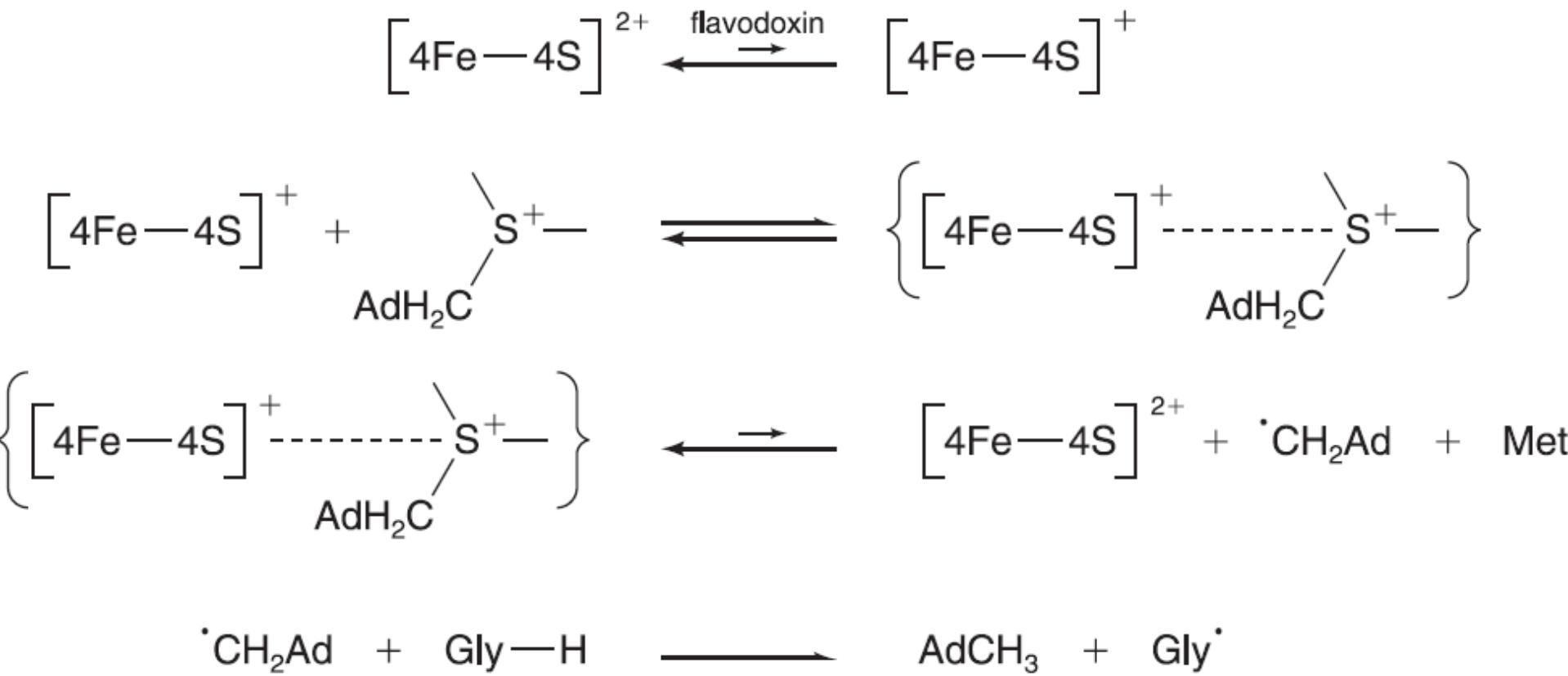
- A **transient Cys radical** is also formed in the active site by reaction with the Gly radical.
- The R2 component has a **[4Fe-4S]^{2+/+} cluster & S-adenosylmethionine** (so-called AdoMet or SAM), which facilitates the formation of the Gly radical on protein R1.

Proposed Mechanism



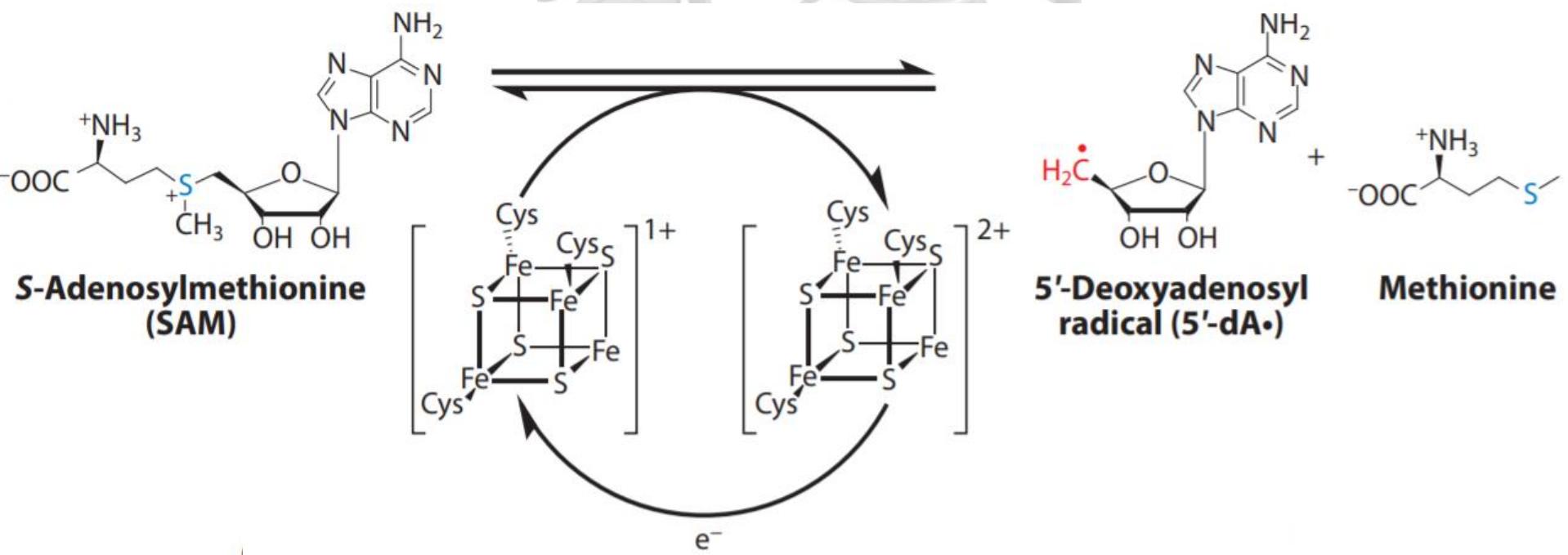
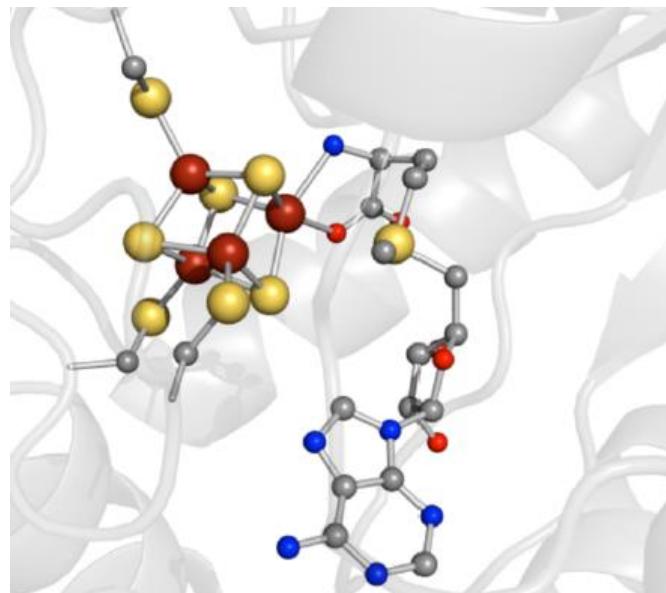
- **AdoMet:** not a strong oxidant, but can **accept 1e^-** to form a short-lived sulfuranyl radical, which cleaves homolytically into **Met & Ado radical**.
- Then, the Ado radical possibly abstracts the hydrogen atom of the Gly.





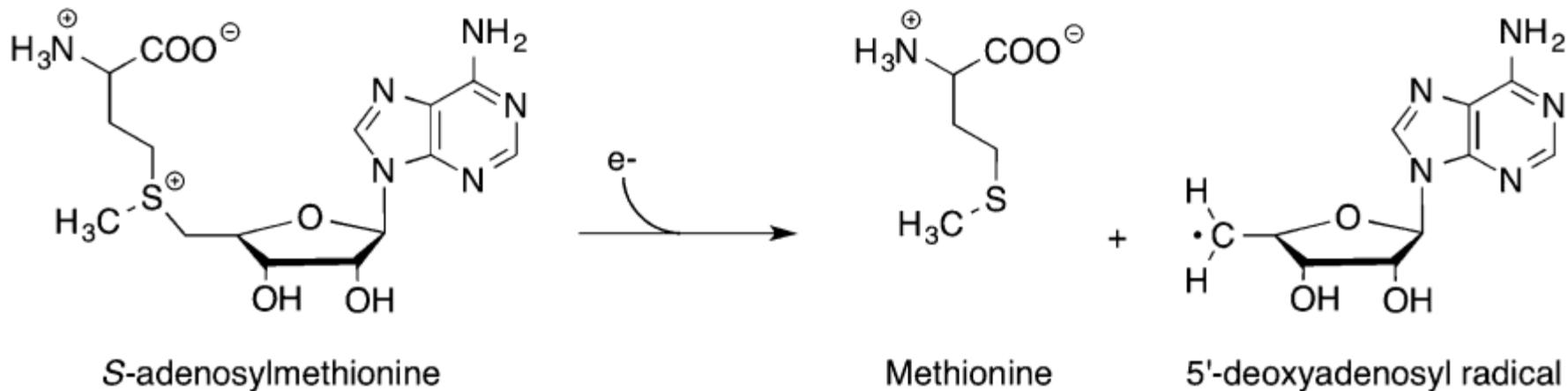
- The $[4\text{Fe}-4\text{S}]$ cluster mediates ET to AdoMet, **likely via $[4\text{Fe}-4\text{S}]\text{-AdoMet complex}$** .
- The **(thermodynamically unfavorable)** reduction is coupled to the irreversible cleavage & formation of the Gly radical steps to drive the reaction.

4. Radical-SAM Enzymes (with Fe-S Clusters)

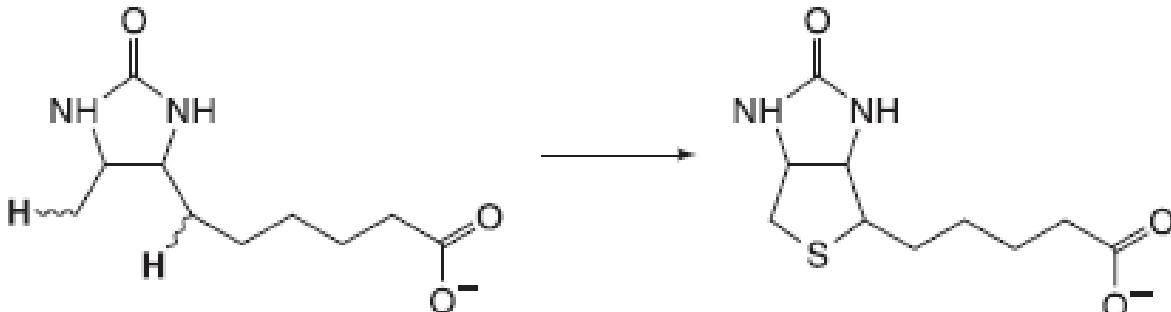
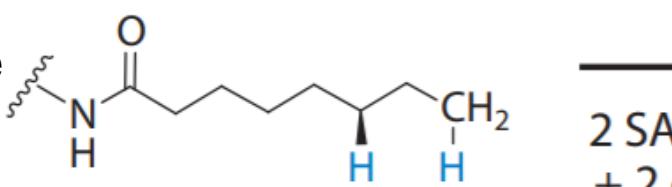


Fe-S Clusters

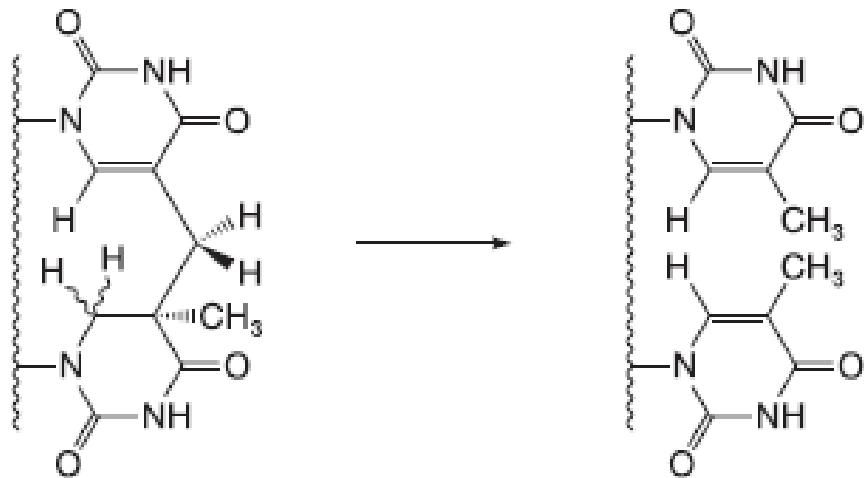
- Ubiquitous in biological systems & different functions.
- The first-reported & most common function: **ET**.
- Other functions: **enzymatic catalysis, structural stabilization, regulation of gene expression & unexpectedly, generation of radicals.**
- Novel **radical-SAM protein superfamily** uses **S-adenosylmethionine** (SAM or AdoMet) as a **cofactor or co-substrate** to generate a **radical**.



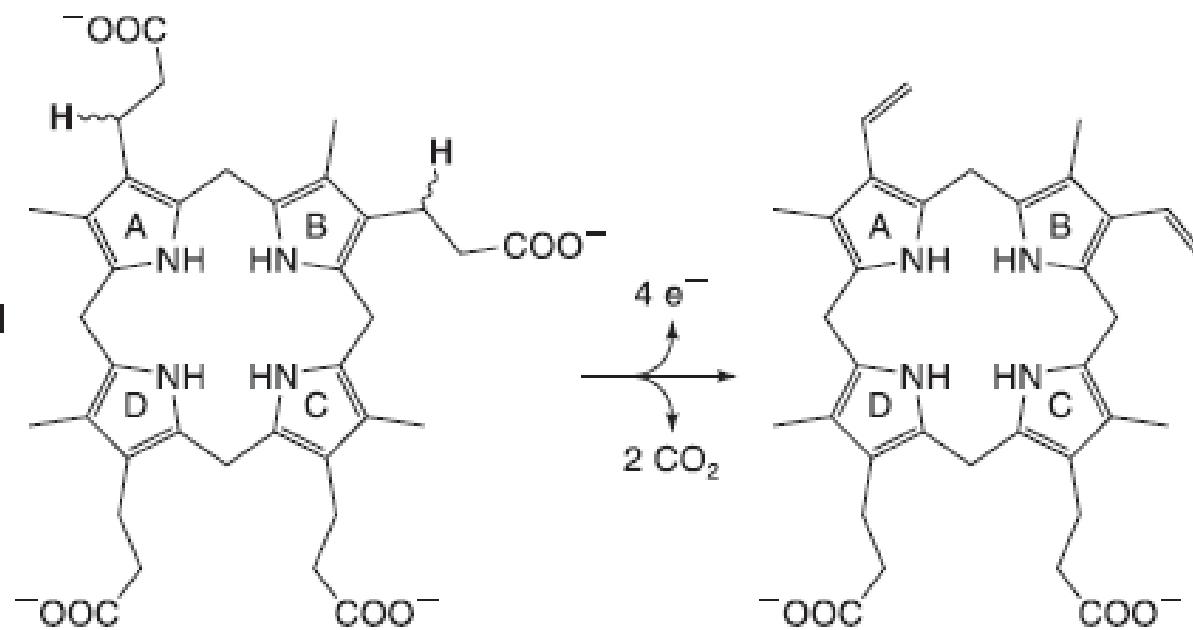
- These SAM enzymes: Gly radical formation, rearrangement, cofactor biosynthesis, DNA repair.
- The *common key mechanistic step*: **reductive cleavage** of SAM to form **Ado radical** & Met.

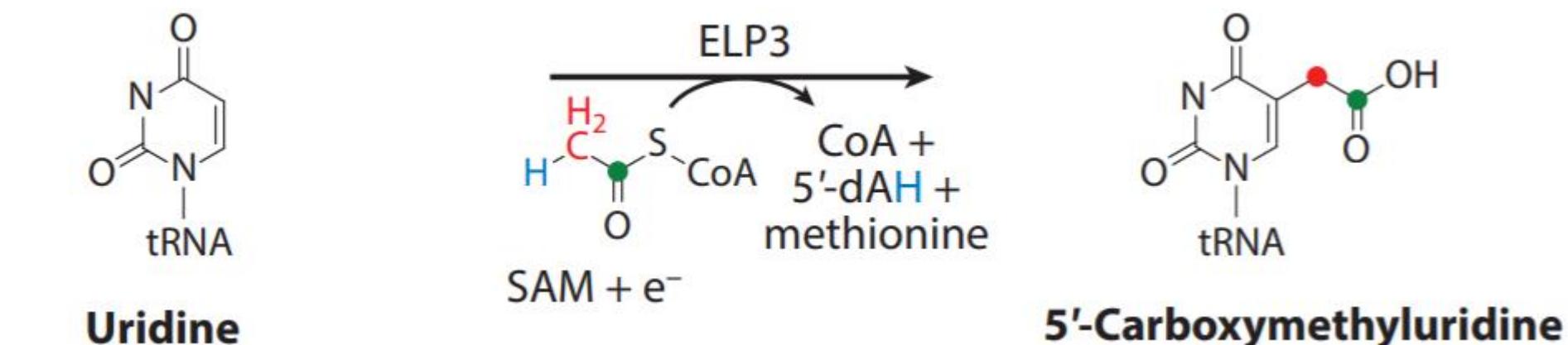
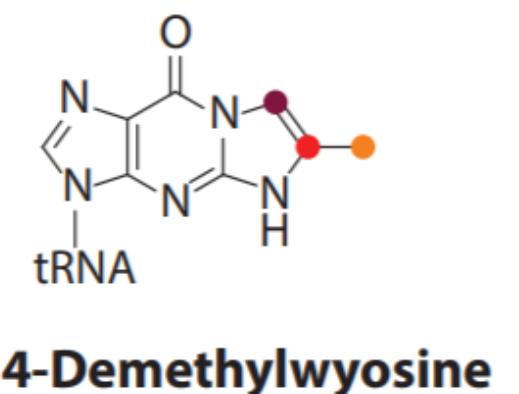
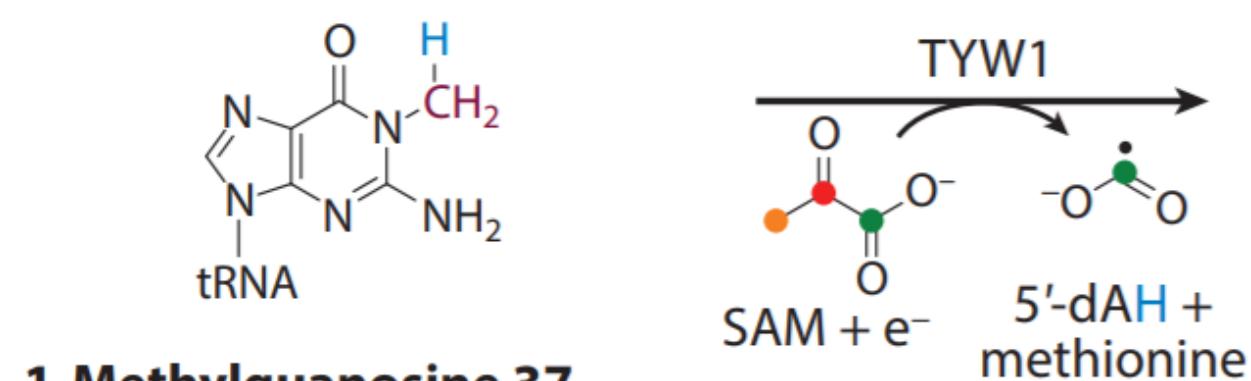
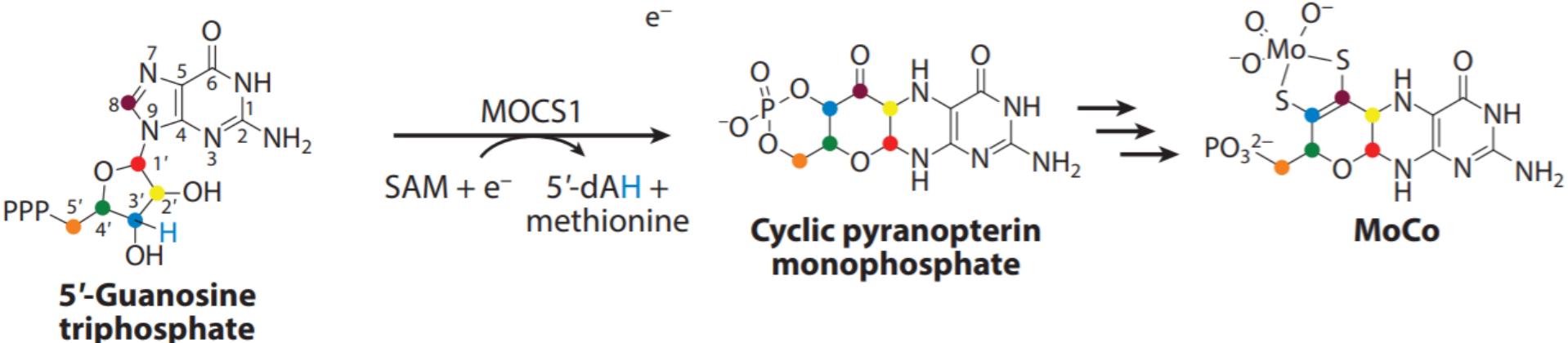
Enzyme	Reaction Catalyzed	Role of SAM
Activating enzymes		Substrate
Lysine aminomutase		Cofactor
Biotin synthase		Substrate
Lipoyl synthase	 <p>LIAS</p> <p>$2 \text{ SAM} + 2 \text{ e}^- \rightarrow 2 \text{ 5'-dAH} + 2 \text{ methionine}$</p>	Substrate

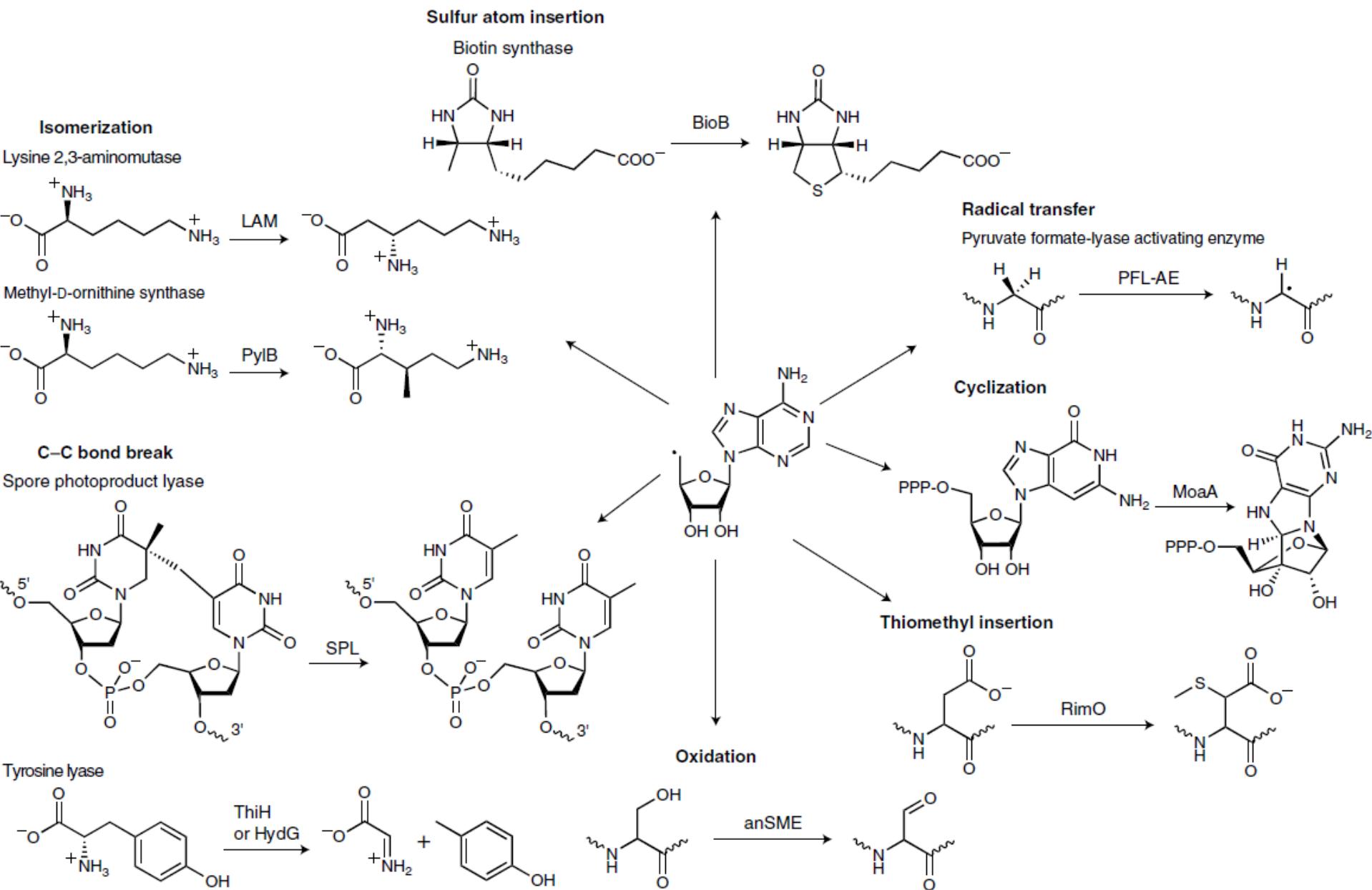
Spore photoproduct lyase



Coproporphyrinogen III oxidase

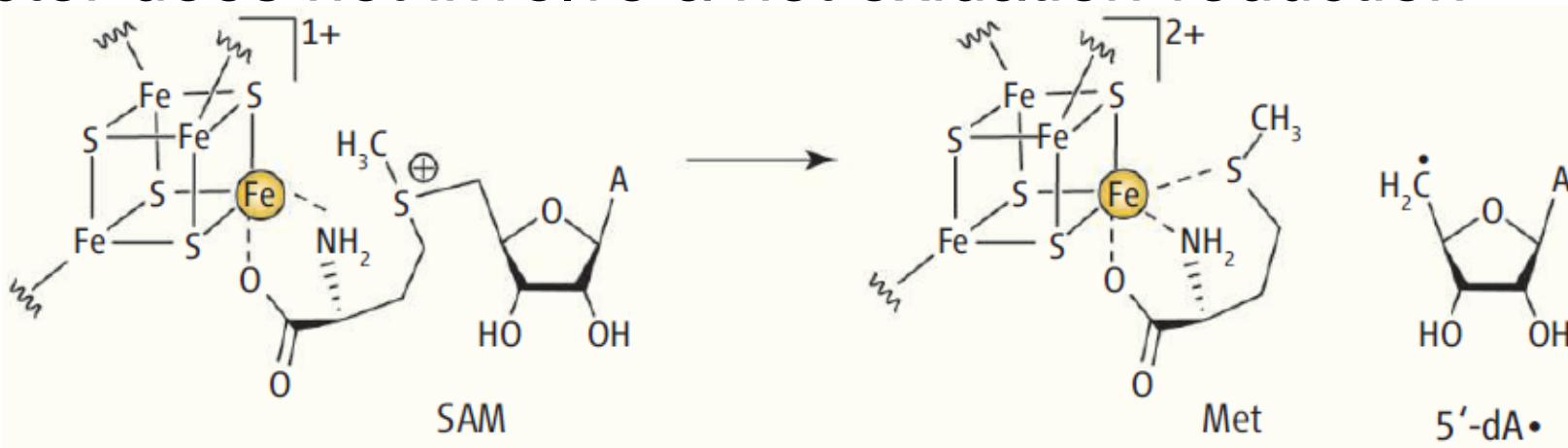






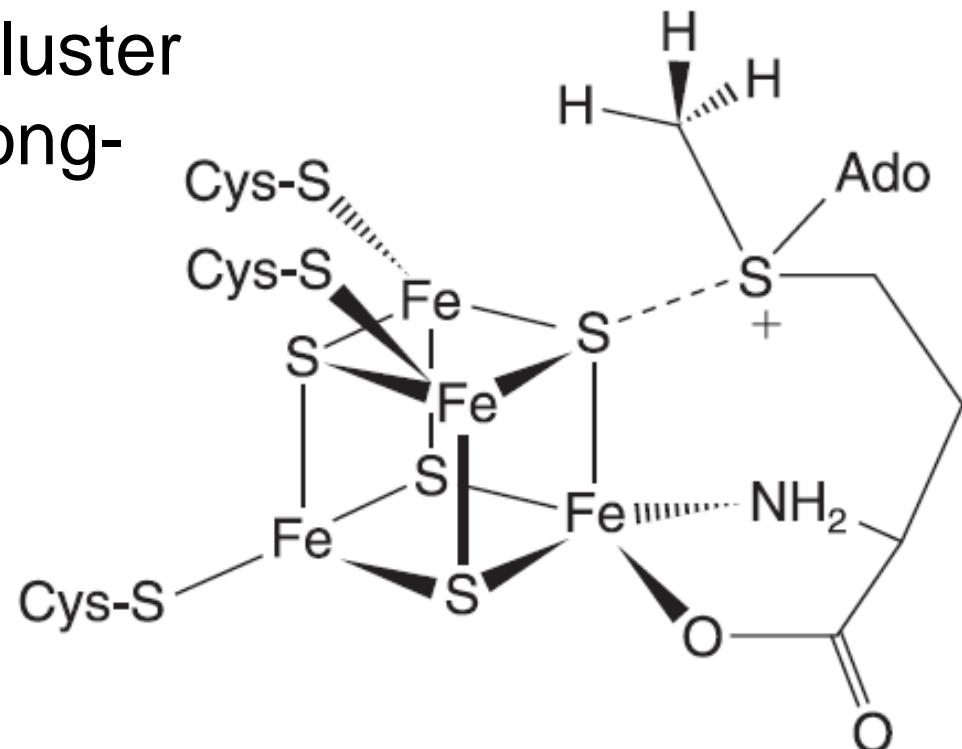
Radical-SAM Enzymes

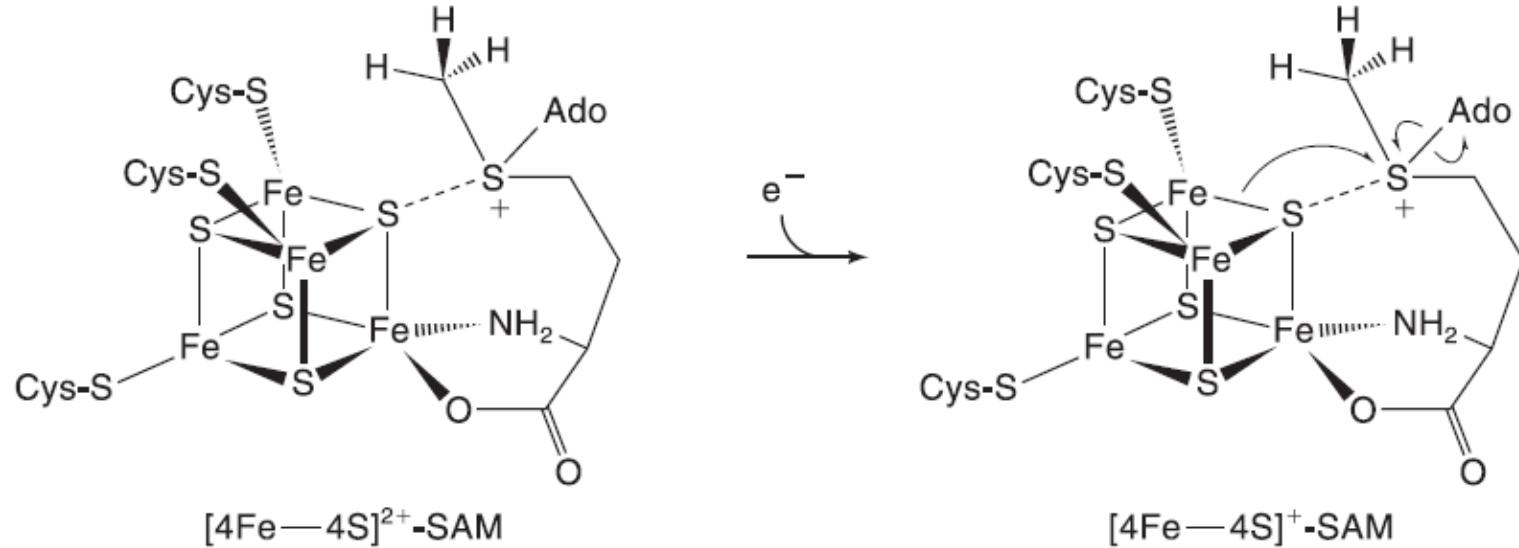
- Can be grouped into 2 types:
 1. **SAM as a substrate** (e.g., the activating enzymes & biotin synthase); stoichiometrically convert SAM to Met & Ado radical.
 2. **SAM as a catalytic cofactor** (e.g., lysine 2,3-aminomutase & spore photoproduct lyase): The **Ado radical can be regenerated** after each reaction.
- SAM as a substrate catalyzes a net redox reaction, (SAM is reduced & the substrate is oxidized); SAM as a cofactor does not involve a net oxidation-reduction.



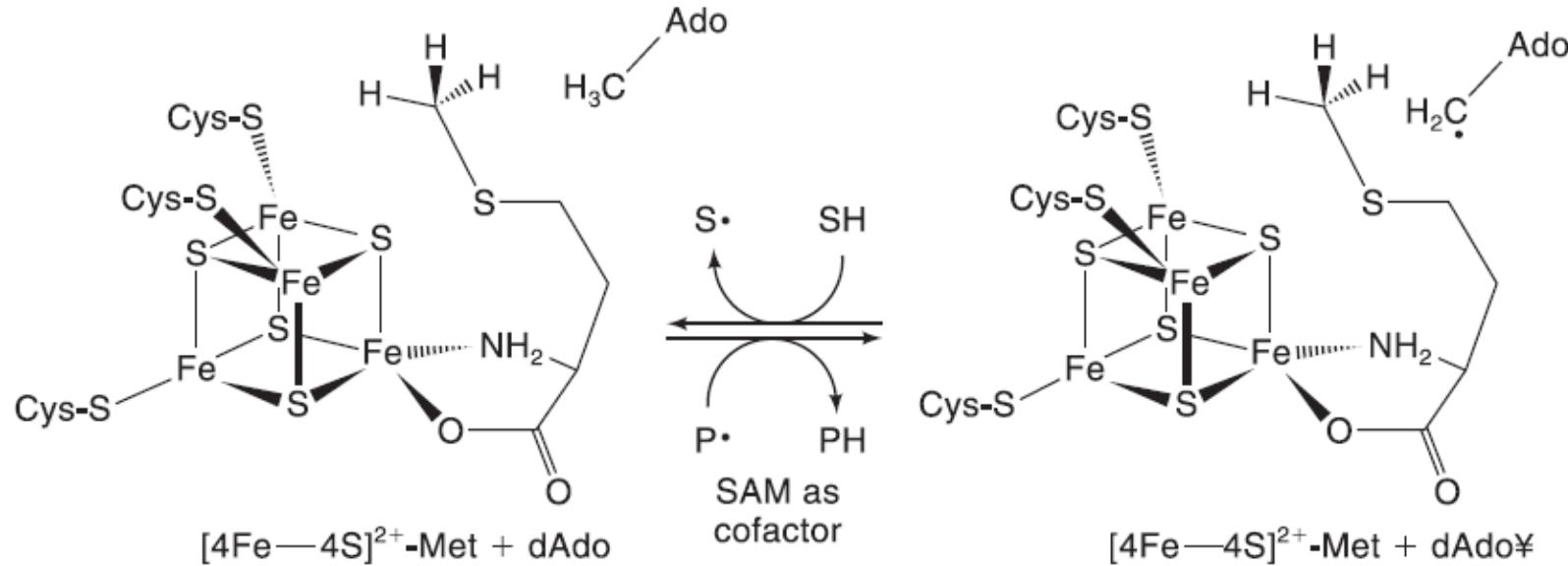
- The **reduced [4Fe4S]⁺** form: the electron source for reductive cleavage.
- A conserved sequence (CXXxCXXC) with **only 3 Cys** to coordinate to the cluster: a site-differentiated cluster with **one coordination of Fe by a non-Cys**.

• ET from a reduced Fe-S cluster to SAM (poor oxidant) via long-range ET is not likely, but **via direct interaction** with SAM. The S orbital of SAM overlaps with the cluster for **inner-sphere ET**.



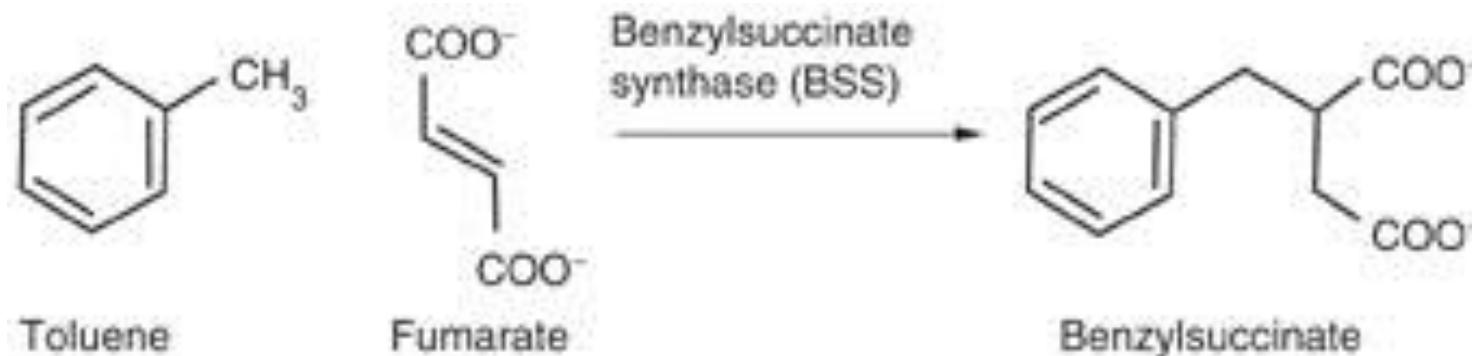
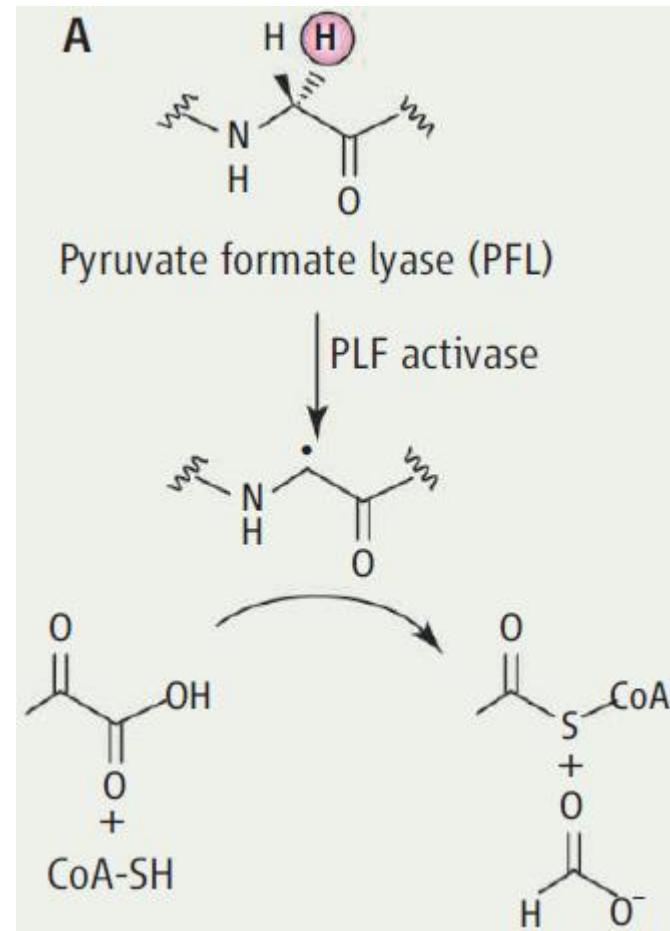


Proposed Mechanism



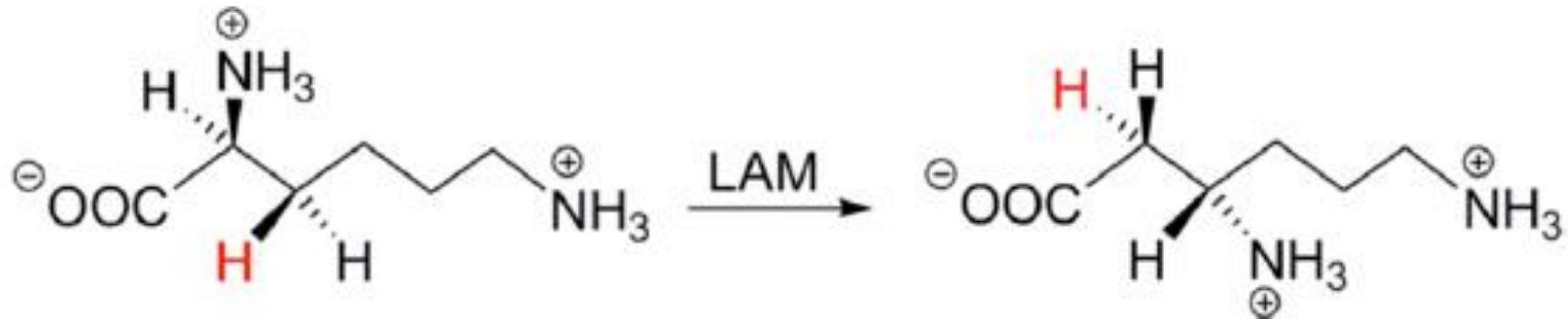
Gly Radical Formation

- The activating component of the Class III RNR, pyruvate formate lyase (PFL), & benzyl-succinate synthase enzymes.
- They form a Gly radical, which is stable under anaerobic conditions; but it reacts quickly with O_2 .

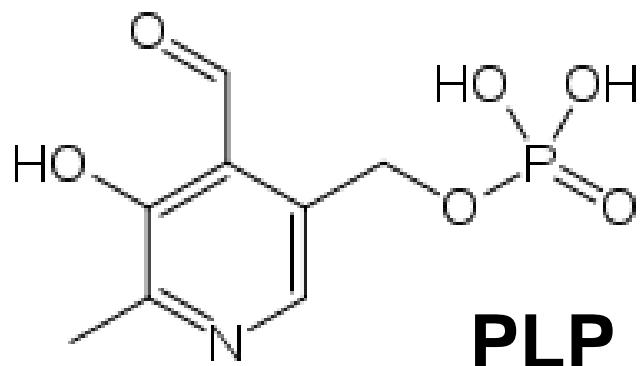


Lysine 2,3-Aminomutase (LAM)

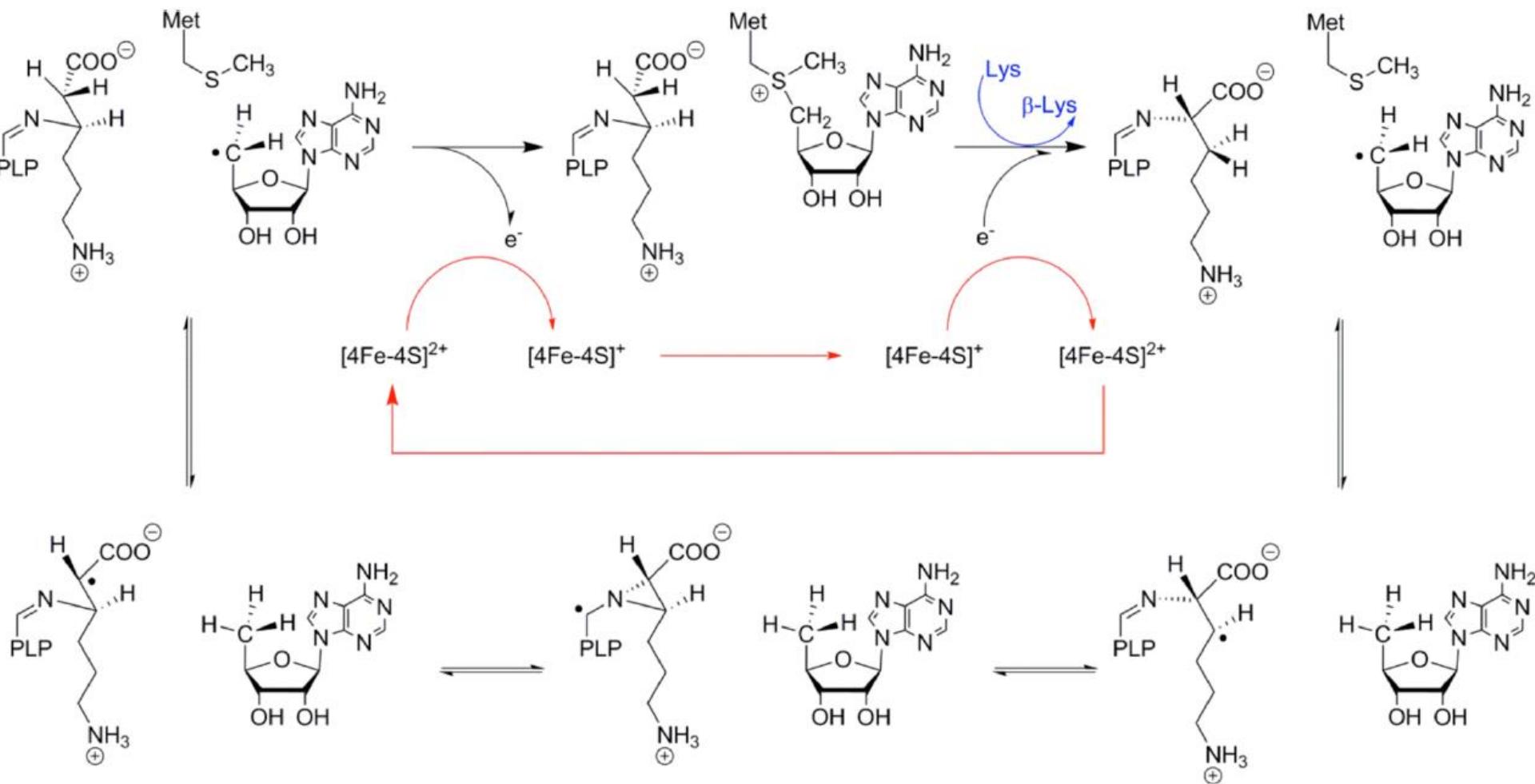
- Catalyzes reversible isomerization of L-Lys & L- β -Lys.
- SAM is required as a cofactor.



- Pyridoxal 5'-phosphate (PLP) is required, as in Cbl-dependent aminomutases.

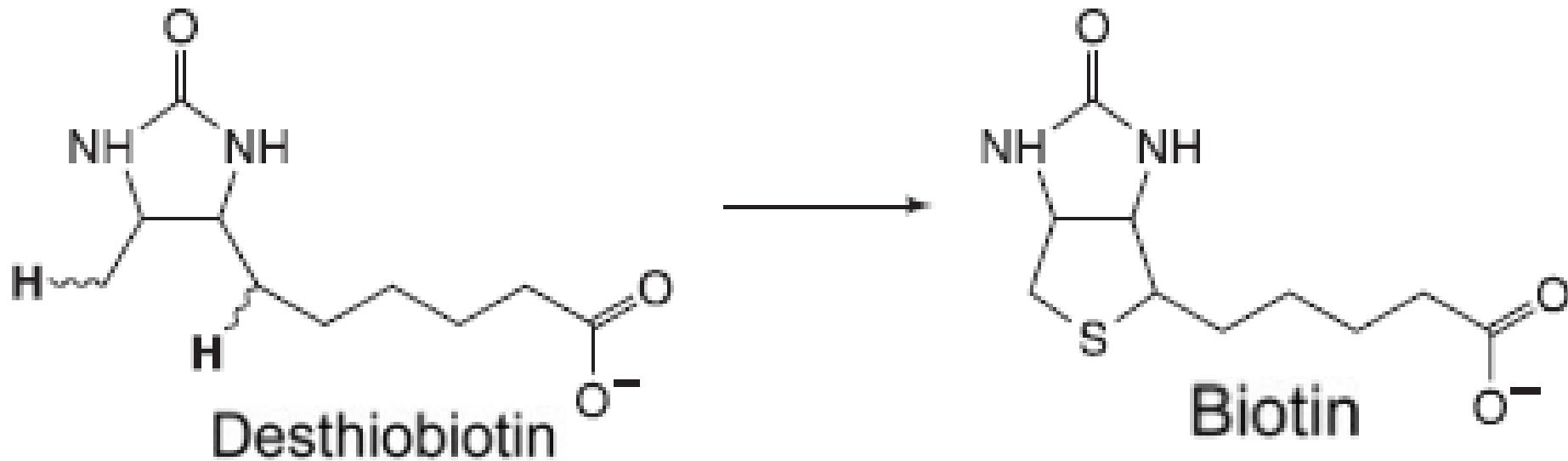


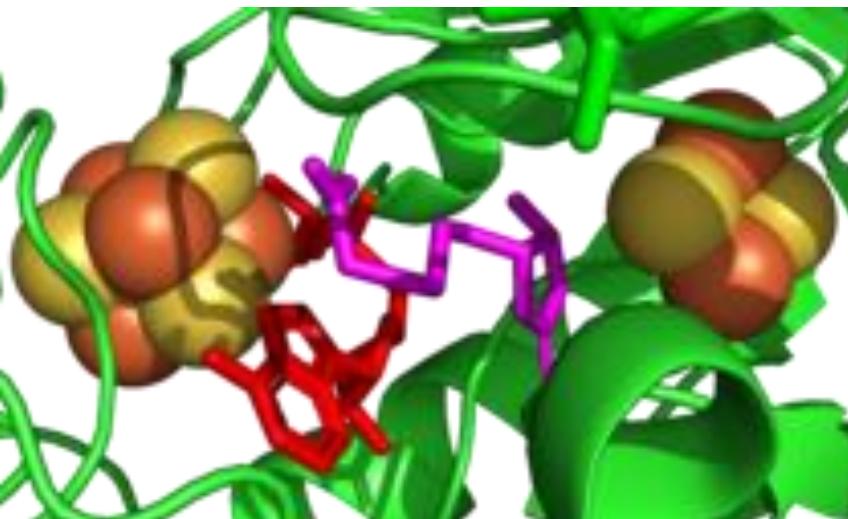
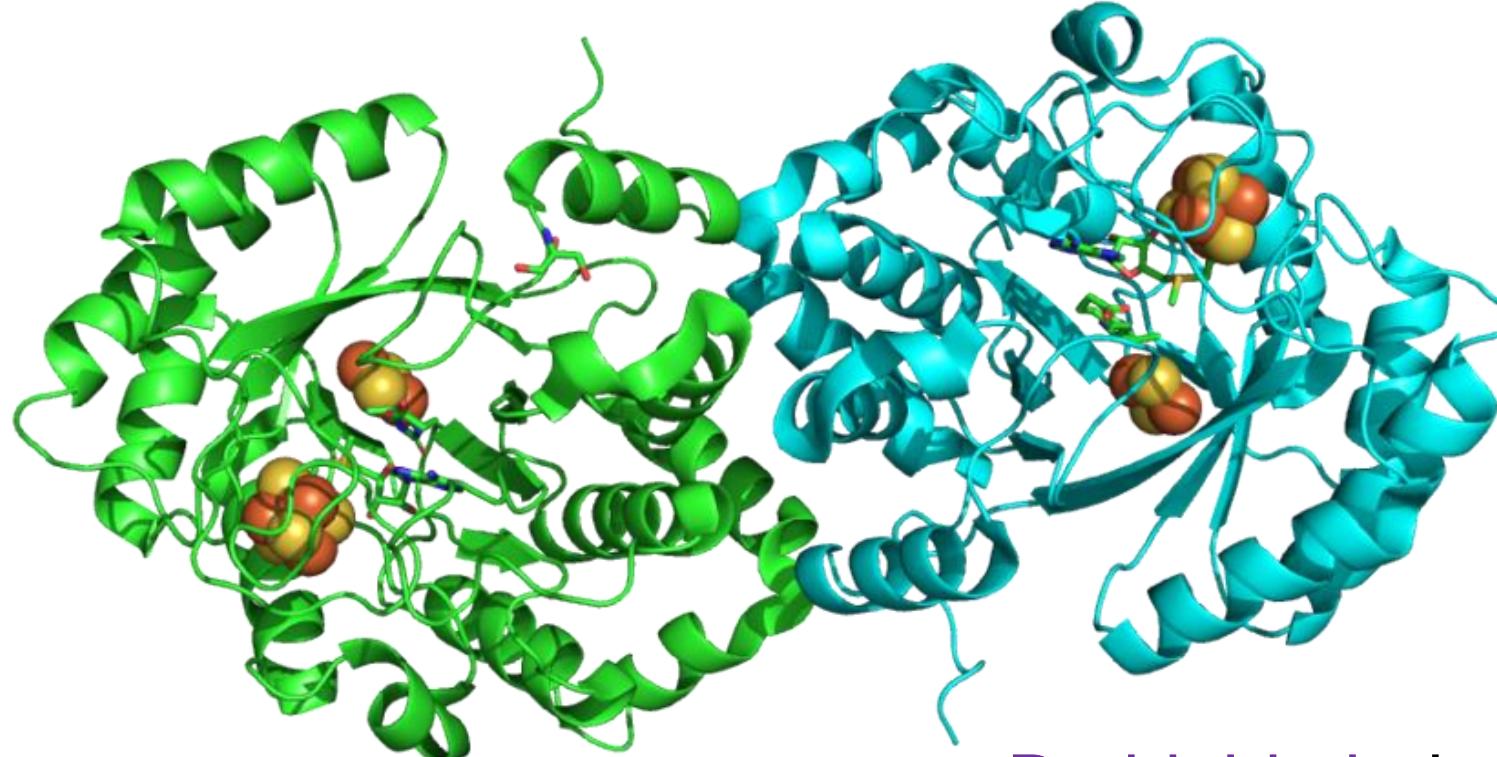
Proposed Mechanism



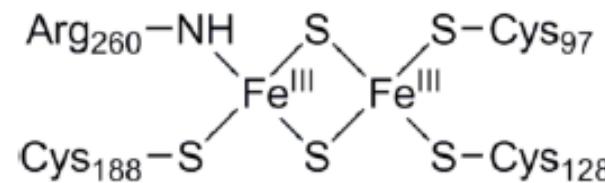
Biotin Synthase

- Biotin: Vitamin B₇.
- Catalyzes the **S atom insertion** into dethiobiotin to **form biotin** (the final step in the biotin biosynthetic pathway).
- SAM & NADPH are required.
- **[2Fe-2S] cluster** was proposed **as the S donor** (substrate).

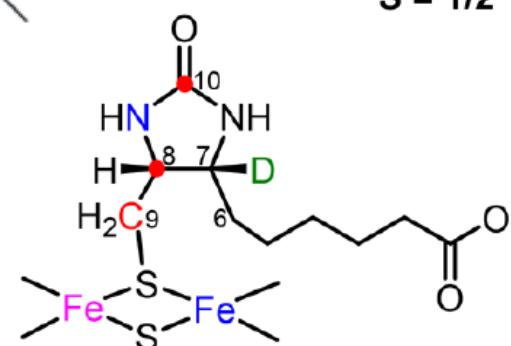
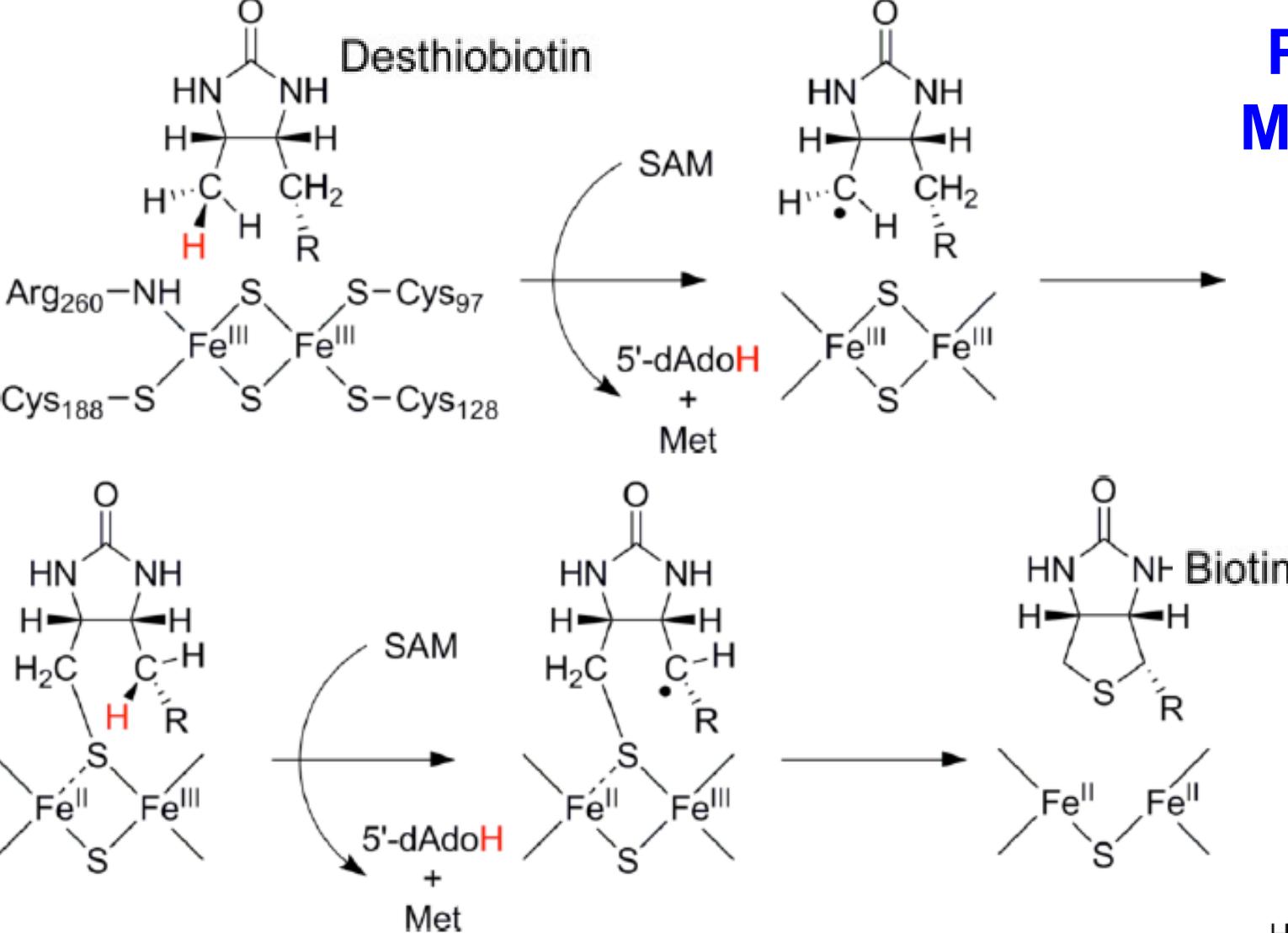




- Dethiobiotin between SAM & [2Fe-2S] cluster for H abstraction & S insertion.
- The [2Fe-2S] cluster with 3 Cys & 1 Arg.

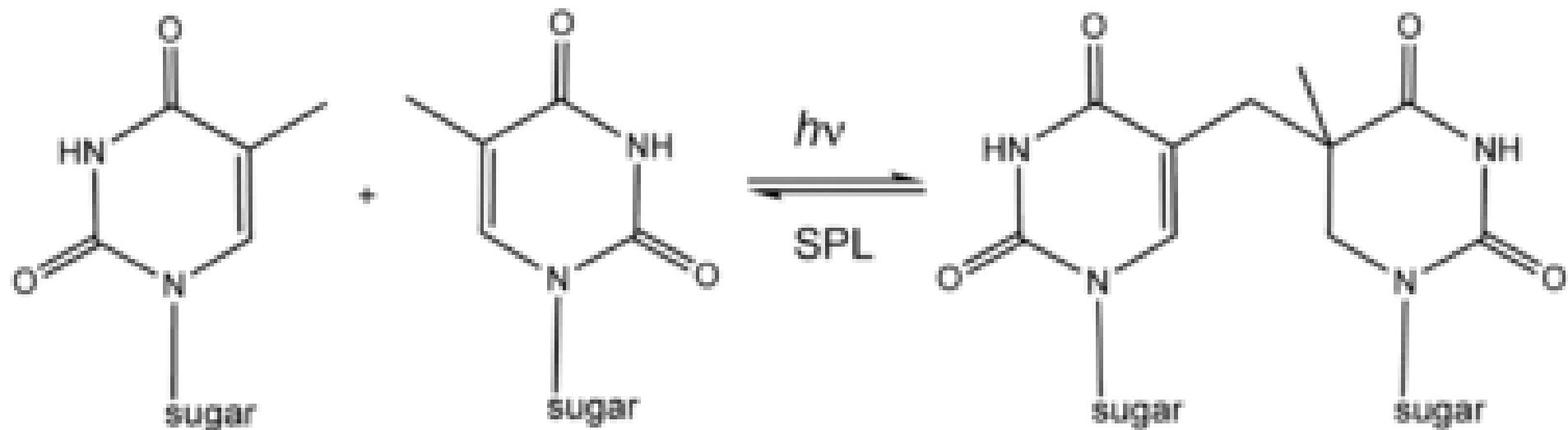


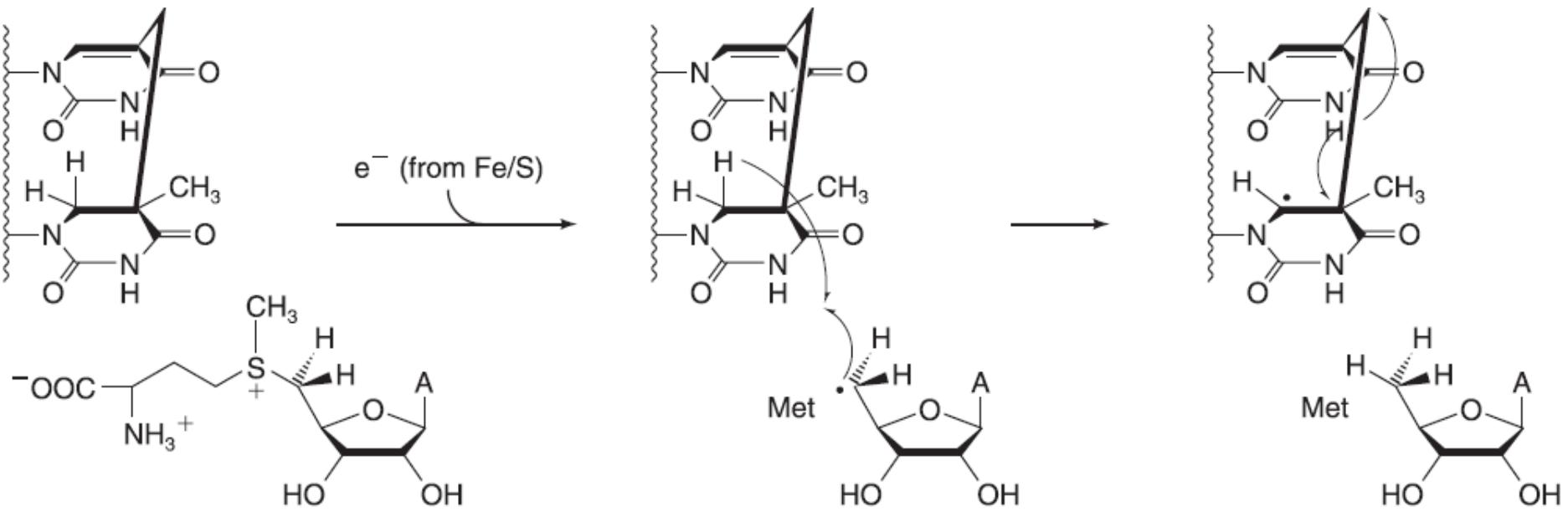
Proposed Mechanism



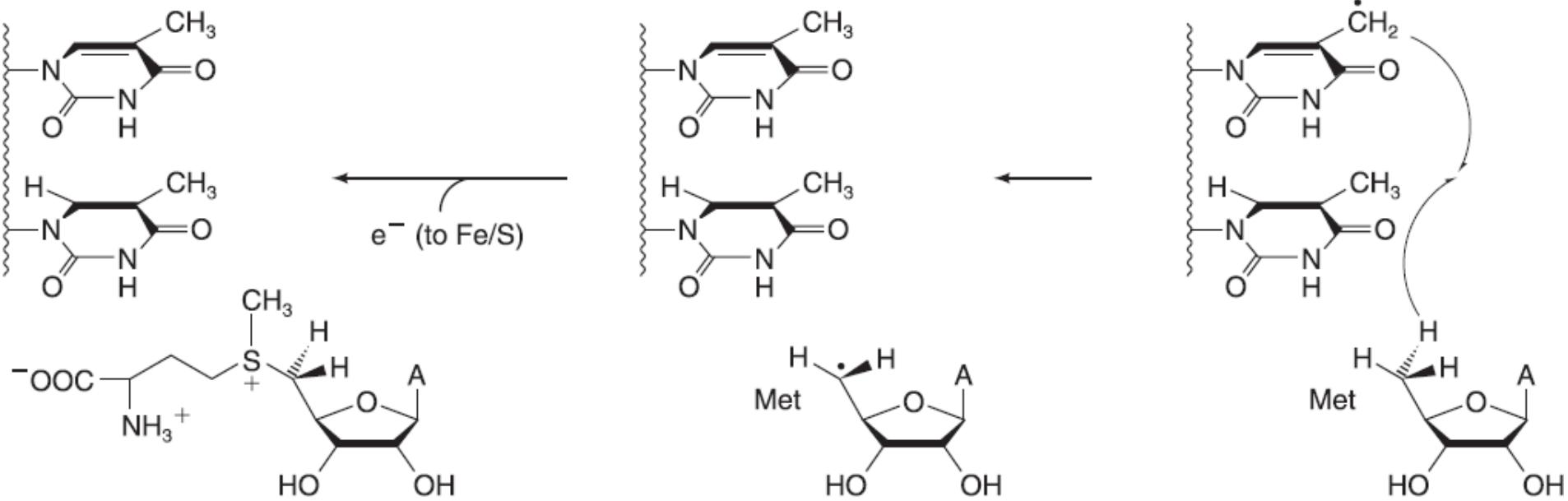
Spore Photoproduct Lyase: DNA repair

- Upon UV irradiation, bacterial spores form **DNA damage** (spore photoproduct (SP)). This DNA damage is repaired by a SP lyase.
- SAM is required as a catalytic cofactor to reversibly generate an **Ado radical**.
- The repair process is initiated by **H abstraction (at the C6)** from the **damaged DNA**.

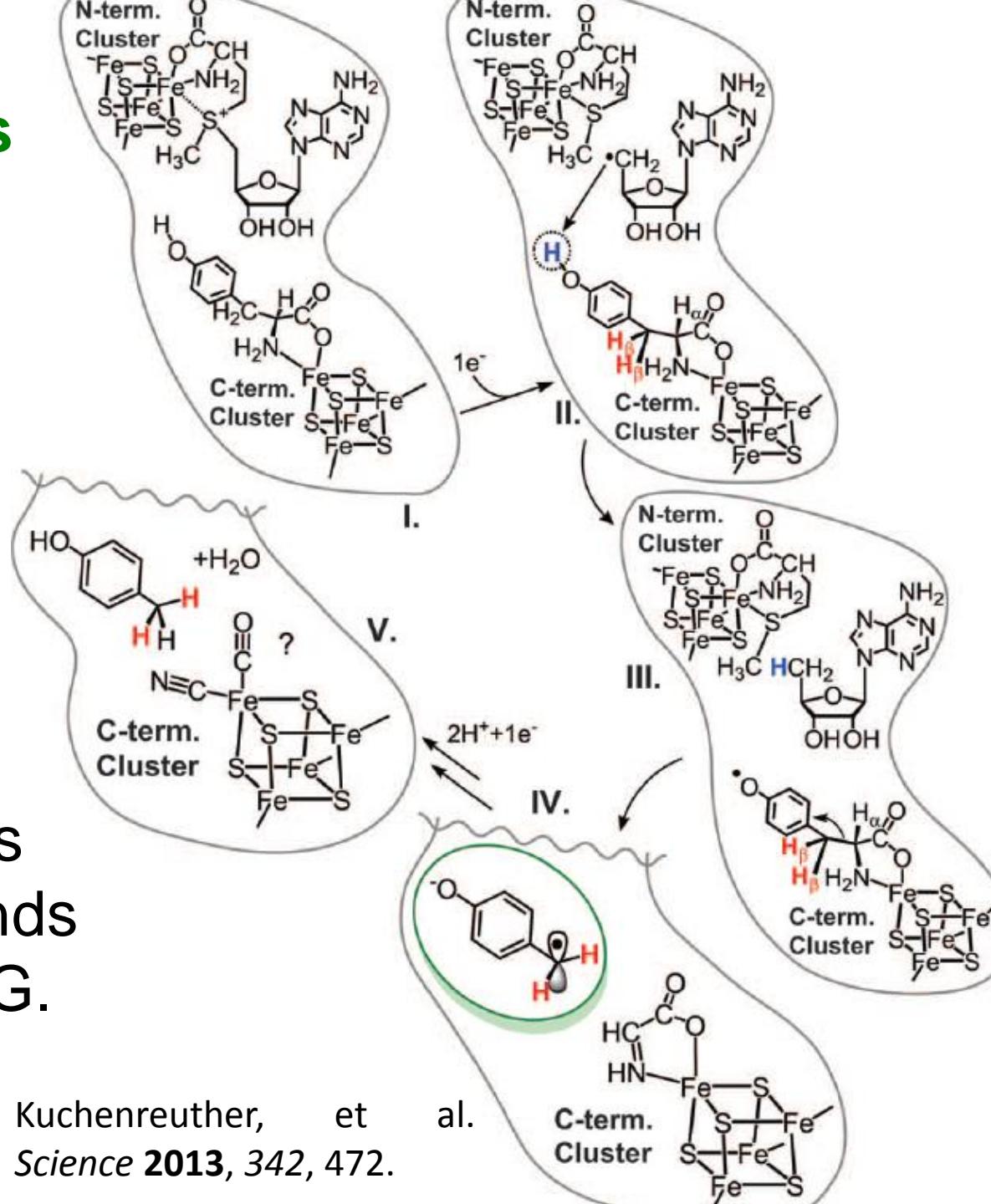
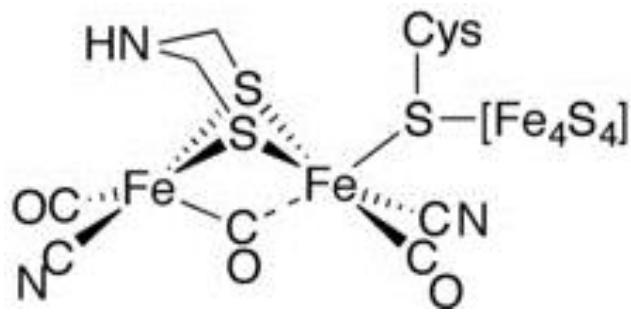




Proposed Mechanism



[FeFe] H₂ase's H-cluster biosynthesis in HydG

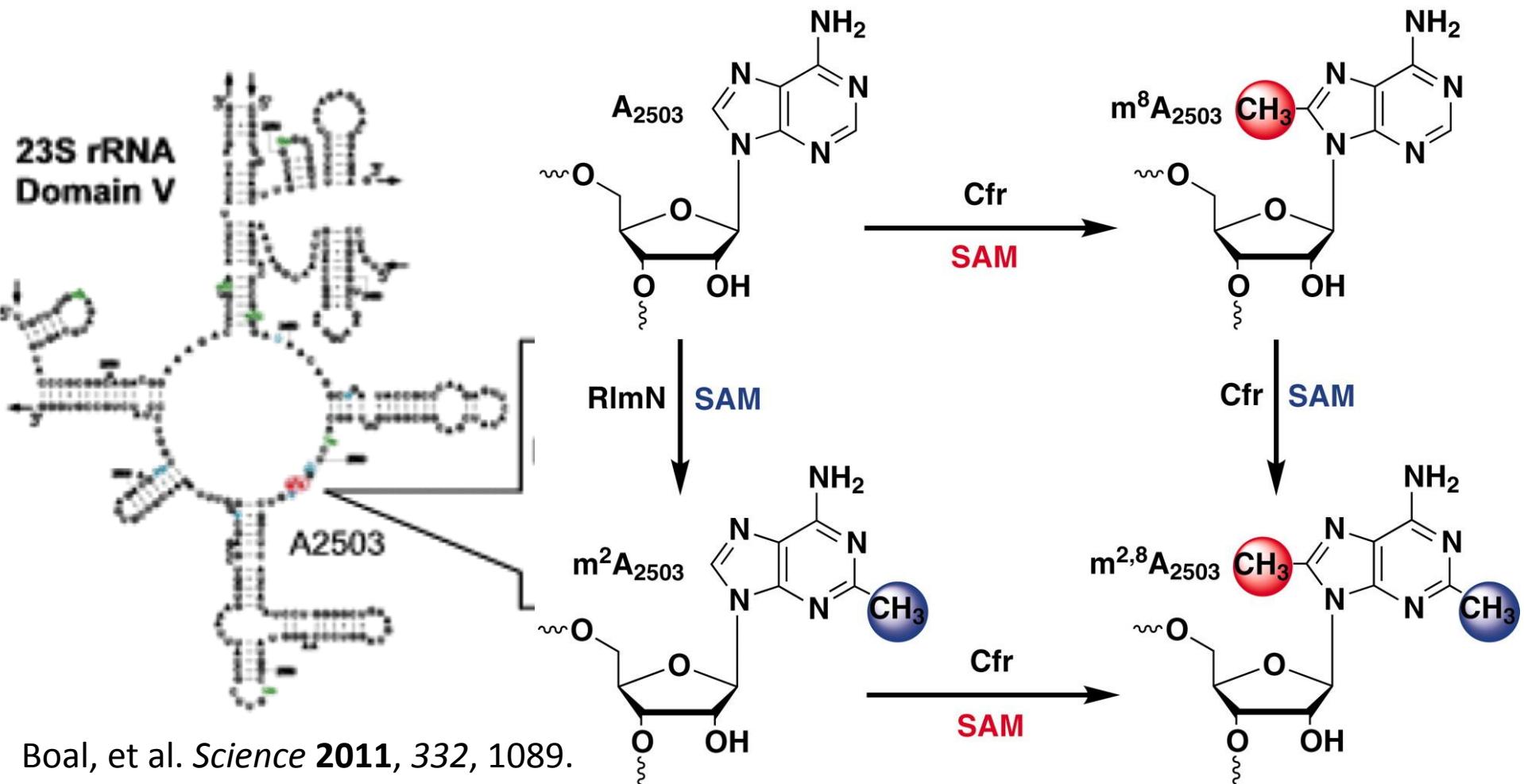


Free tyrosine releases
the **CO** and **CN⁻** ligands
by SAM enzyme HydG.

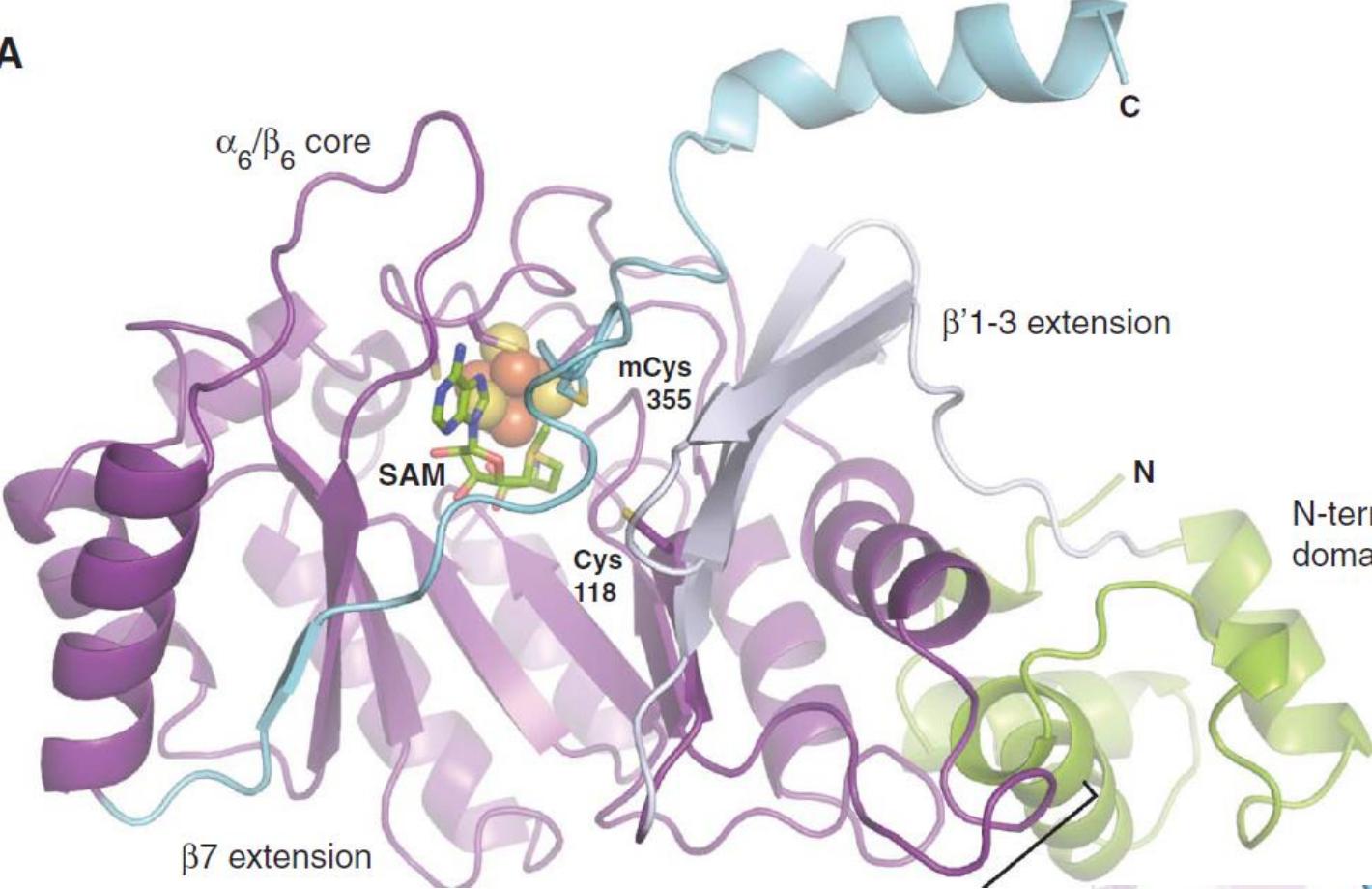
Kuchenreuther, et al.
Science **2013**, *342*, 472.

Methyl Transfer: ribosomal RNA modification enzyme (RlmN)

- Catalyze methylation of a 23S rRNA on the C2 position of adenosine 2503 (A2503) via radical pathway.

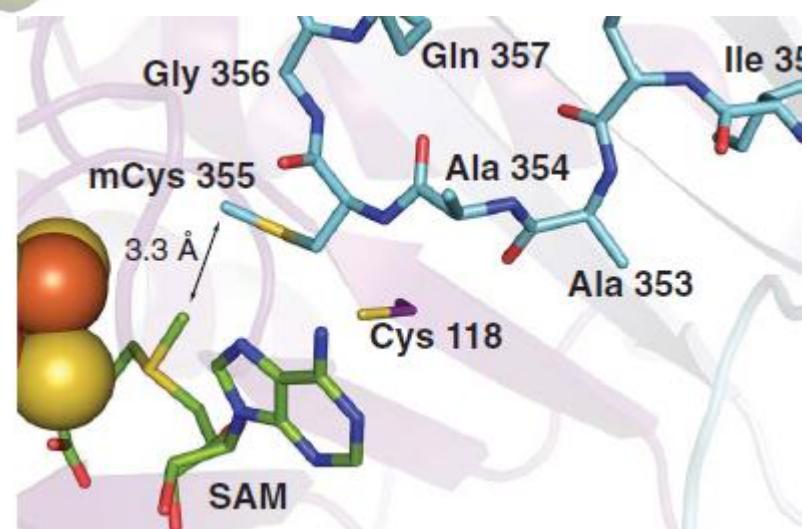


A

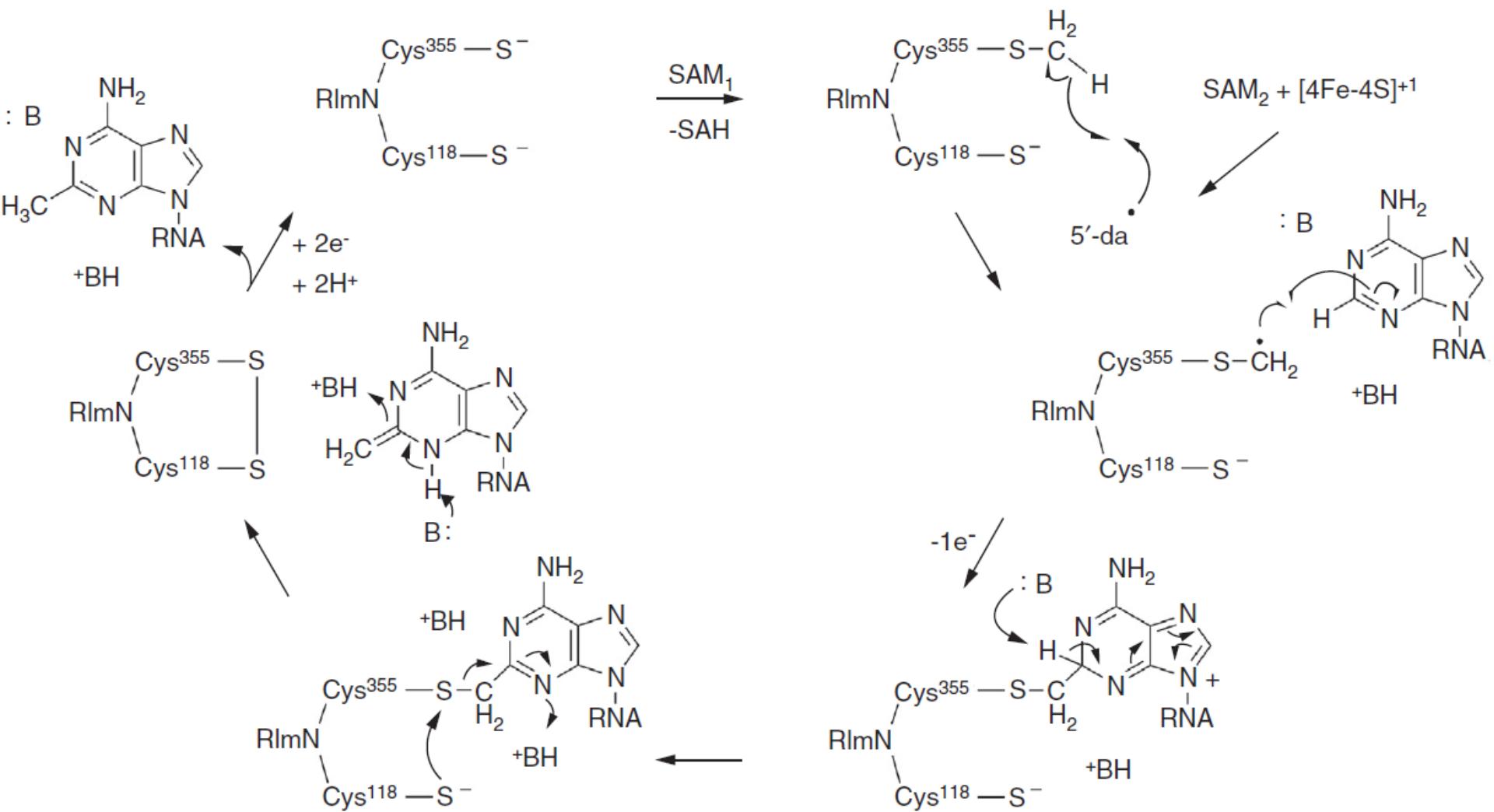


Boal, et al. *Science*
2011, 332, 1089.

Methylated Cys355 (mCys355)

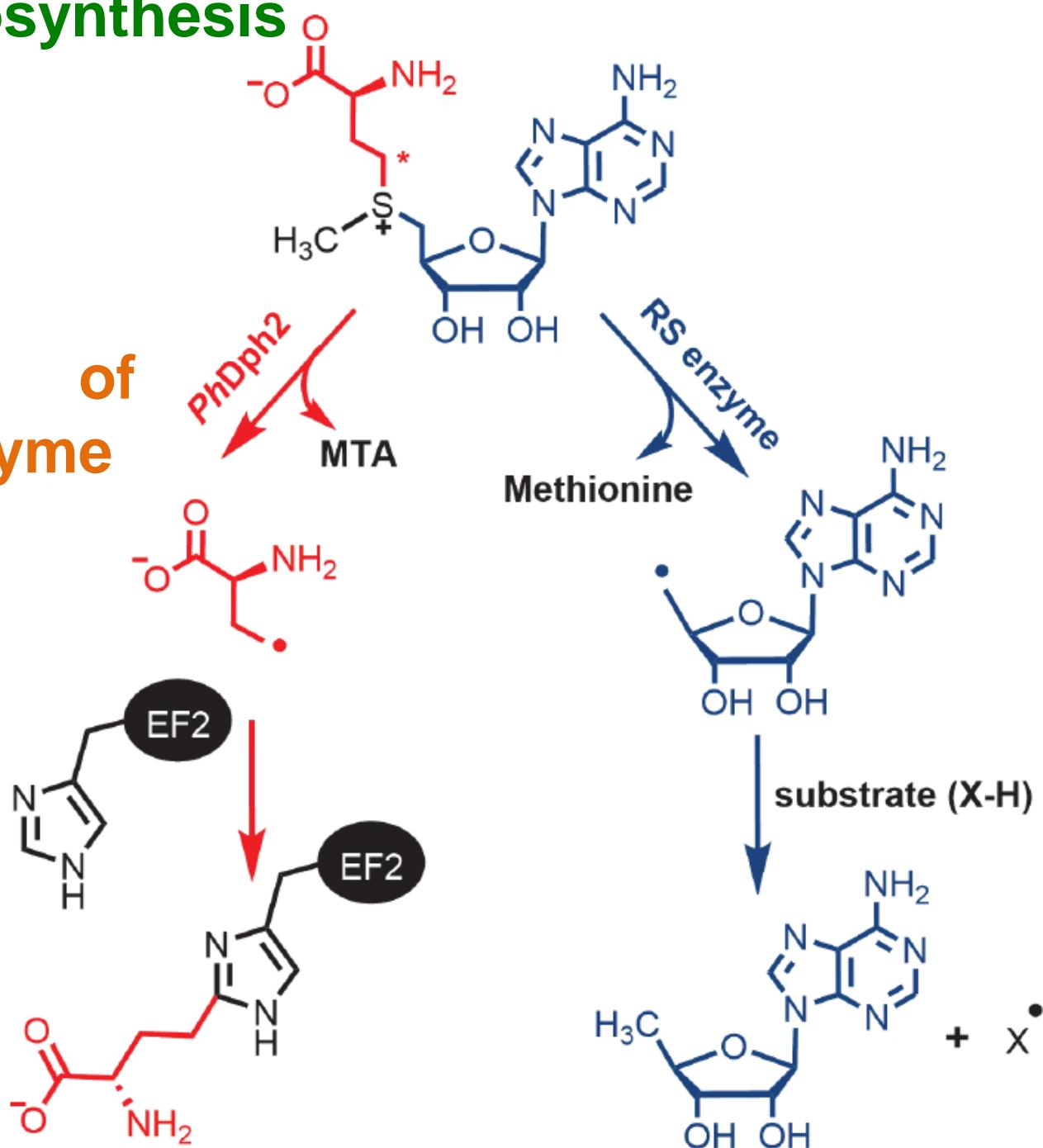


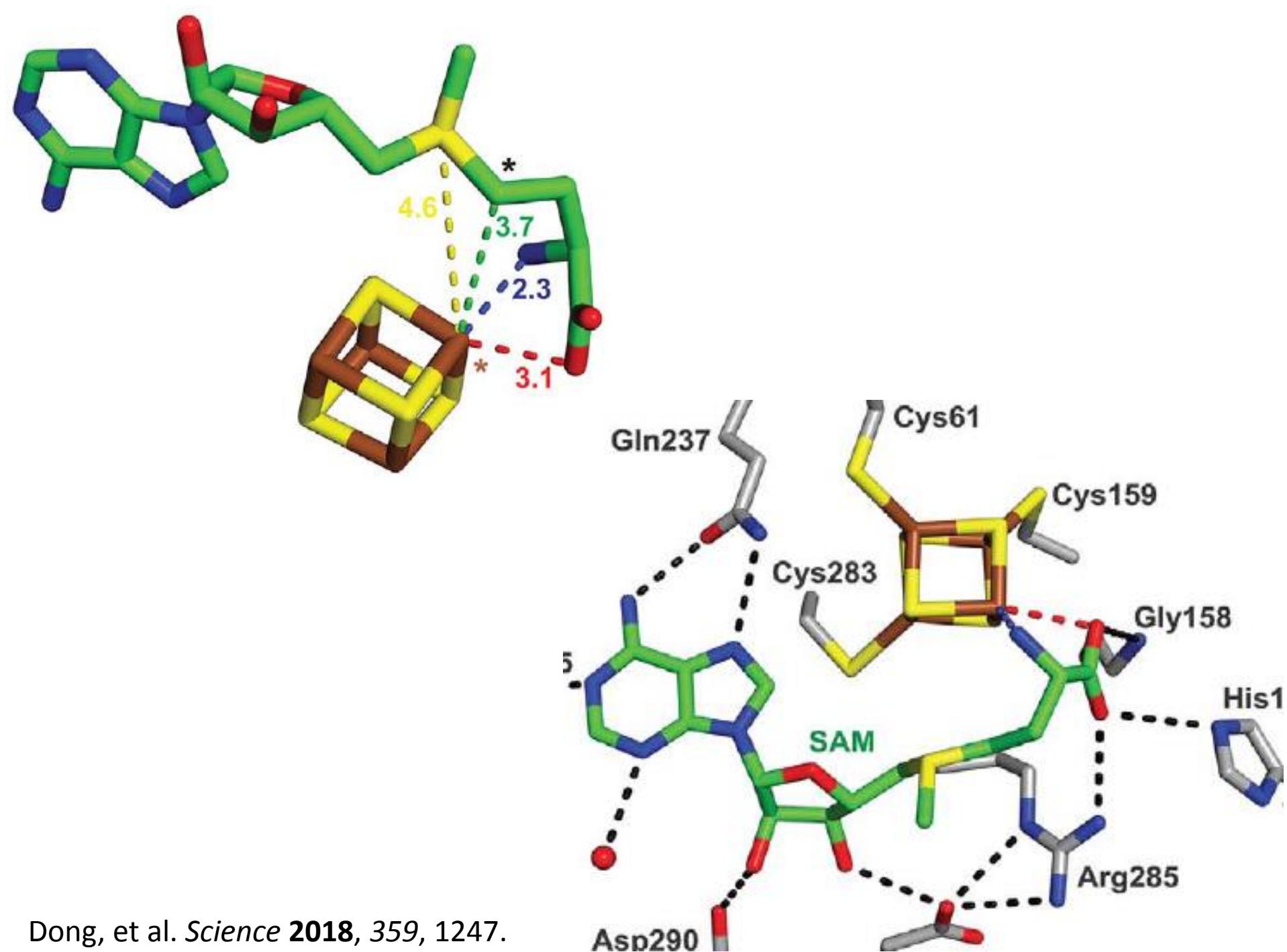
Proposed Mechanism for C8 Methylation by Cfr

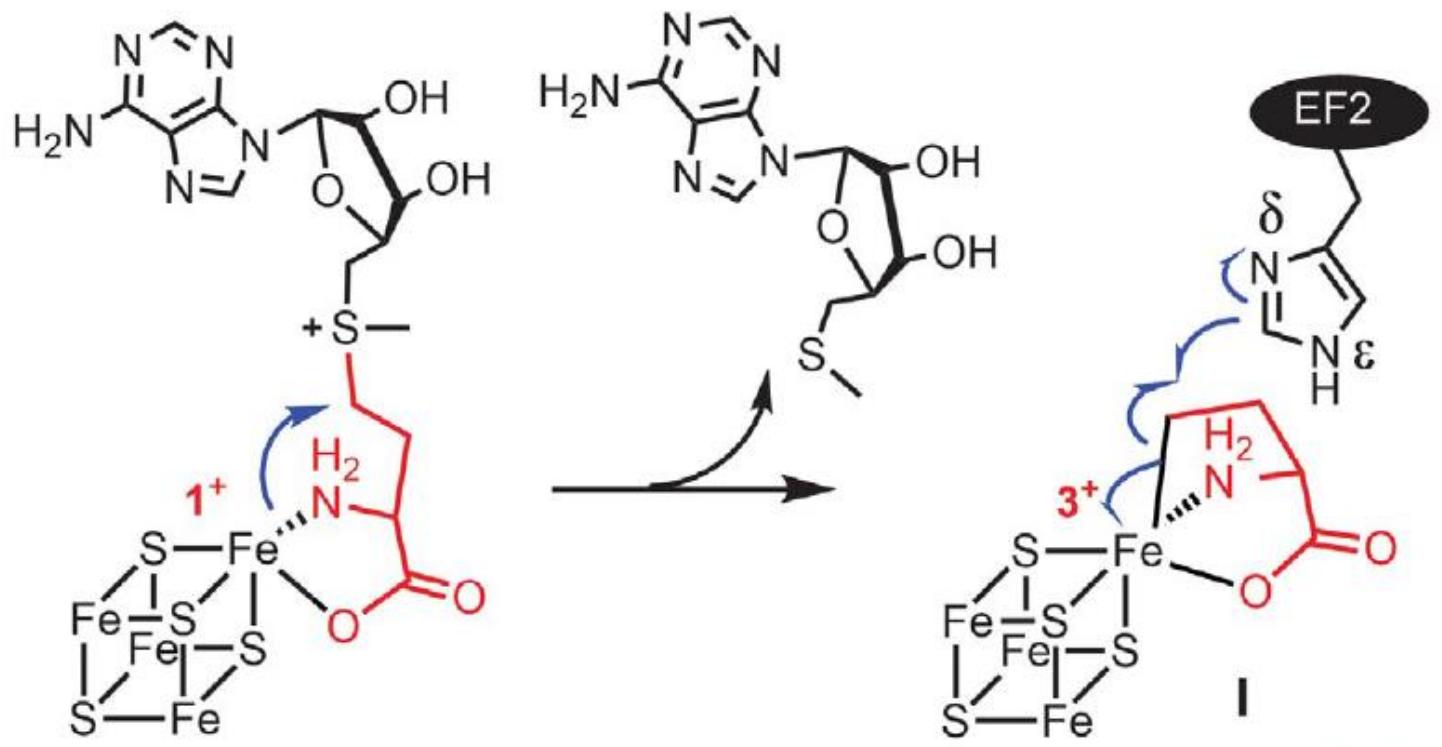


Diphthamide Biosynthesis

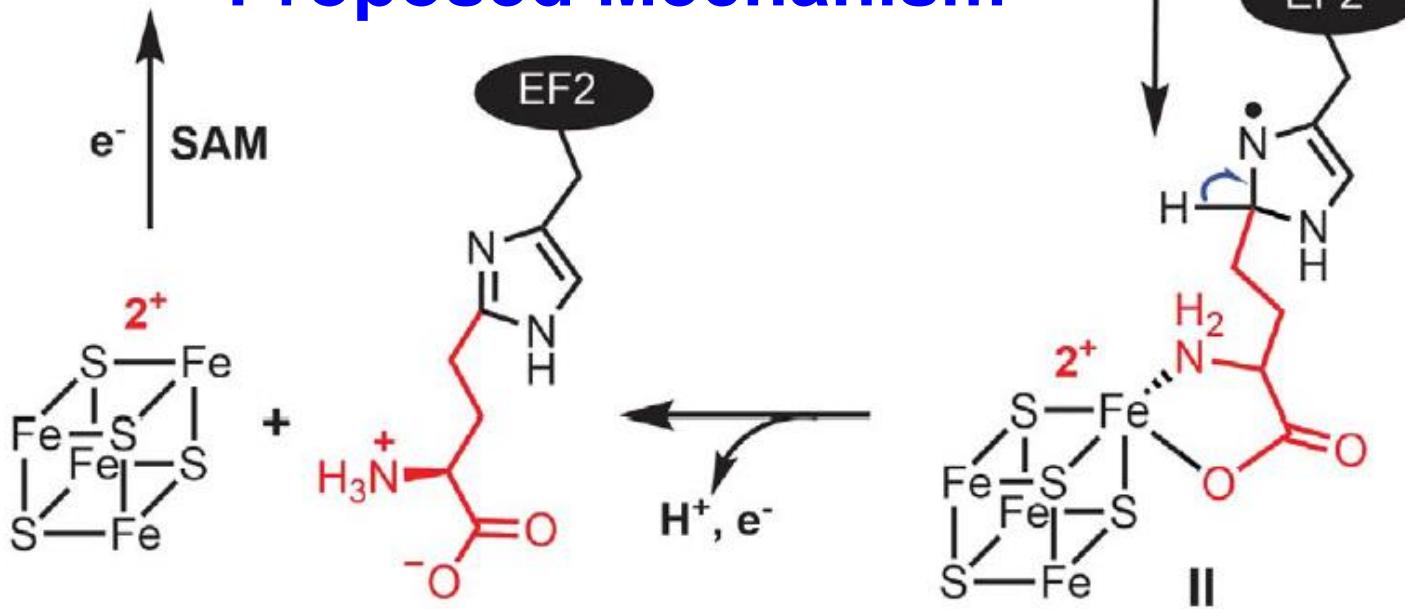
A new type of radical-SAM enzyme







Proposed Mechanism



5. Galactose Oxidase & Amine Oxidases (Cu)

Galactose Oxidase

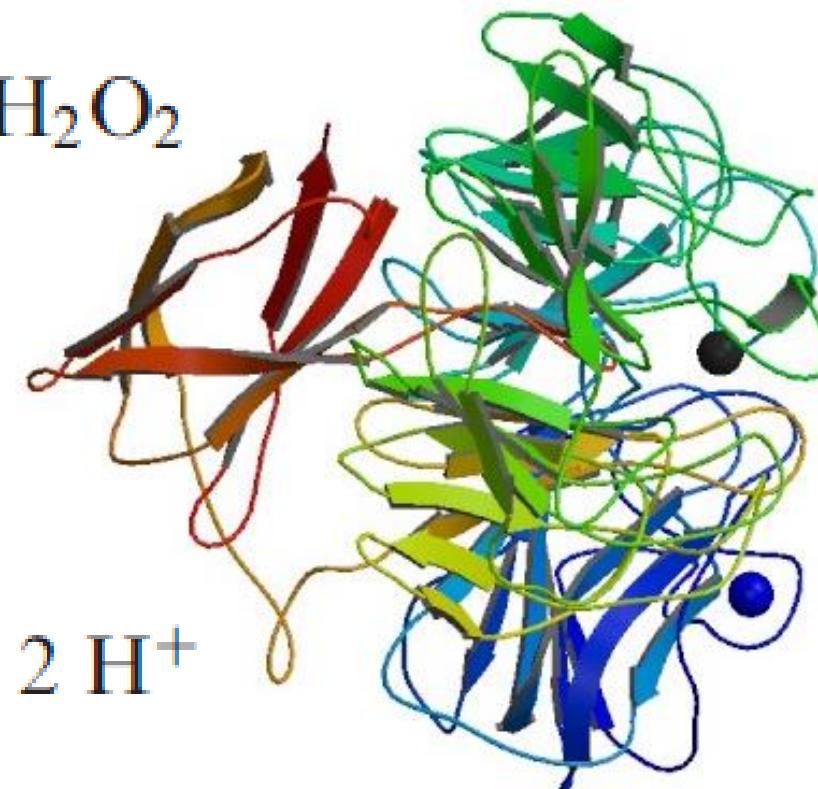
- Catalyzes oxidation of primary alcohols to the related aldehydes:



- 2 steps with 2 half-reactions:

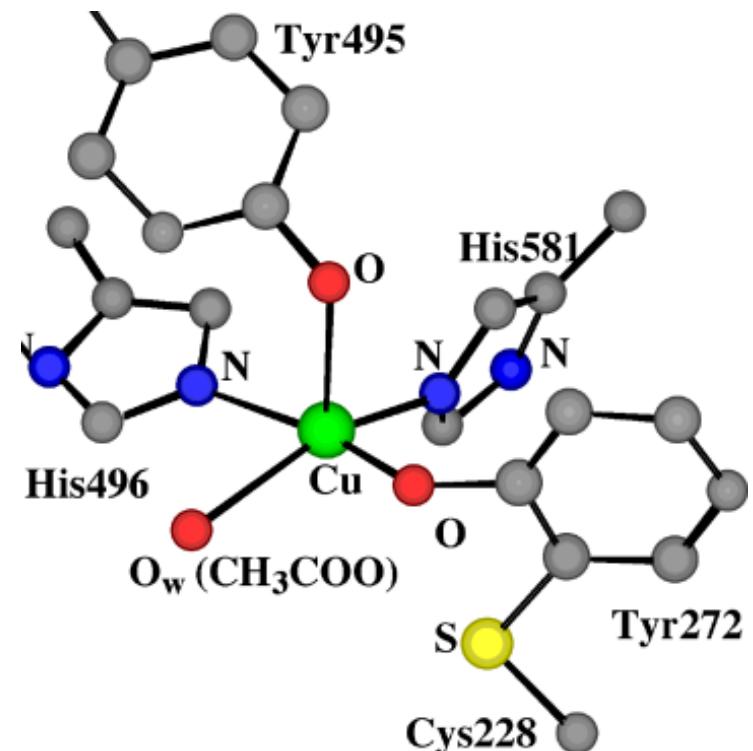
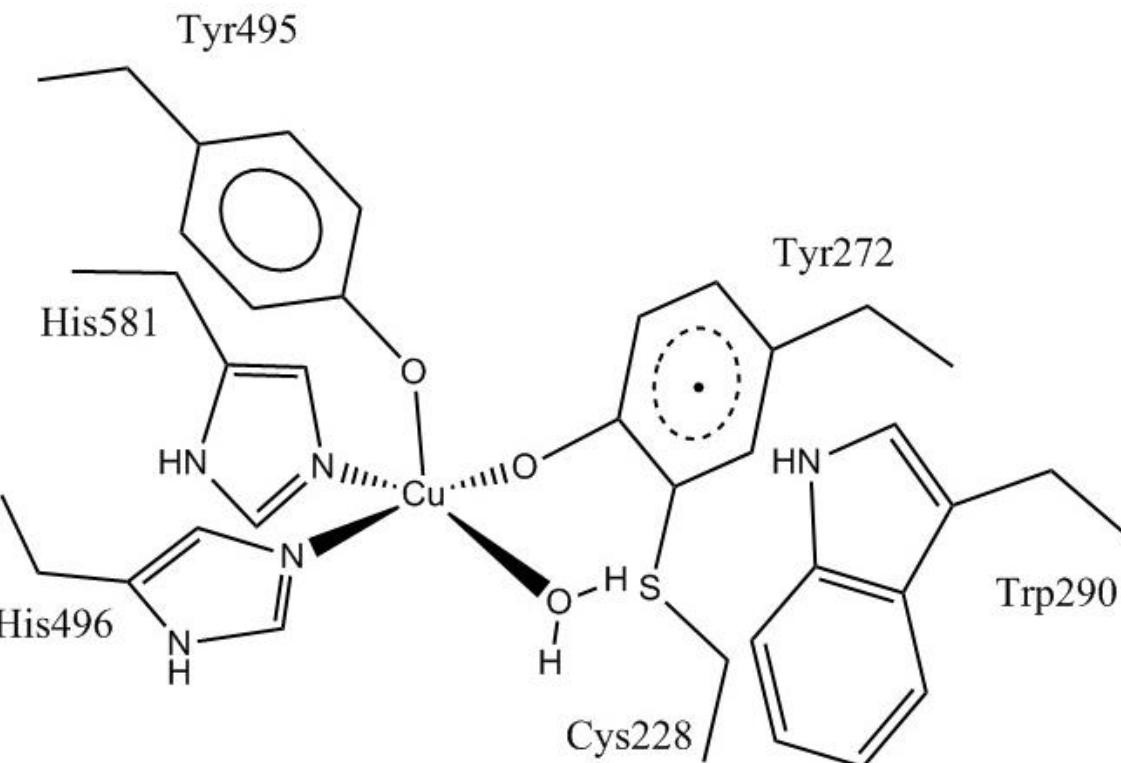
2e⁻ oxidation of RCH_2OH &

2e⁻ reduction of O_2 :



- These **2e⁻** processes occurs in the active site with **ONLY a Cu metal**. The second electron is associated with a protein side chain radical.

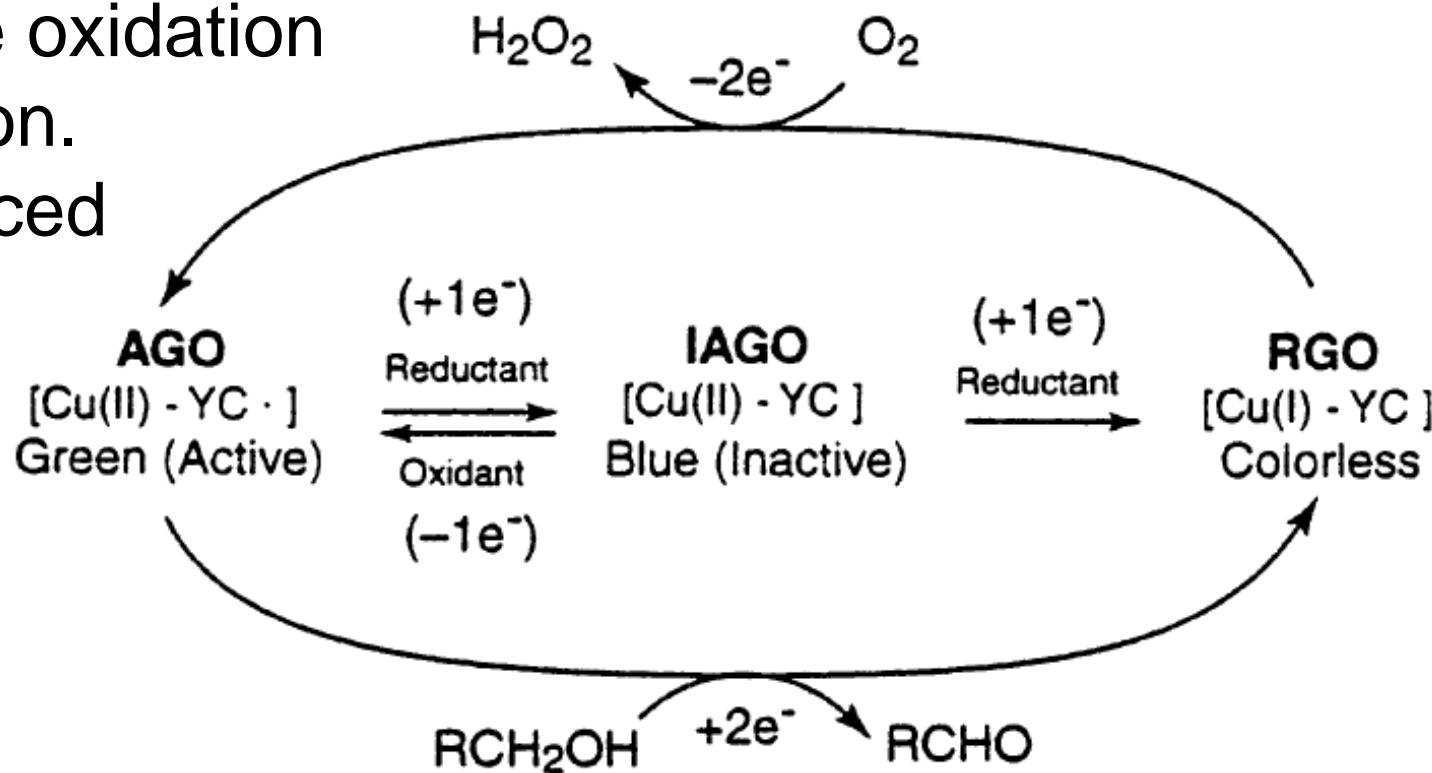
- Square-pyramidal Cu coordination with 2 His, 2 Tyr & 1 H₂O. The **key redox-active Tyr272** phenolic ring forms a **covalent linkage with** the S of **Cys (YC)**. Trp290 protects the Cys-Tyr.
- The Tyr272 radical is **strongly coupled** (anti-ferromagnetically) with the Cu ion by sharing electrons to facilitate the 2e⁻ redox reactions.



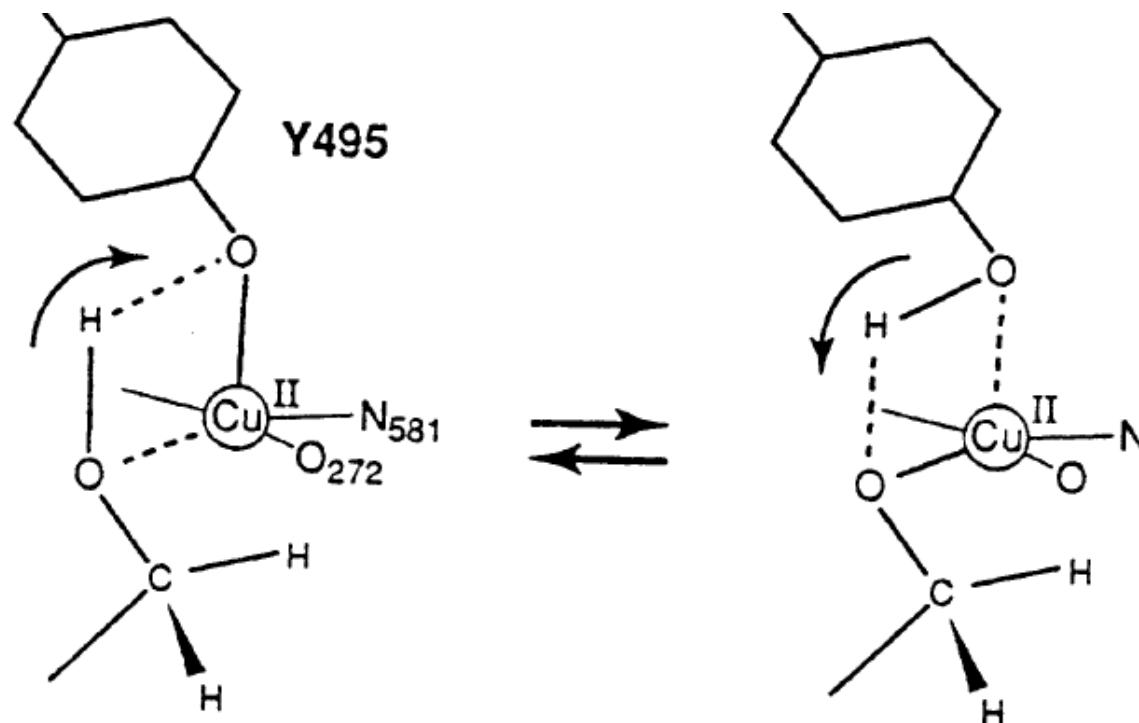
- The 2 redox centers (1 YC & 1 Cu) → 3 different oxidation states: (1) the **fully oxidized** form (**Cu(II)** + a **Cys-Tyr radical** (**YC•**)); (2) **1e⁻** reduction gives a **Cu(II)** + **YC**; (3) the fully reduced form: **Cu(I)** + **YC**.

- The **fully oxidized & reduced forms: catalytically active** & are interconverted by **2e⁻** redox processes for the substrate oxidation & O₂ reduction.

The **1e⁻** reduced complex is catalytically **inactive**.

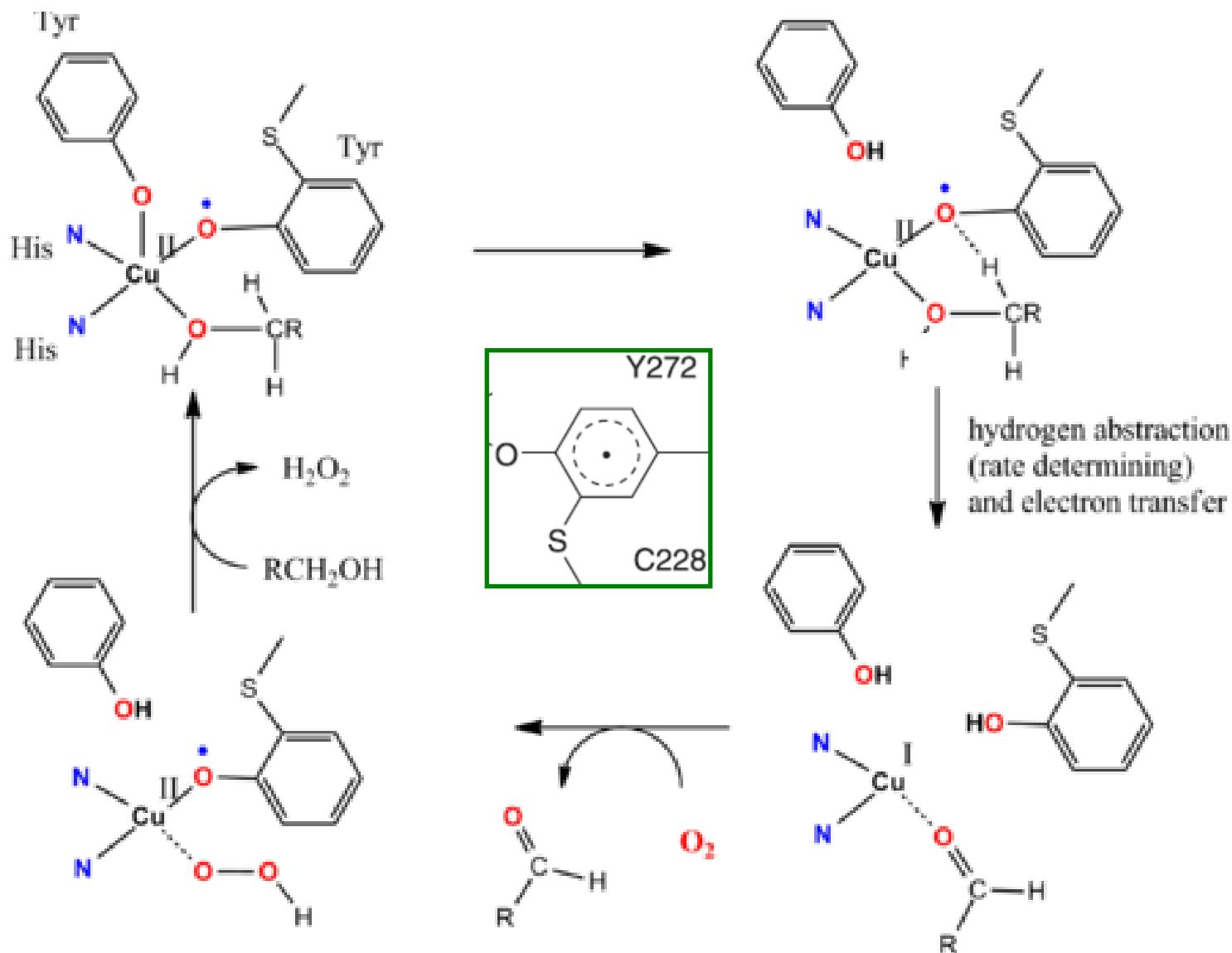


- The alcohol coordinates to the Cu center & forms a H-bond with the deprotonated axial Tyr495.
- **proton transfer** (acid-base reaction);
- activates the substrate by forming a reactive alkoxide intermediate.



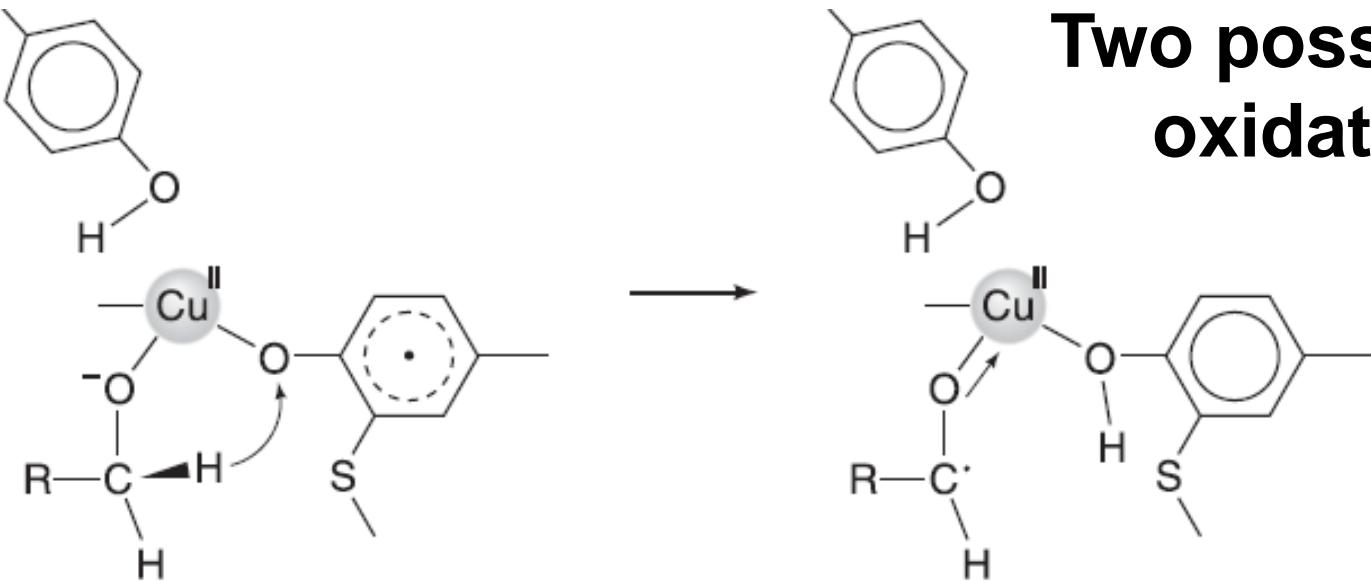
- Isotopic labeling study: the pro-S H of the substrate is stereospecifically cleaved.

Proposed Mechanism



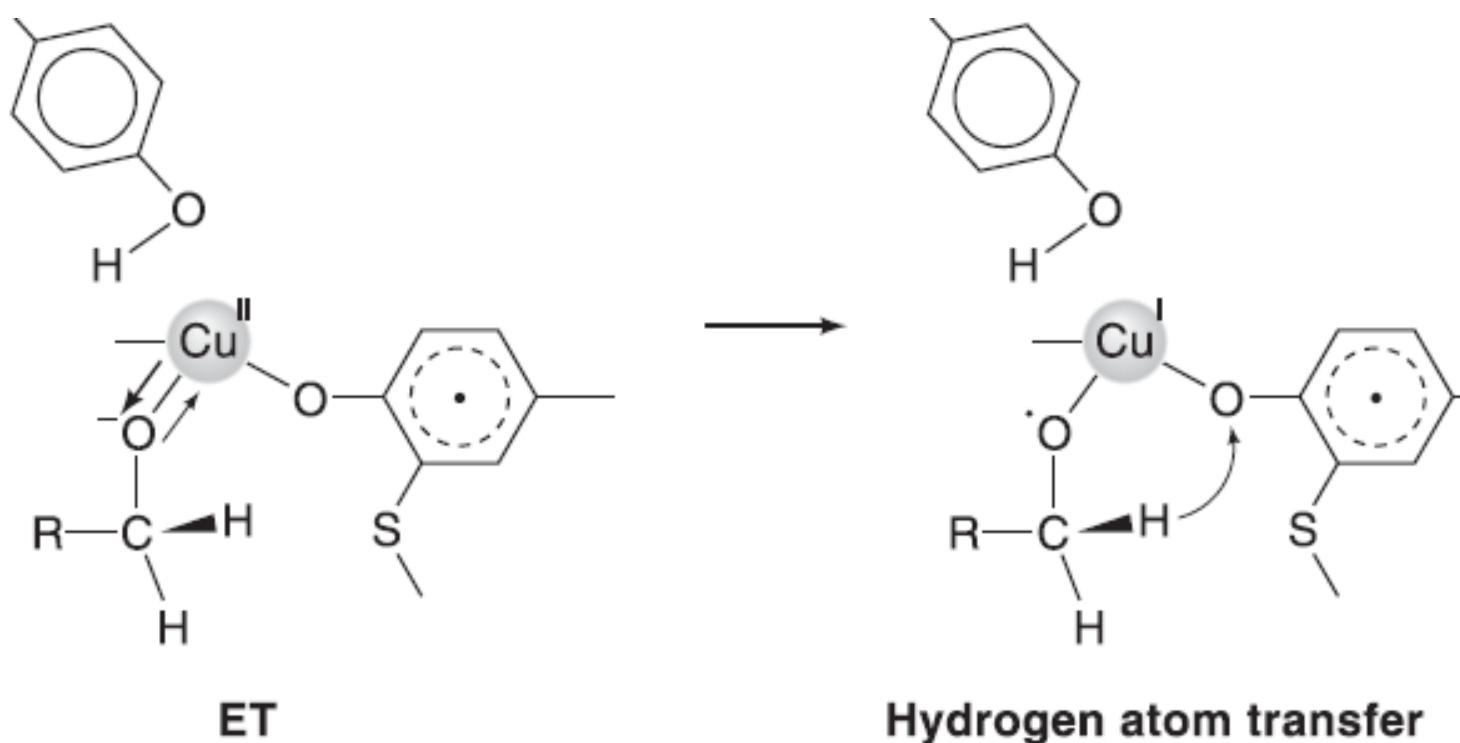
- The Cys-Tyr radical (**H atom acceptor**). Proton-coupled electron transfer (PCET) to the YC radical forms a phenol. The other e⁻ from the substrate reduces Cu(II).

Two possible substrate oxidation processes



Hydrogen atom transfer

ET

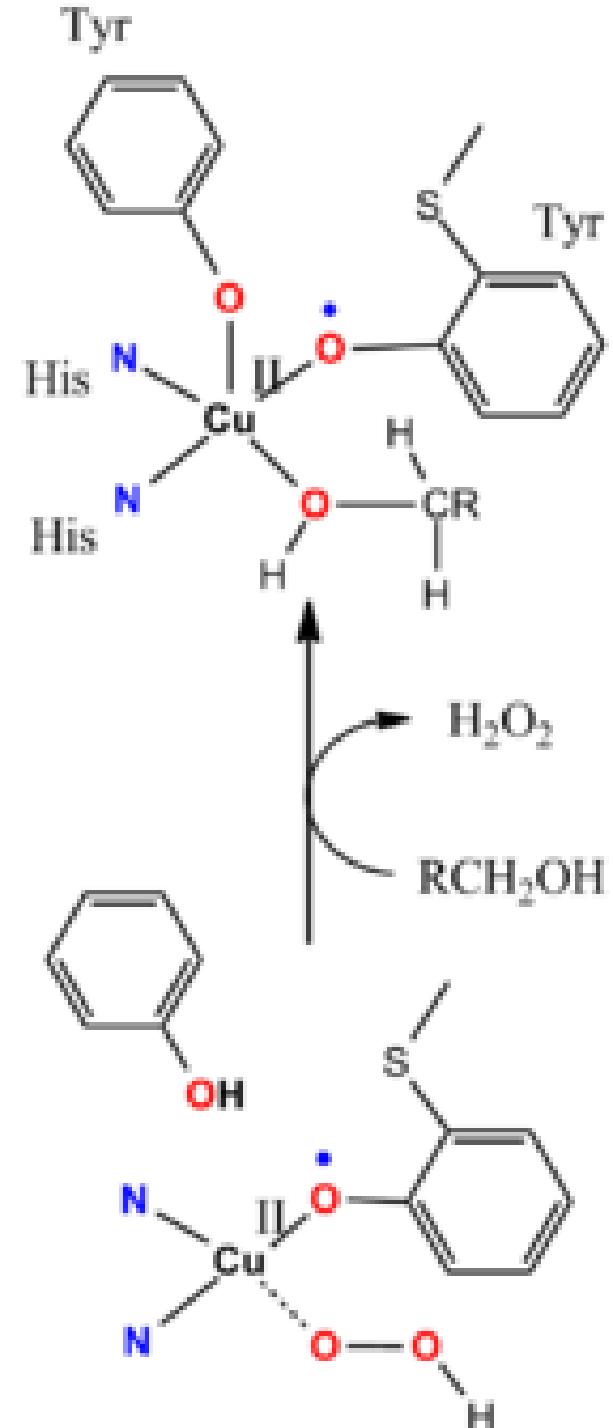


ET

Hydrogen atom transfer

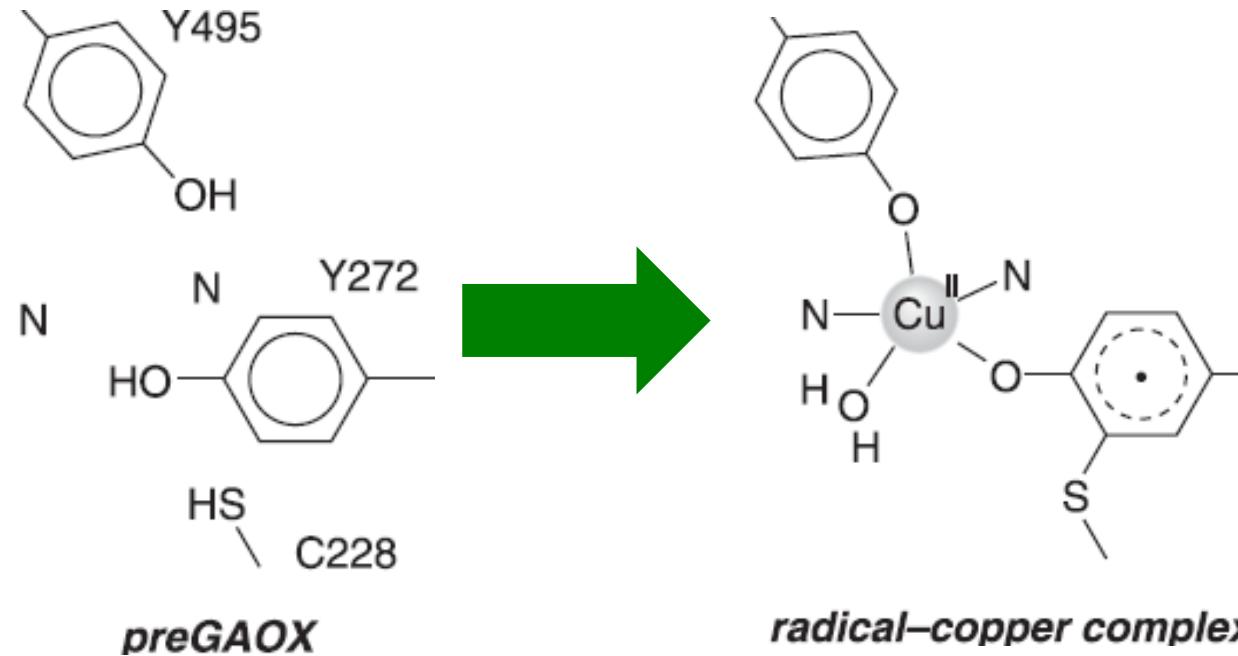
The O₂ reduction step

- After dissociation of the RCHO product, the reduced enzyme complex reacts with O₂ to form Cu(I)-O₂ rapidly.
- Then, undergoes inner-sphere ET to form a Cu(II)-superoxide intermediate, which abstracts a H atom from Tyr272 phenol to form Cu(II)-OOH.
- Proton transfer from axial Tyr495 to Cu(II)-OOH gives H₂O₂ & deprotonated Tyr495, which re-coordinates to the metal center.



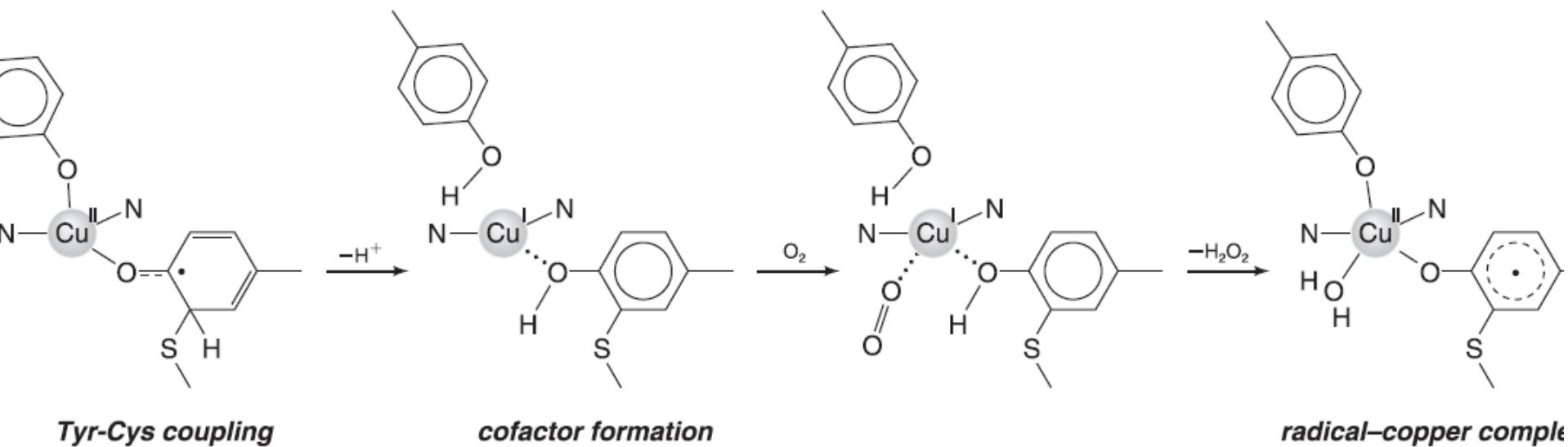
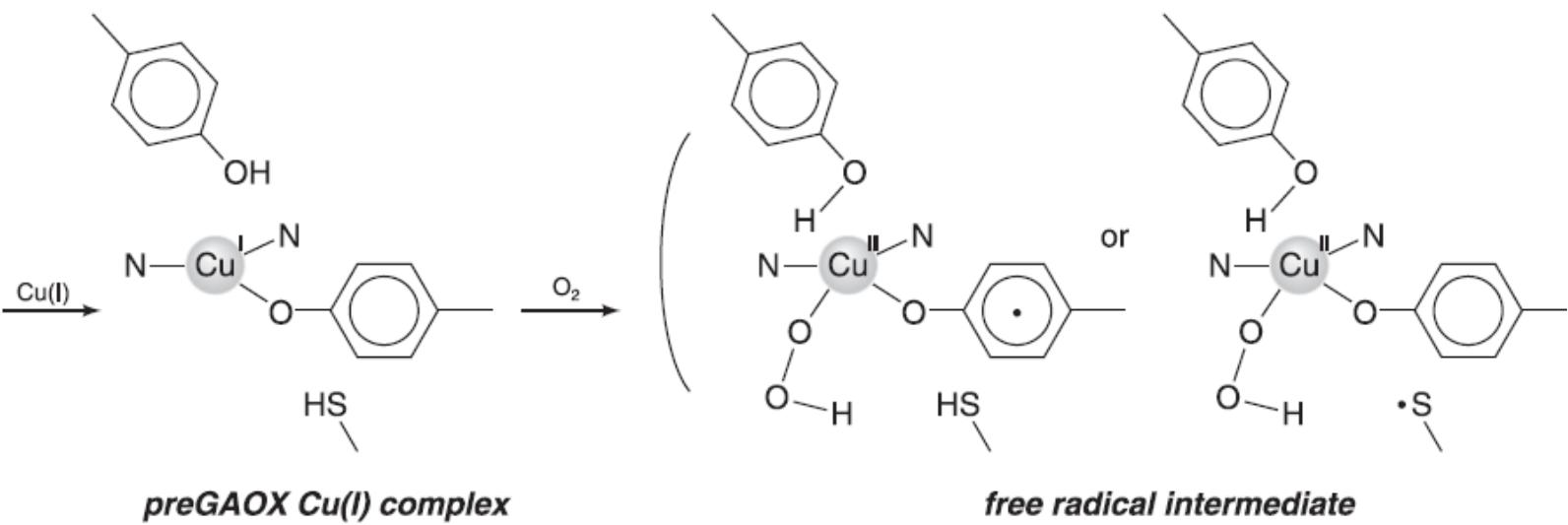
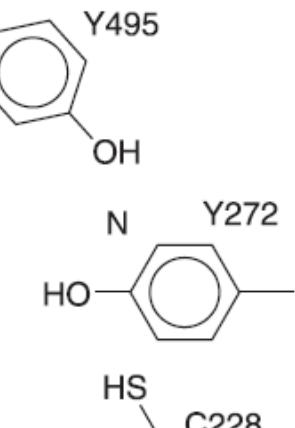
Mechanism of Cofactor Biogenesis (生源论)

- The Cys-Tys side-chain linkage requires a post-translational modification for the enzymatic activity.



- The protein is expressed as a precursor pregalactose oxidase that reacts with Cu(I) & O₂ (4e⁻ process; 2e⁻ for O₂ reduction) to form the Tyr-Cys linkage cofactor.
- Self-processing reaction of the redox cofactor.
- The reaction with Cu(I) is ~4000 faster than Cu(II).

Proposed Mechanism

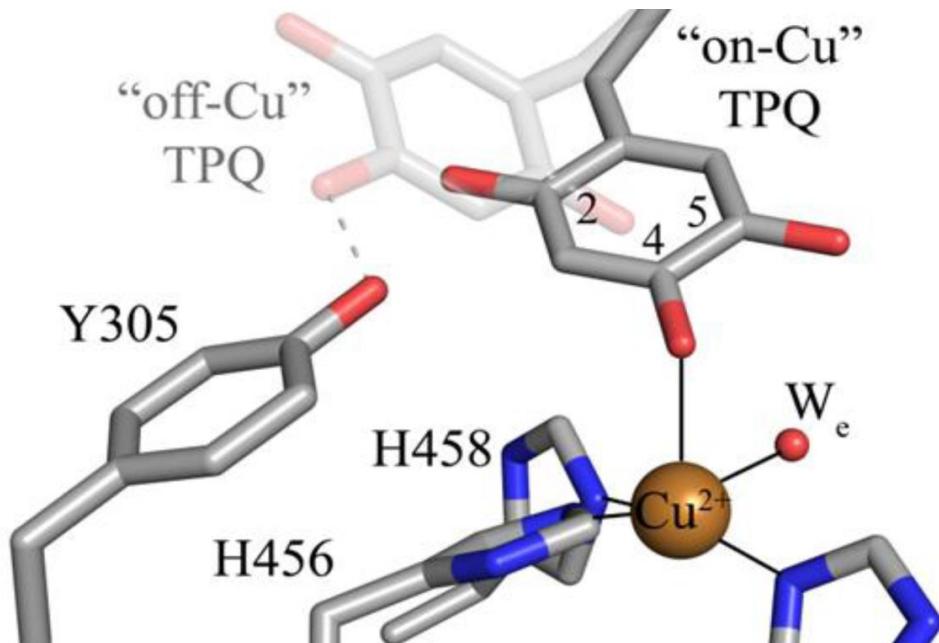
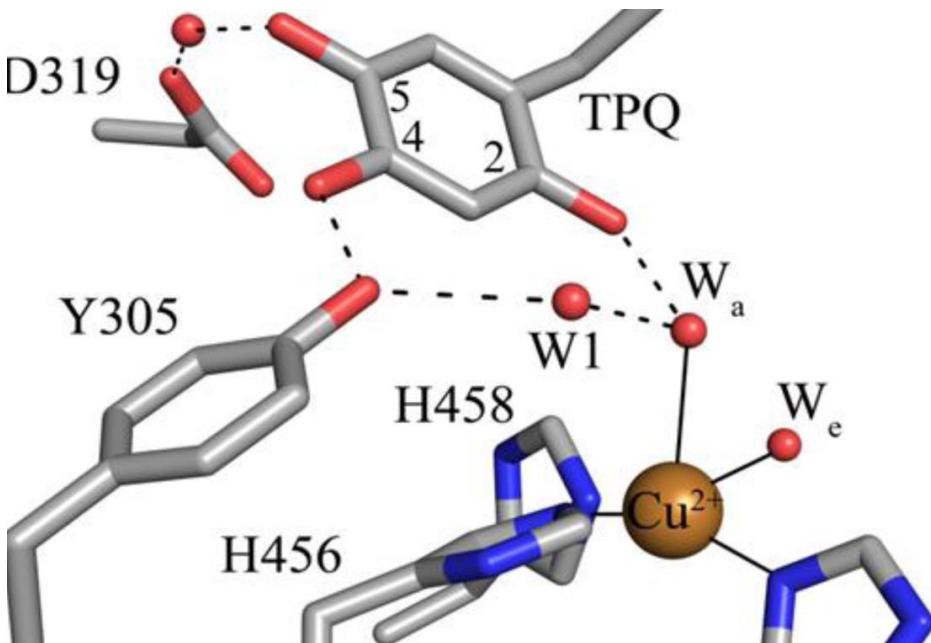


Amine Oxidases

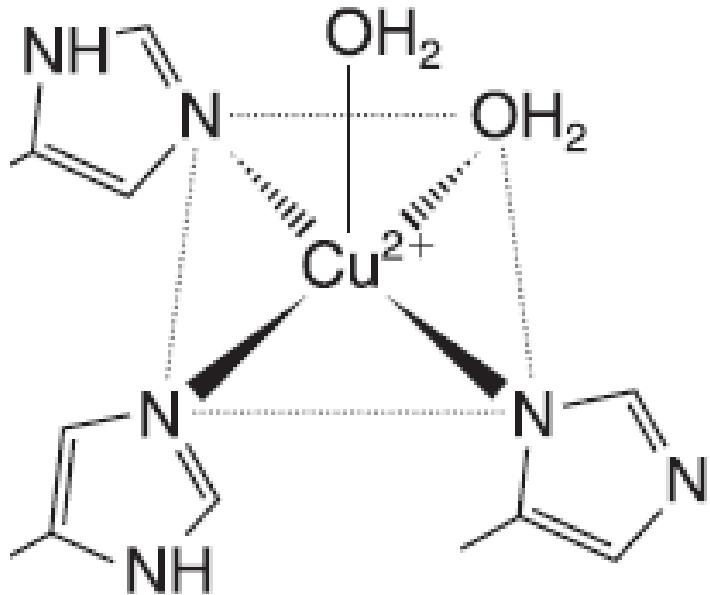
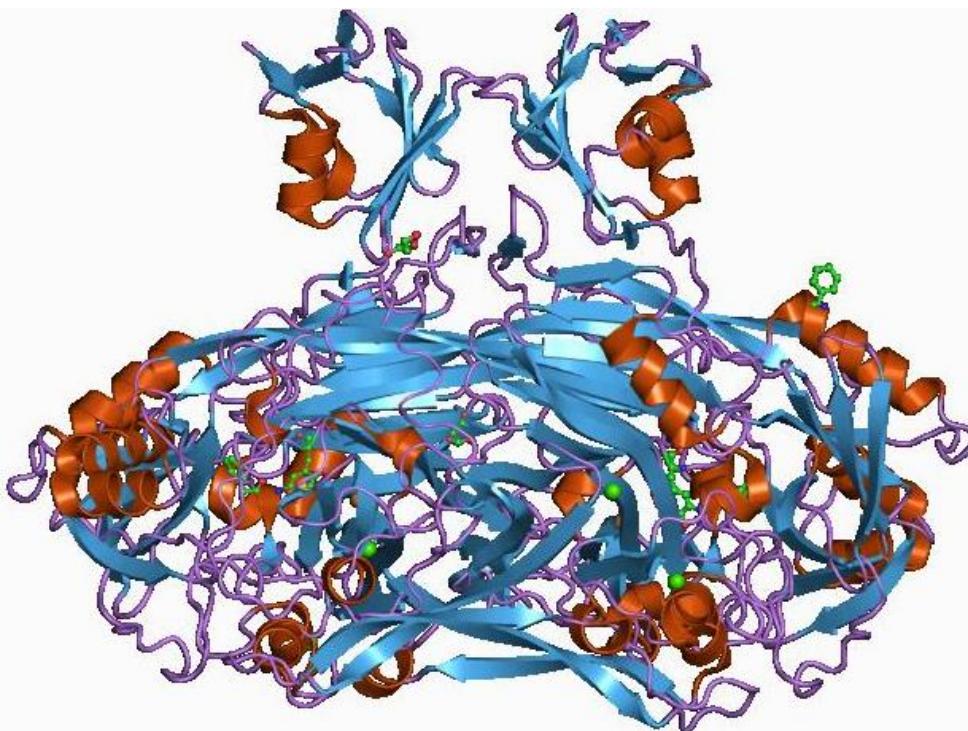
- Catalyze **oxidation of primary amines to give aldehyde & NH₃:**



- Contain a **Cu ion & a quinone** (2,4,5-trihydroxyphenylalanine quinone, **TPQ**), which is derived from a Tyr.

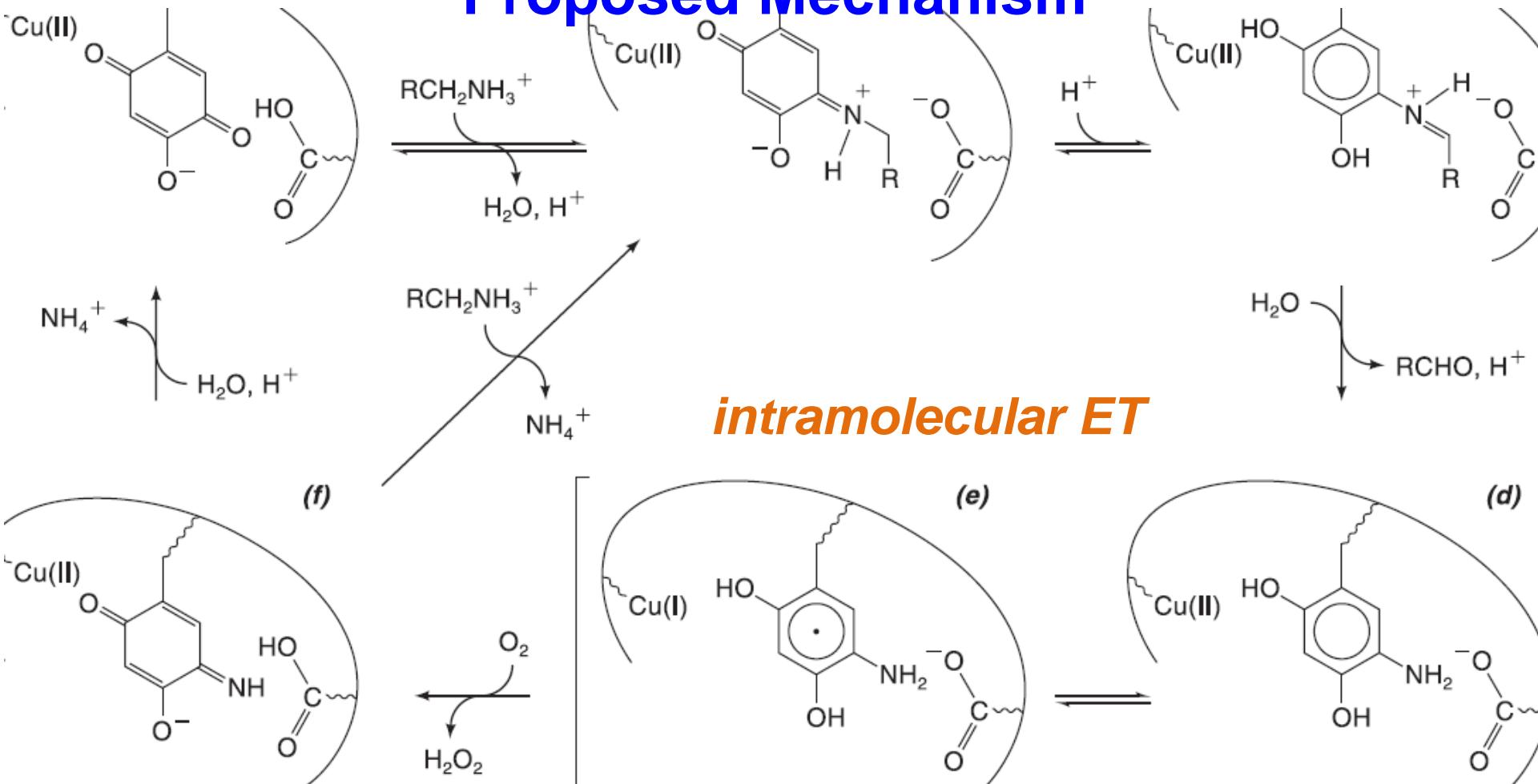


- EPR, X-ray absorption spectroscopy (XAS), paramagnetic NMR & CD/MCD: the 5-coordinate (square-pyramidal) Cu(II) center.

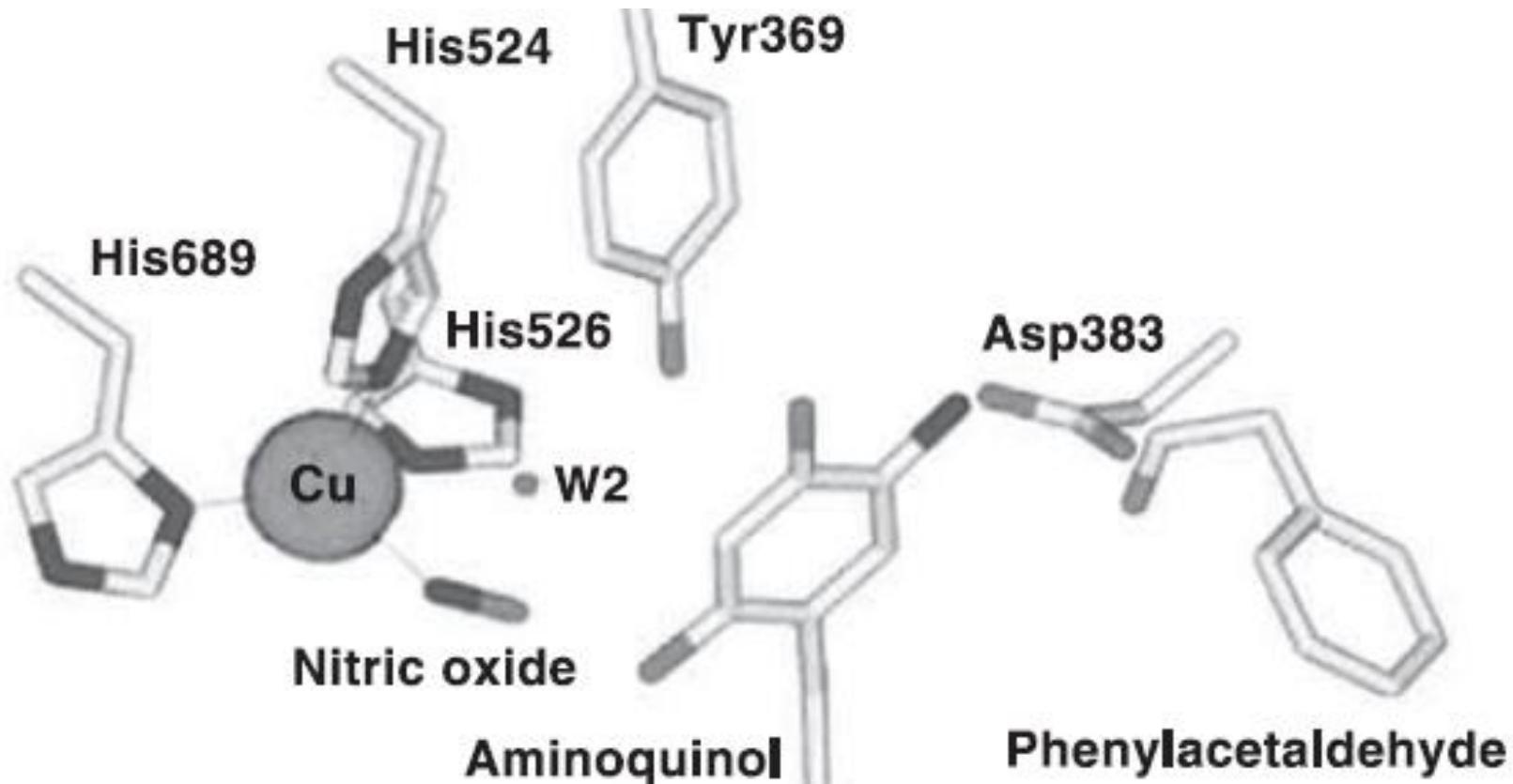


- Reactions at TPQ affects the Cu(II) coordination mode.
- As TPQ could react with all primary amines, the substrate specificity is controlled by the electrostatic & steric properties of the active site.

Proposed Mechanism



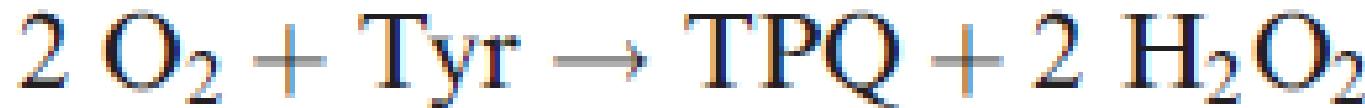
- TPQ **oxidizes the amine & the reduced catechol amine**, then **reduces O_2** to form NH_3 & H_2O_2 .
- A conserved Asp acts as the base. Both H^+ to form H_2O_2 are derived from the reduced TPQ.



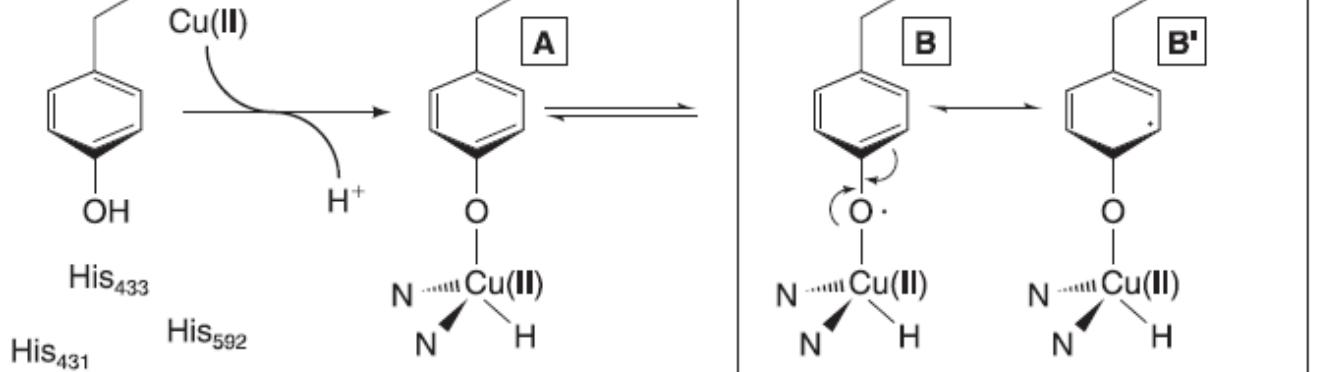
- The NO is positioned between Cu & TPQ, & forms a H bond to the reduced aminoquinol form of TPQ. The nonlinear NO geometry: Cu(II)-NO predominantly.
- the O₂-binding & reaction site is likely located between Cu & TPQ.

Mechanism of Cofactor Biogenesis

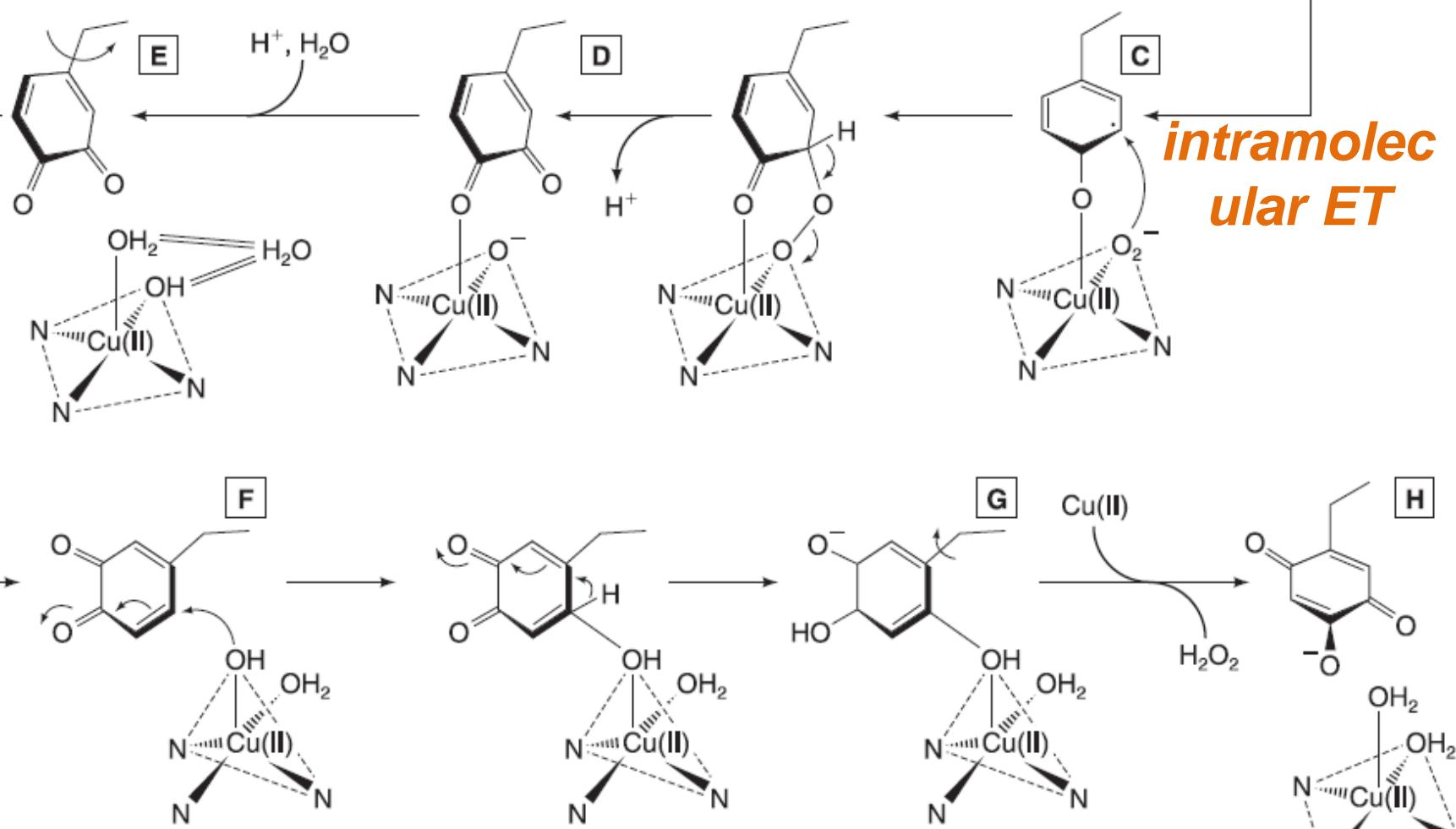
- $6e^-$ oxidation of a Tyr in the active site forms TPQ: post-translational, self-processing reaction:



- Coordination of TPQ to Cu(II) suggested that Tyr can coordinate to Cu(II) to activate the phenol ring of Tyr for oxidation.
- Self-processing redox enzymes (e.g. Galactose Oxidase & Amine Oxidases) use O_2 to modify few amino acids (e.g. Tyr, Trp, Cys, & His) to form new redox cofactors.

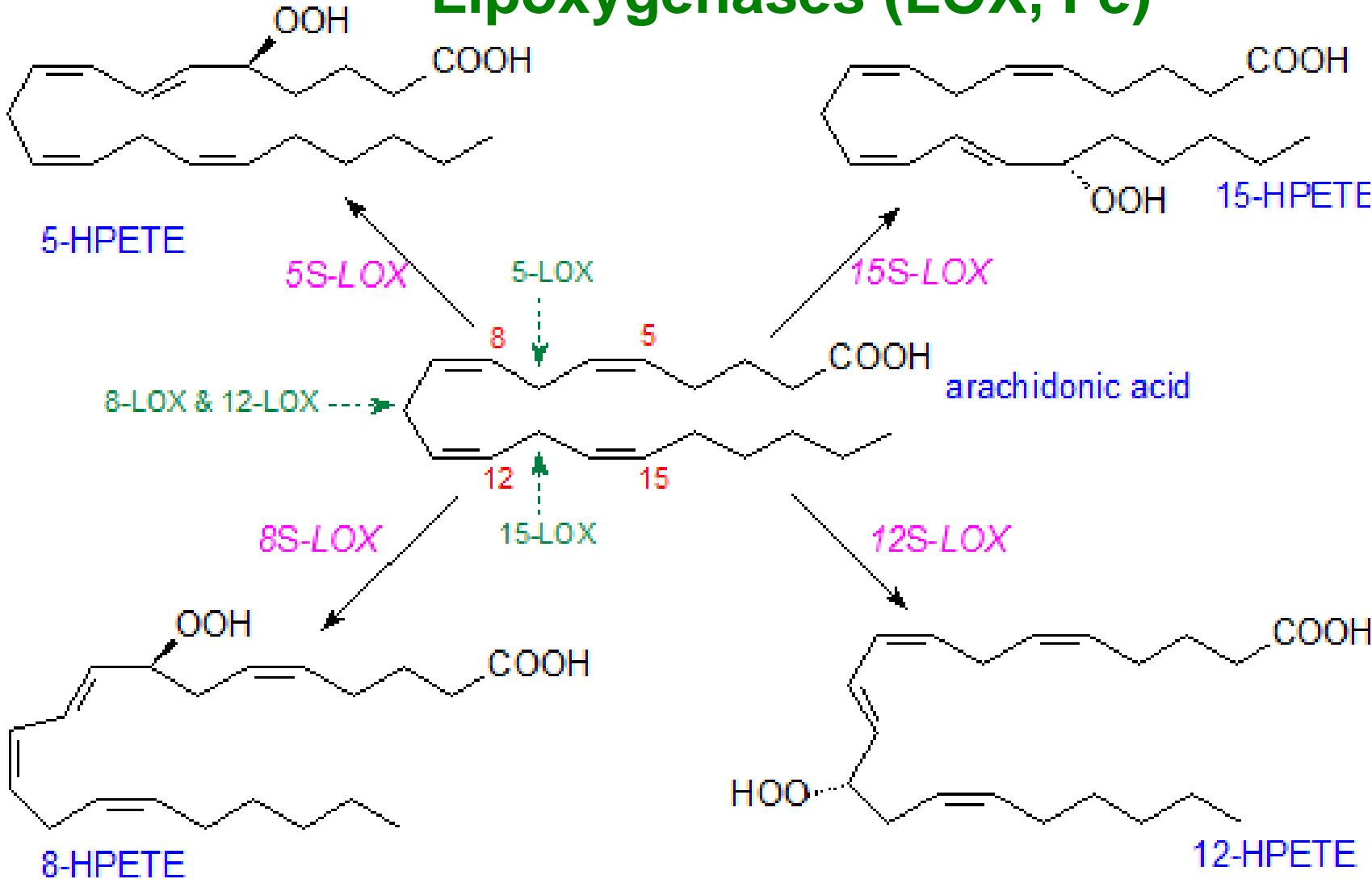


Proposed Mechanism



6. Lipoxygenase

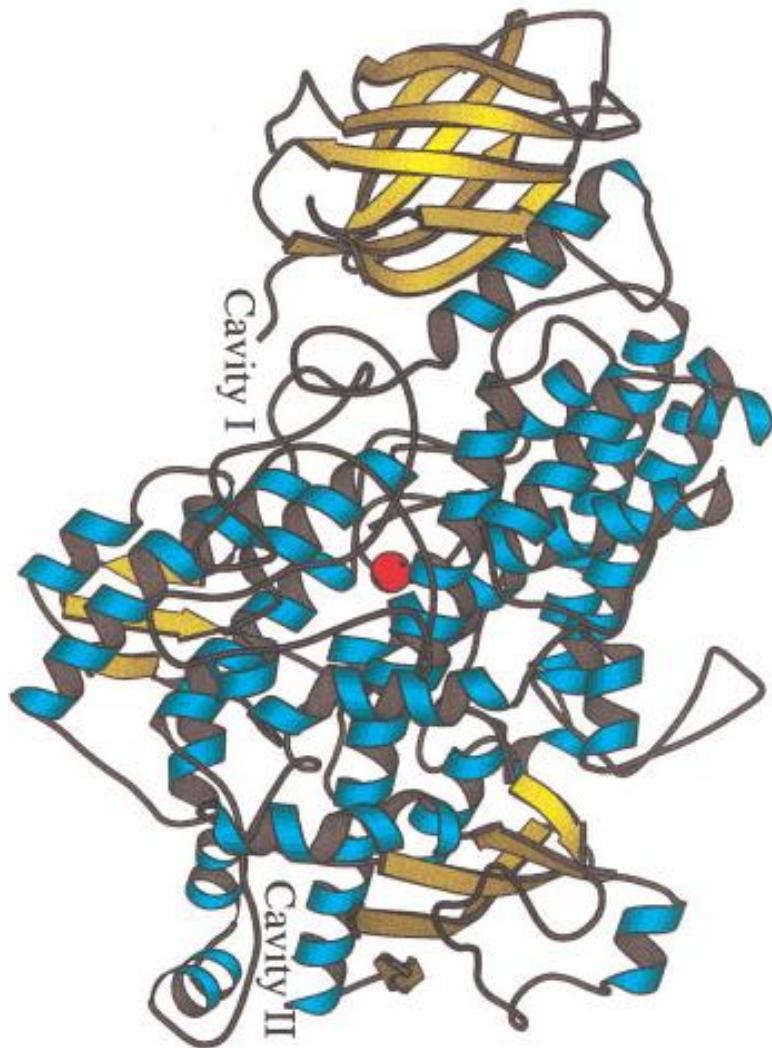
Lipoxygenases (LOX, Fe)



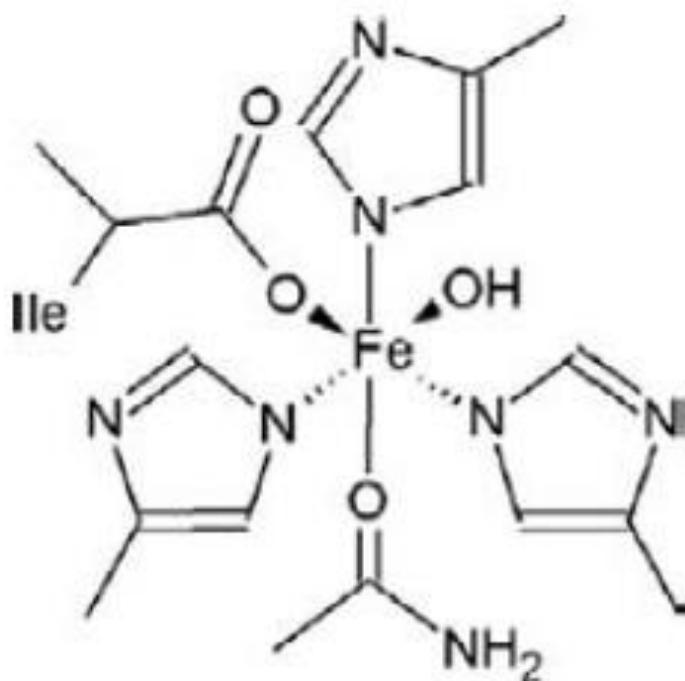
- Catalyzes peroxidation of unsaturated fatty acids.
- Found in many organisms (from cyanobacteria to higher plants & animals).

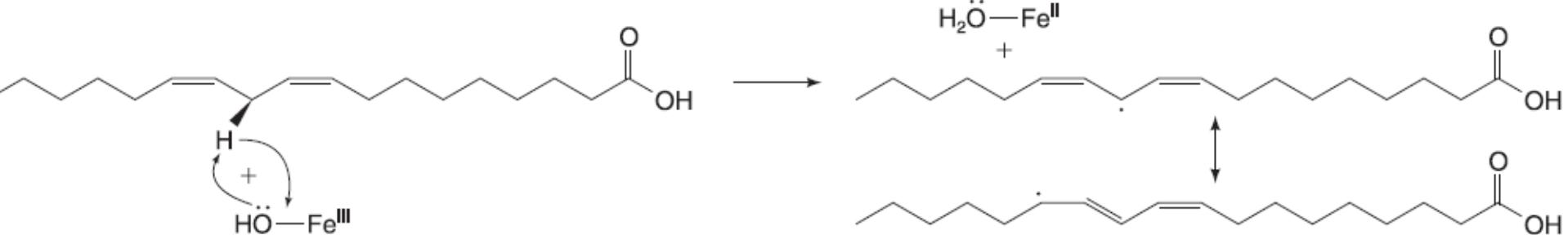
- Key biological processes: formation of many compounds affect inflammation process. E.g. leukotrienes & lipoxins attract white blood cells to sites of infection or injury; response to injury & infection in plants with the different products.

- Crystal structures of the soybean & a mammalian LOXs: (1) the N-terminal domain (mainly β -sheet) was suggested to extract the substrates from a membrane; (2) the C-terminal domain (mainly α -helical) contains the **non-heme Fe** & putative substrate binding site.



- The Fe site is adjacent to a long, narrow, hydrophobic cavity (possible substrate binding pocket).
- The resting state: Fe(II) with 3 His, the C-terminal carboxylate, a H₂O, & a Asp side chain (Asp → His in mammal).

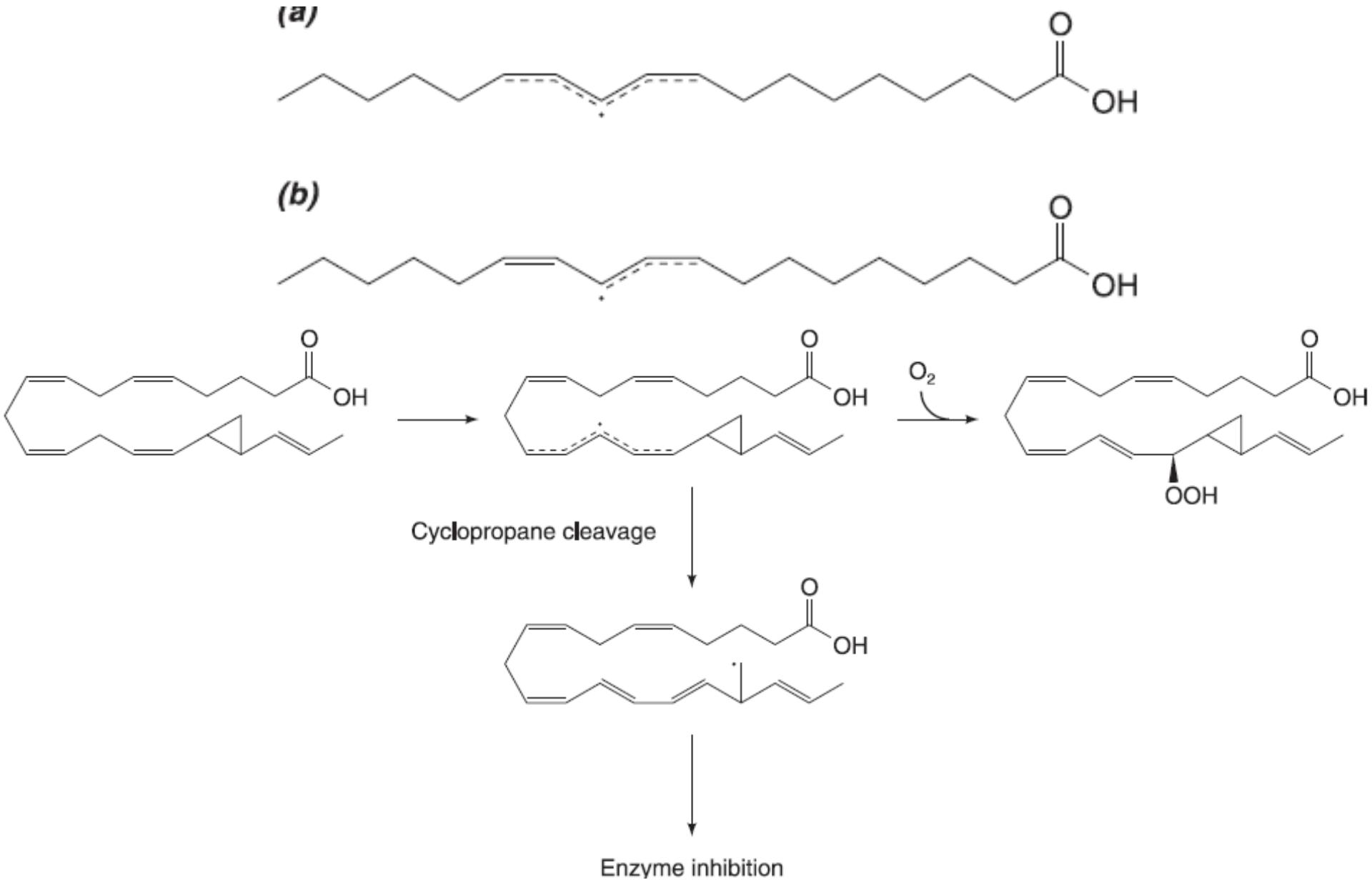




Proposed Mechanism



- By molecular modeling, the Fe site was suggested to be close to the unsaturated part of the substrate.
- The **active form: Fe(III)-OH** undergoes irreversible **H atom abstraction** (a weak C-H) to form an **pentadienyl radical intermediate & reduce Fe**.
- Then, **reacts with O₂** to form a **peroxy radical**, which is reduced by the Fe center to regenerate Fe(III) state.

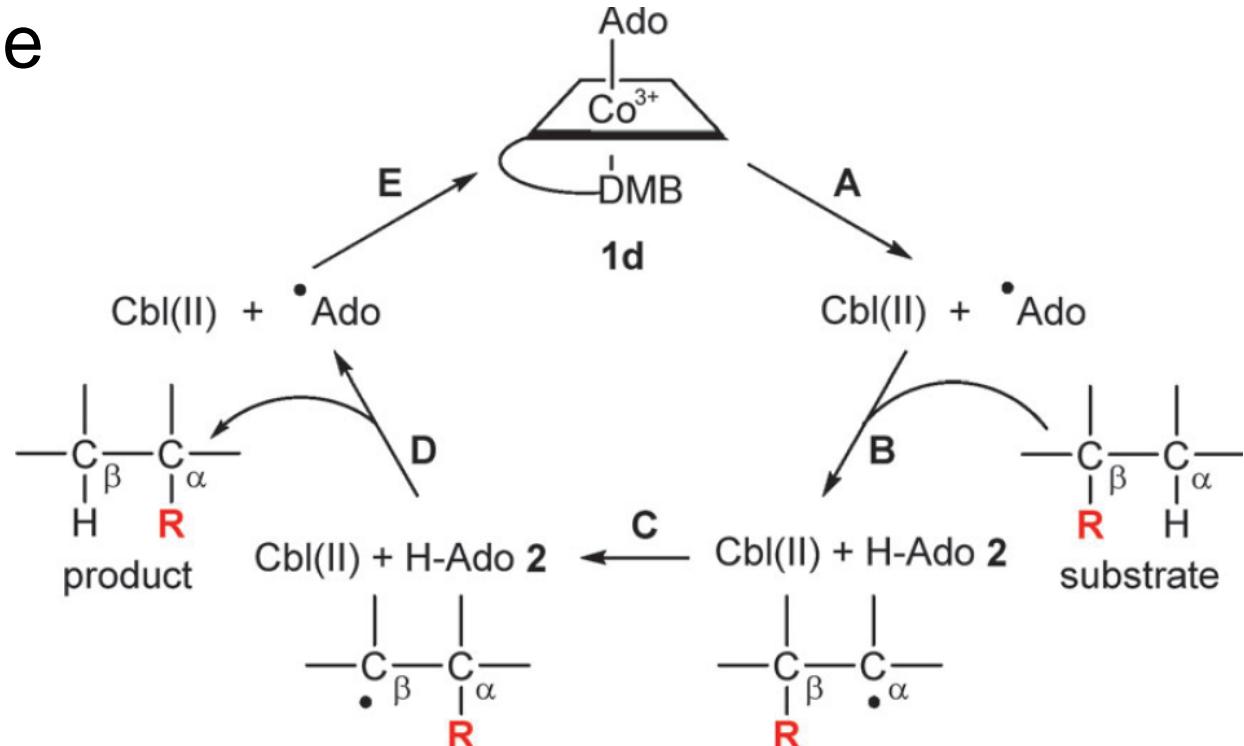


- The substrate radical was supported by formation of rearranged product from the radical clock substrate.

- Fe(II) cannot be oxidized to Fe(III) by O_2 , while lipid hydroperoxides can oxidize Fe(II) enzyme to form the active Fe(III) state.
- Remarkable kinetic isotope effects (KIE) was observed with deuterated substrates ($k_H/k_D = \sim 80$), much larger than the other enzymes → **Quantum tunneling**.
- Lipoxygenase use different strategy for oxygenation of an organic substrate: activation of the substrate by the **Fe(III)-OH** followed by O_2 attack.
- While, most other oxygenases catalyze a H atom abstraction by a more reactive high-valent species (e.g. **iron(IV)-oxo**, $Fe=O$).

Key Summary

- Free **radical** contains **unpaired electron(s)**.
- Radical reactions are **well controlled** & reactive **radical intermediates** are **protected in enzymes**.
- Carbon radical can be formed from **homolysis of the Co-C bond** of **AdoCbl (B₁₂)**, which can trigger **H atom abstraction** with the substrate & then substrate **skeleton rearrangement**. A significant protein effect on the weakening C-Co bond cleavage.



- Ribonucleotide reductases (NRs) catalyze reduction of ribonucleotides to form deoxyribonucleotides. All NRs involve a **transient Cys radical** for H abstraction of the substrate.
- 3 classes of NRs: **Class I: a Tyr radical & non-heme diiron center;** **Class II: a Ado radical & AdoCbl;** **Class III: a Gly radical, [4Fe4S] & adenosylmethionine (AdoMet or SAM).**
- Apart from common ET role by the Fe-S clusters, radical-SAM enzymes with **Fe-S clusters & SAM** can undergo **reductive cleavage** of SAM (as a **cofactor or co-substrate**) to **generate Ado radical & Met.** Also, the **[2Fe2S]** can be a **S atom donor** in botin synthase.

- Galactose oxidase & amine oxidases are Cu-containing enzymes: **oxidation** of primary alcohols (galactose oxidase) or amines (amine oxidases) followed by **O₂ reduction**.
- Galactose oxidase & amine oxidases are self-processing redox enzymes, which **use O₂** to modify amino acids to form **new redox cofactors**.
- Galactose oxidase: a **Cu metal & a redox-active Cys-Tyr (radical)** cofactors.
- Amine oxidases: a **Cu metal & a quinone** (2,4,5-trihydroxyphenylalanine quinone, **TPQ**) cofactors.

- Lipoxygenases (LOXs) are **non-heme** enzymes and catalyze **peroxidation** of unsaturated fatty acids.
- The **active form** in LOXs: **Fe(III)-OH** undergoes irreversible H atom abstraction of a weak C-H bond followed by reaction with **O₂**.
- Very **large** kinetic isotope effects (**KIE**, $k_{\text{H}}/k_{\text{D}} = \sim 80$) → **Quantum tunneling**.

**Thank You for Your Kind
Attention, Attending BIC
Course & Exploring BIC
Basics! Hope U Enjoy BIC!**

(Many BIC Qs still need to be
asked & solved by various
experimental and computational
ways)