How neutron crystallography has revealed hidden secrets of enzyme mechanism

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South Africa
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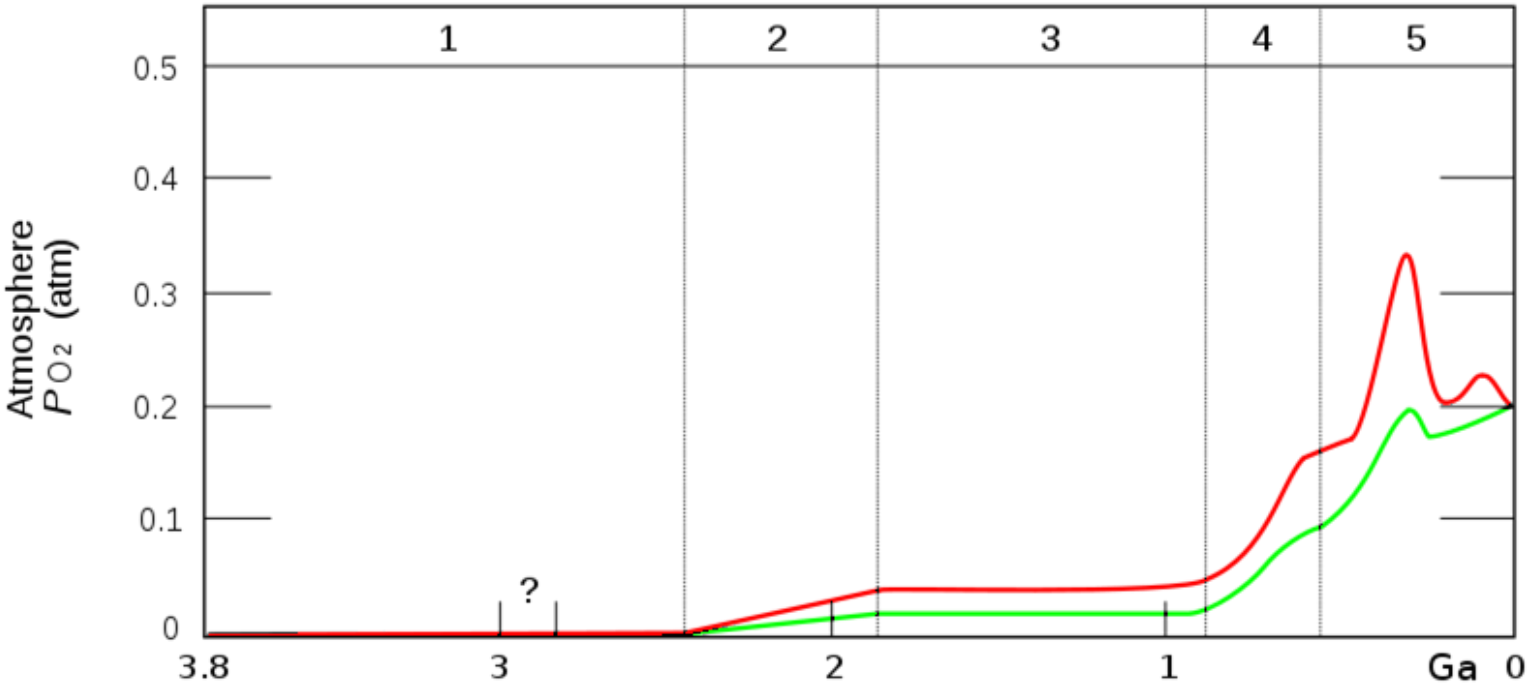
~ 2.4 billion years ago - Great Oxygenation Catastrophe

http://en.wikipedia.org/wiki/Great_Oxygenation_Event



Earth's early atmosphere was practically oxygen-free.

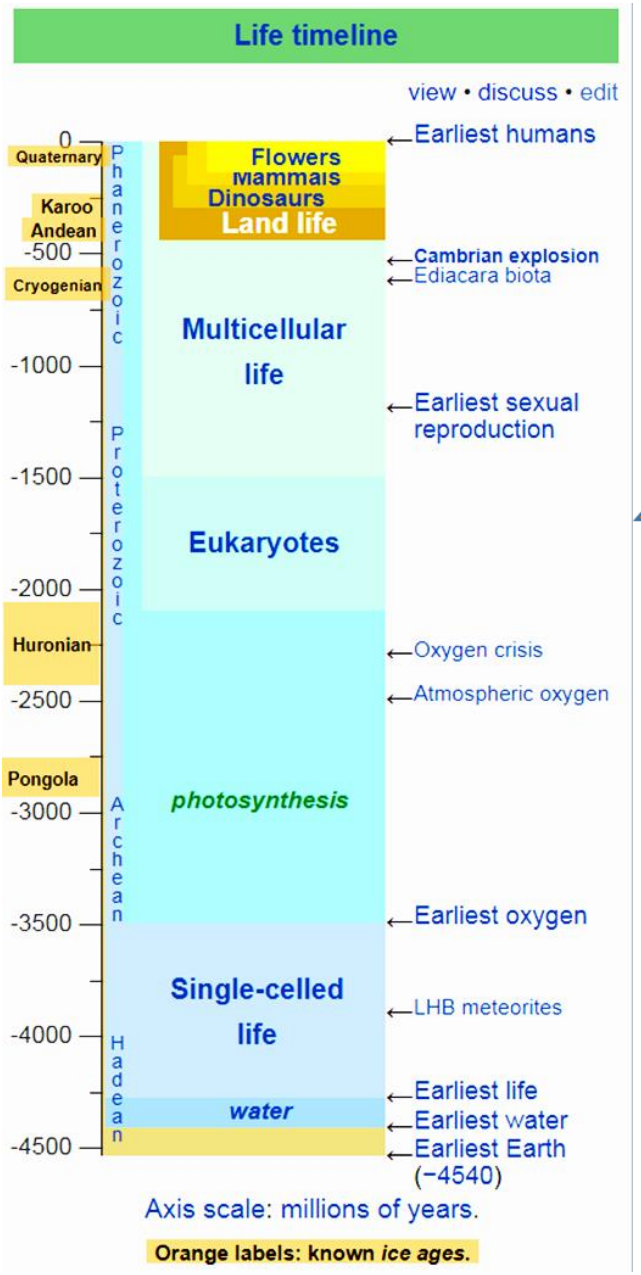
Any free oxygen produced (by photosynthesis by cyanobacteria) was trapped by free iron and organic matter.

When this sink exhausted, levels of oxygen increased to **catastrophic** levels

>Mass extinctions

>Global cooling

>Snowball Earth (650Ma)

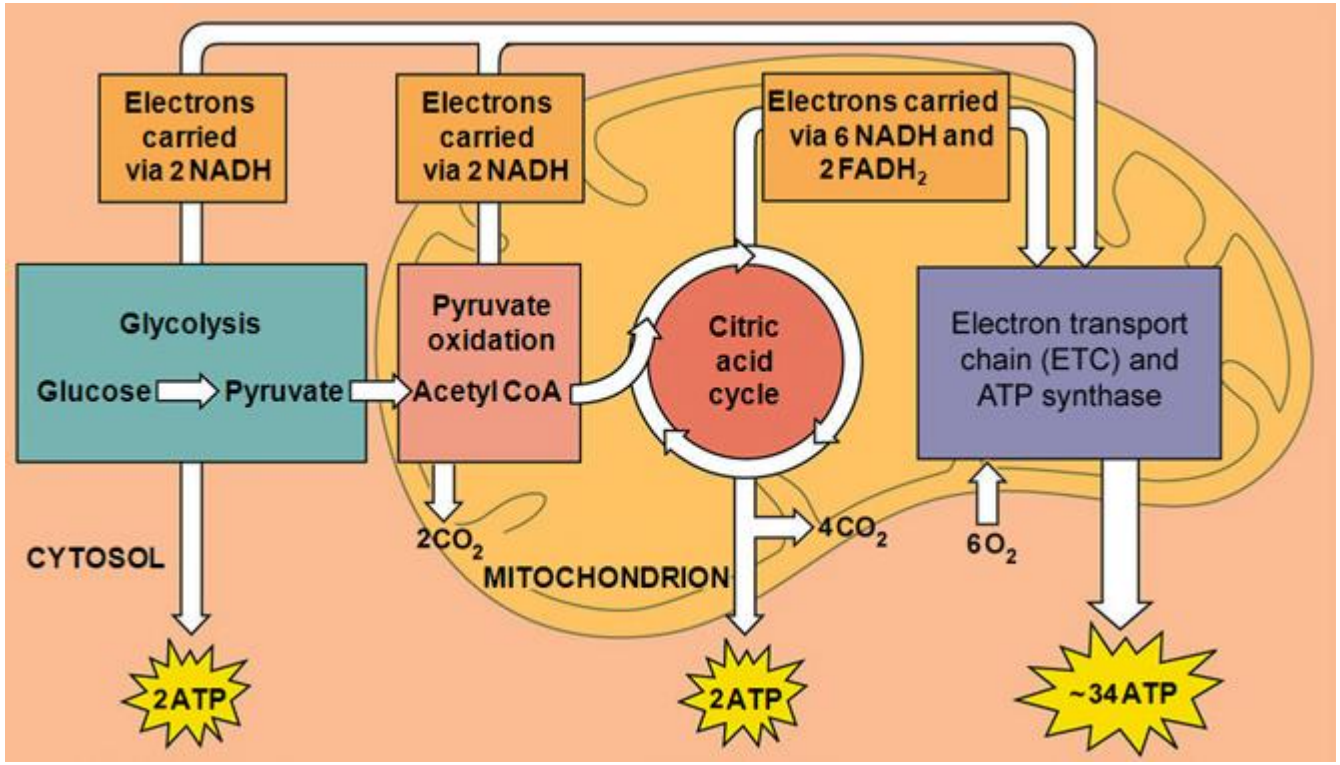


https://en.wikipedia.org/wiki/Template:Nature_timeline

Iron in heme proteins allows aerobic metabolism exploiting oxygen
As well as protection from this corrosive toxin.

Aerobic metabolism allowed multicellular life to develop.

Archaea incorporate bacteria > mitochondria

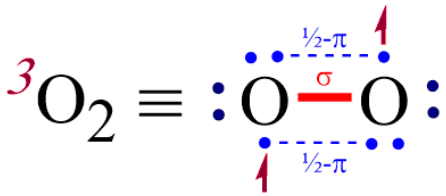


http://faculty.collin.edu/dmcculloch/1406/Notes/Respiration/summary2.htm

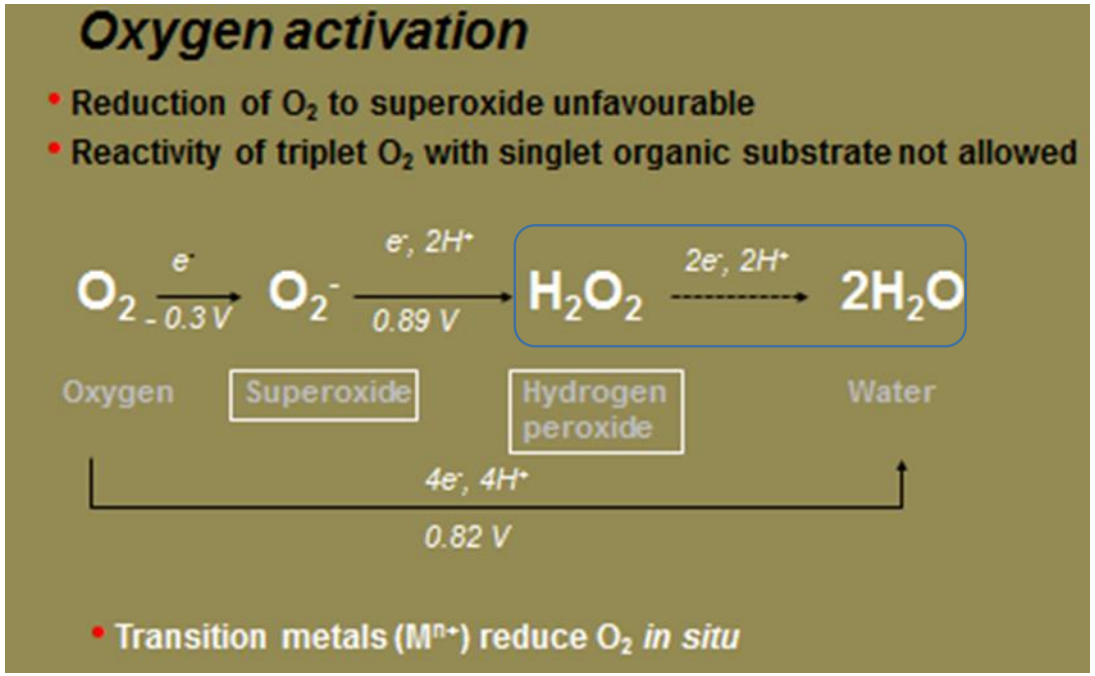
+

Gas sensing/
Signalling
Transcriptional
Regulation
Transcriptional
regulation
Globins
Cytochrome
Catalytic enzymes
Heme chaperones
Circadian control

Oxygen - 4 electron reduction to water



[Ar] 3d6 4s2
Oxidation states of -2 to +6 possible
+2, ferrous, Fe(II)
+3, ferric, Fe(III)



+ 0.82 V overall, but four electron reductions all at once are not easy **in biology.**

How to exploit the oxidising power?

Use one or two-electron steps and transition metals(e.g. Iron) variable oxidation states
Initial one-electron step is not easy (-0.33 V), this limits the reactivity of O_2 in biological systems –
Needs to be overcome - but superoxide and H_2O_2 generated and are not products you want to have around.

H_2O_2 dealt with in various ways including reduction of H_2O_2 to water – catalysed by the **peroxidase enzymes**

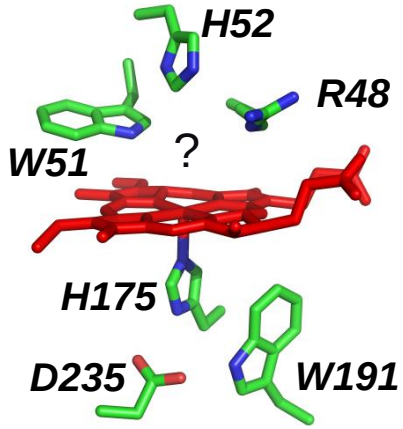
heme peroxidases

- **H_2O_2 reduced to H_2O and substrate oxidized**
- **Common features with P450s, peroxidases, NO synthase *etc.***
- **Oxygen activation through formation of ferryl heme (Compounds I & II)**

Fe(III) P + H_2O_2 → **Fe(IV)-O P·** + H_2O (1)
(Compound I)

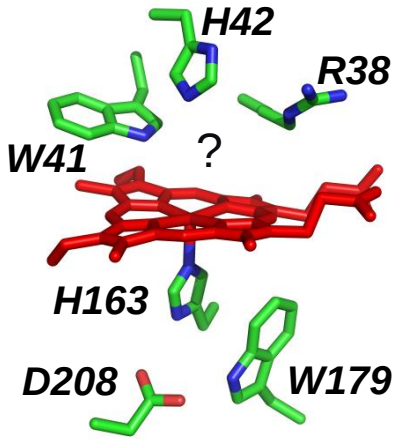
Fe(IV)-O P· + S → **Fe(IV)-O P** + S· (2)
(Compound II)

Fe(IV)-O P + S → Fe(III) P + H_2O + S· (3)



Cytochrome c peroxidase

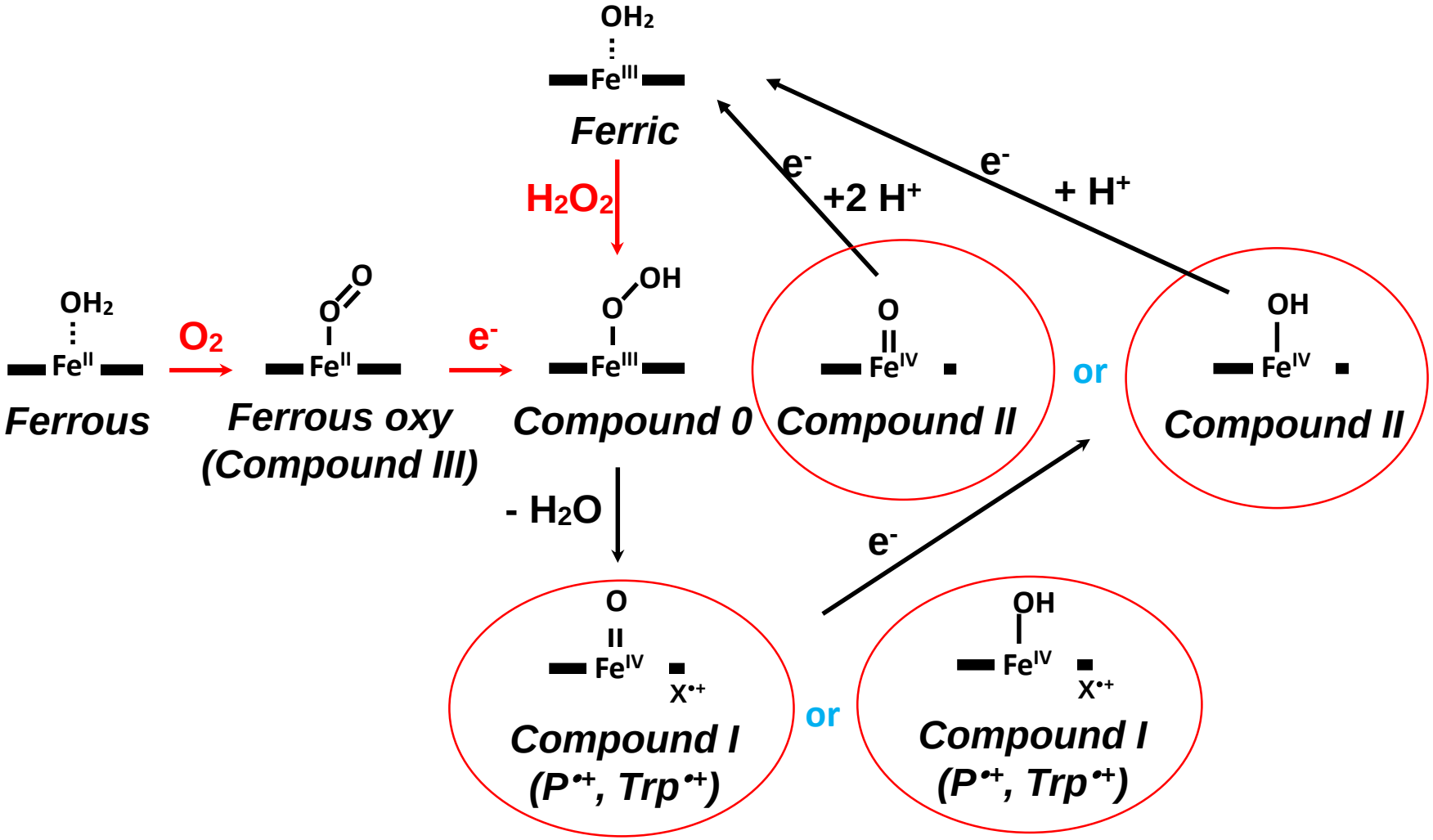
- (meta)stable Compound I ($Trp^{\bullet+}$)
- unstable Compound II



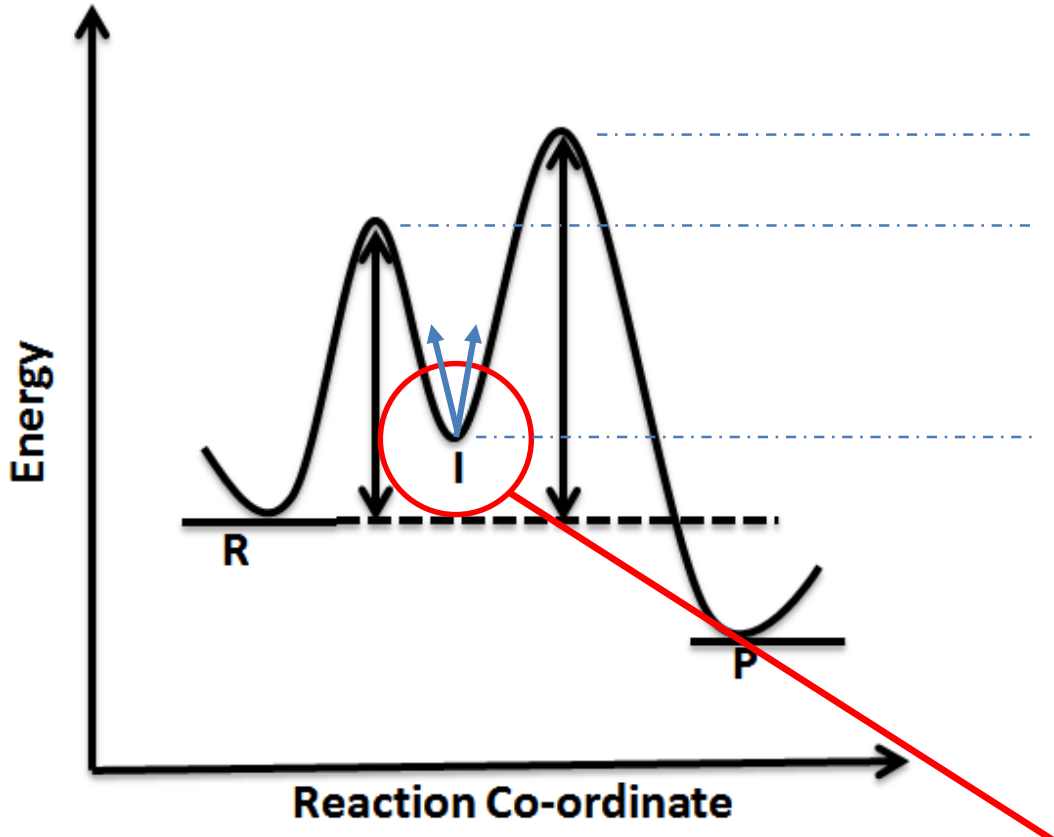
Ascorbate peroxidase

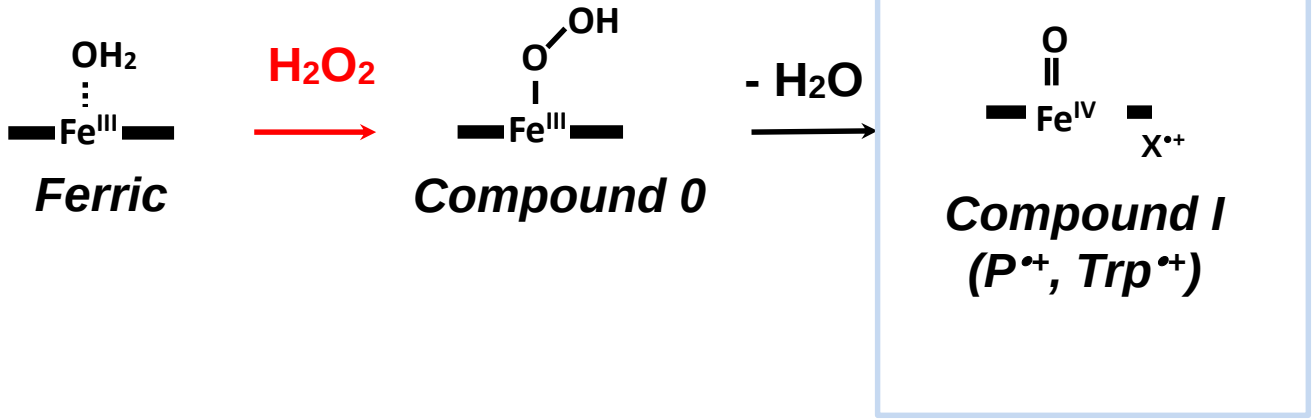
- unstable Compound I ($P^{\bullet+}$)
- (meta)stable Compound II

Ferryl heme in heme peroxidases

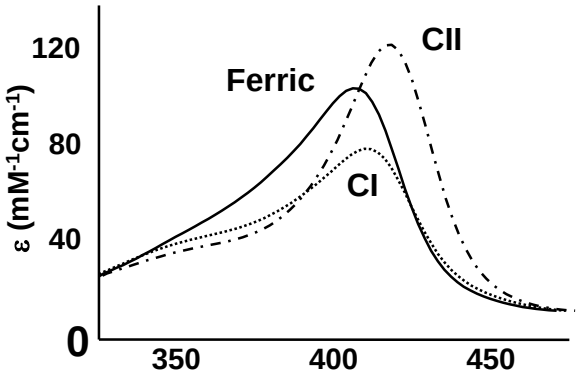


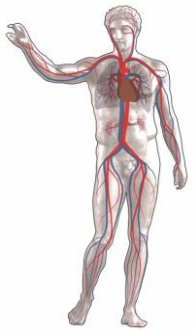
Ligation of heme/heam can be followed spectrophotometrically…





Ferric *Compound 0* ***Compound I (P•+, Trp•+)***

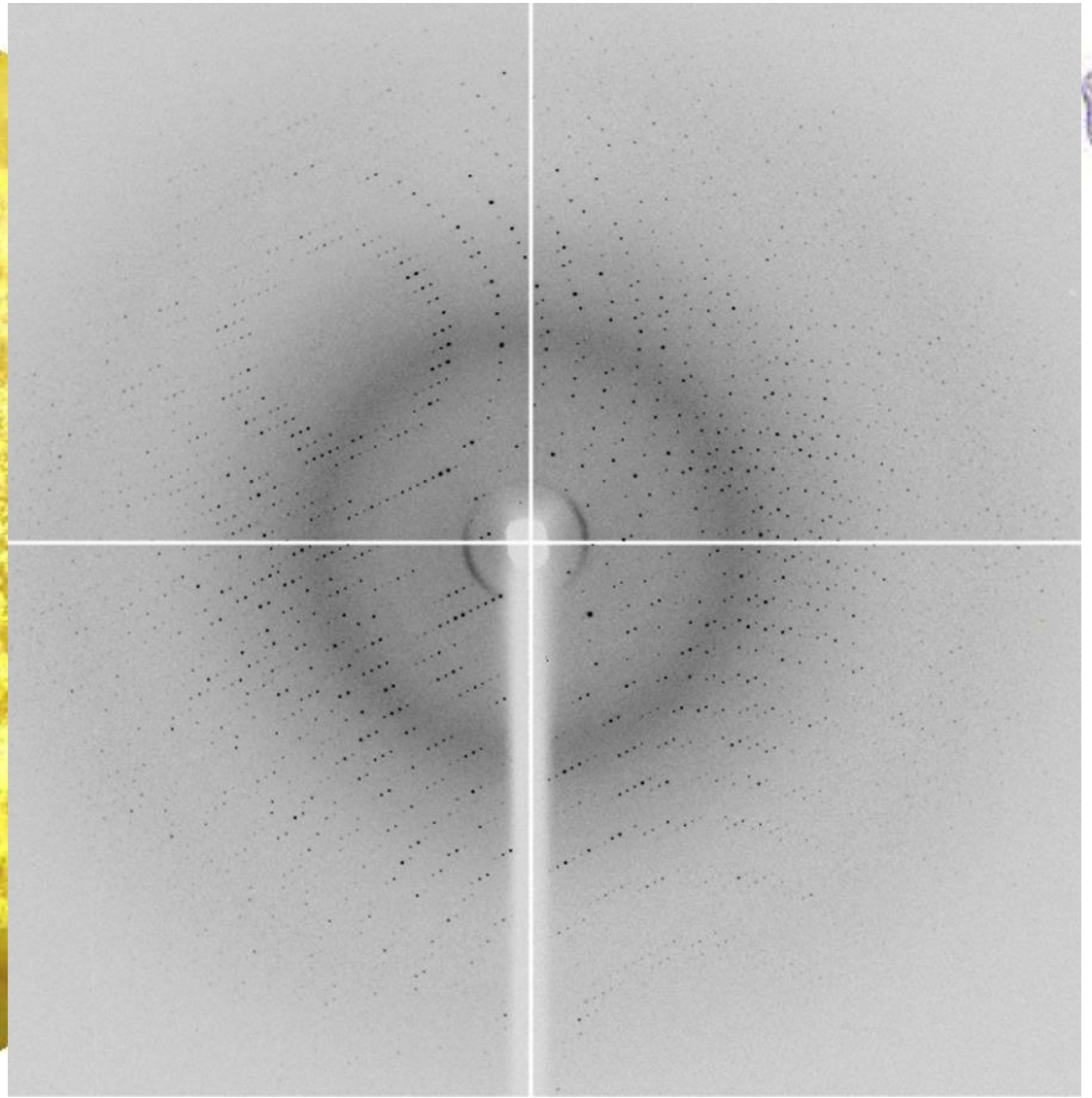


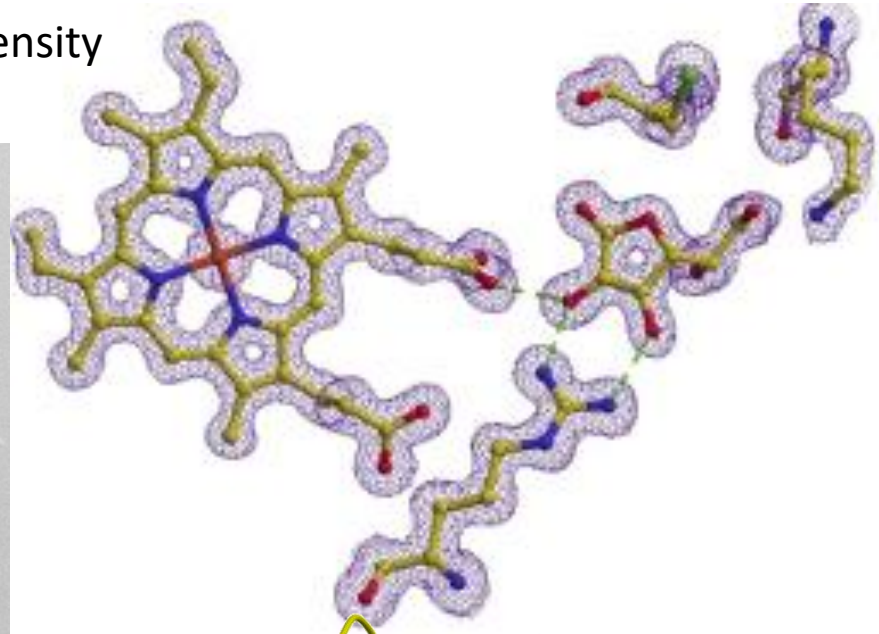


https://en.wikipedia.org/wiki/Energy_profile_(chemistry)

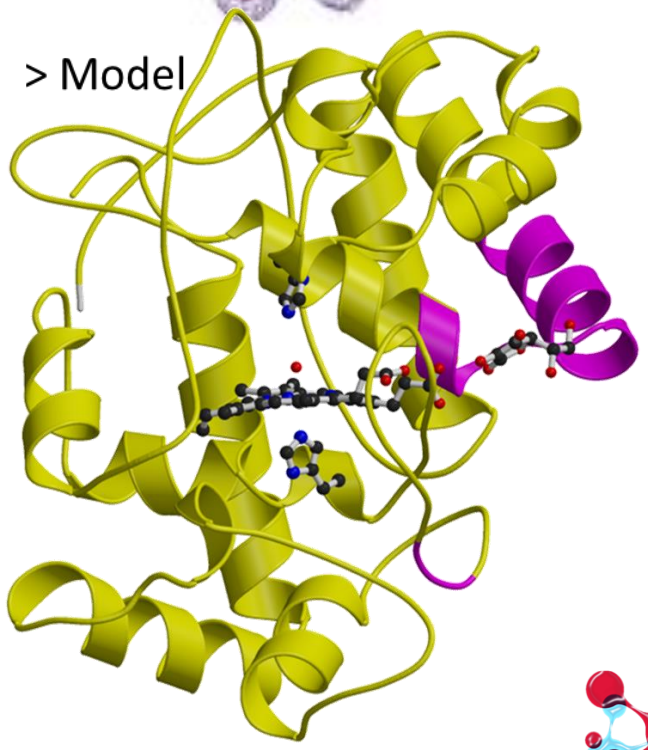
Use 100 K to trap intermediates, spectroscopy to monitor

Crystals > > X-ray Diffraction > > >Electron density





> Model



Nature of ferryl heme

- Mixture of bonds lengths 1.6 – 2.0 Å from EXAFS and X-ray crystallography
- Single (Fe-OH) or double (Fe=O) bond?
- Protonated or not?

Protein	CI	CII	Reference
Horseradish peroxidase	1.6	1.6	*Penner-Hahn, JBC,1983*
	1.64	1.64	*Penner-Hahn, JACS,1986*
	1.67	1.70	*Green, Science, 2004*
	1.67	1.93	*Chance, ABB, 1984*
	1.7	1.8	*Berglund, Nature, 2002*
Cytochrome *c* peroxidase	1.67		*Chance, BC, 1986*
	1.87		*Bonagura, BC, 2003*
	2.0		*Fulop, Structure, 1994*
Chloroperoxidase	1.65	1.82	*Green, Science, 2004* *Stone, PNAS, 2005*
Myoglobin		1.69	*Chance, BC, 1986*
		1.92	*Hersleth, JBIC, 2002* *Hersleth, BBA, 2010*
Cytochrome P450 (CYP 119)		1.82	*Newcomb, PNAS, 2008*

Why the uncertainty?

Species	Fe-O distance (Å)
Compound I	1.65
Compound II	1.80-1.85
Heme-hydroxyl complex	1.95
Heme-water complex	2.30

X-ray‡ **crystallography is wonderful**, *but* it depends on the scattering of **high energy photons** by electrons…….

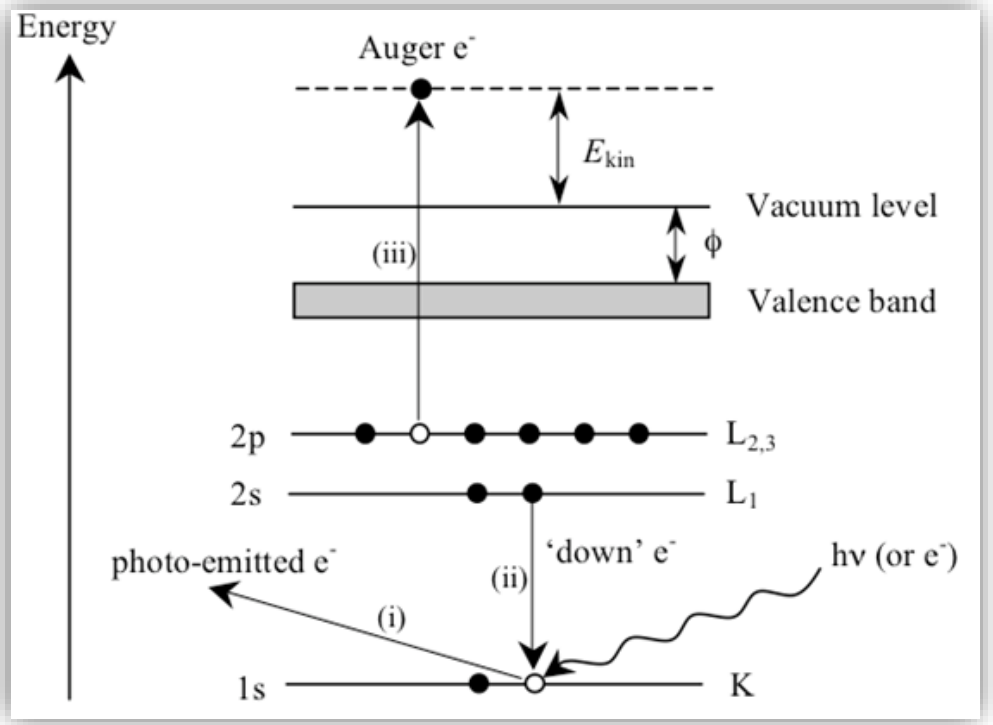
‡ 6-20 keV, ~10^3 x atomic ionisation energy

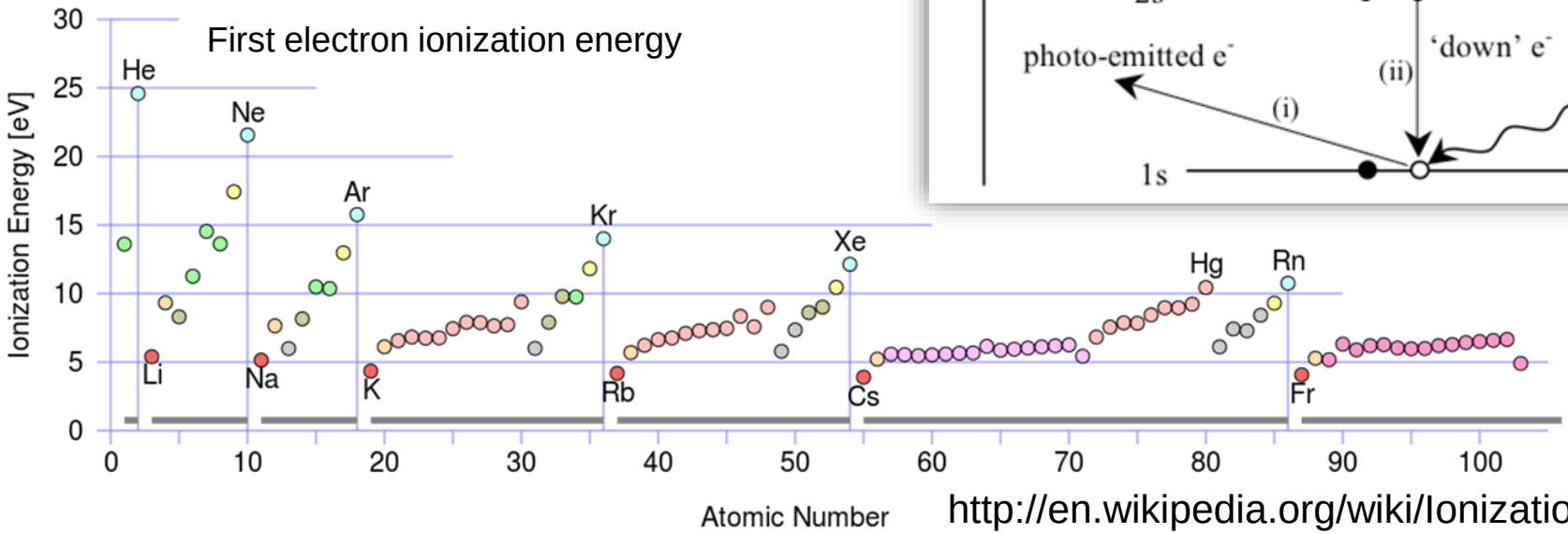
- **The interactions of the photons with electrons ~~may~~ will perturb the system**

X-rays are ionizing

X-rays (8-20 keV)

Photon energy ~10^3 times ionization energy





http://en.wikipedia.org/wiki/Ionization_energy

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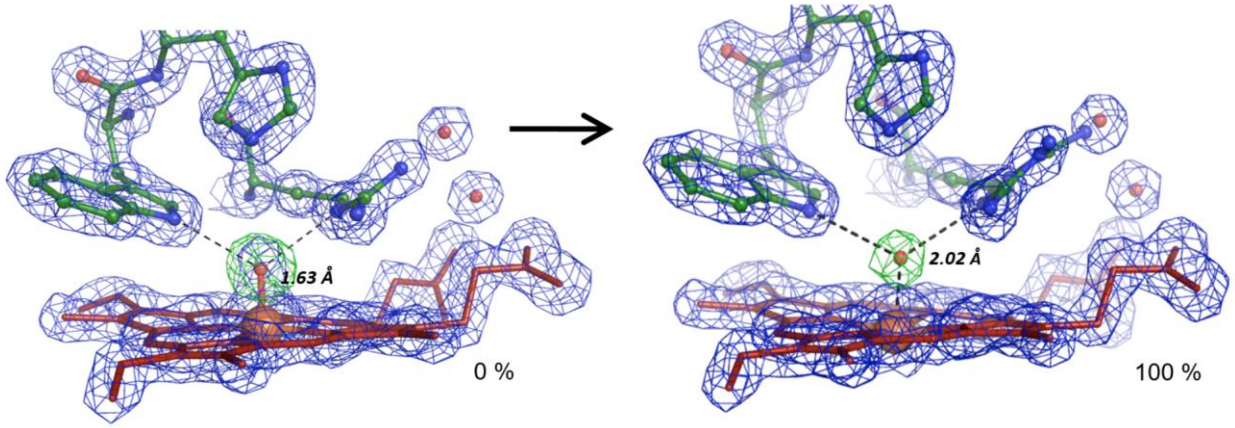
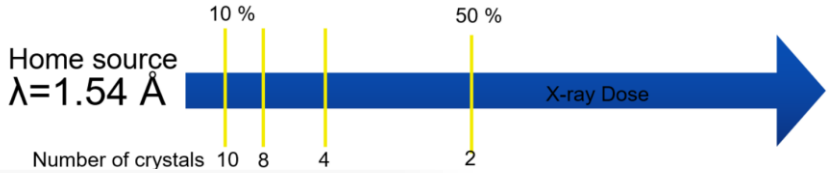
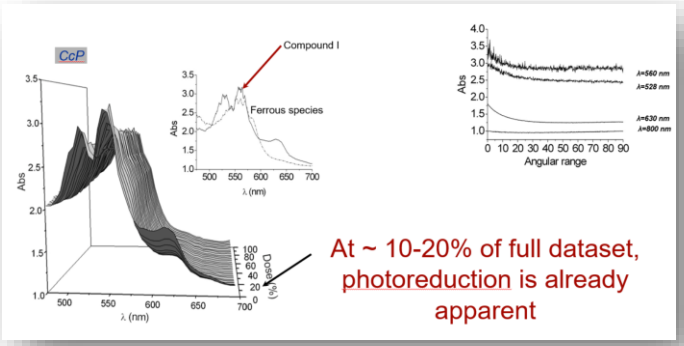
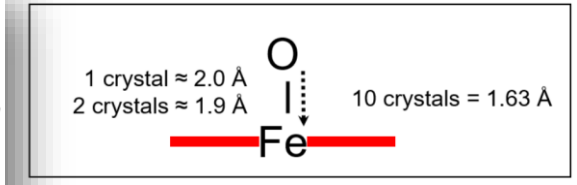
Compound I of CcP: Fe-O distance increases with X-ray dose



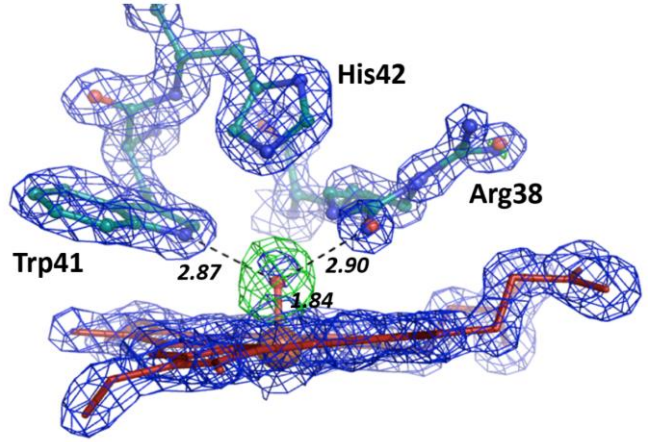




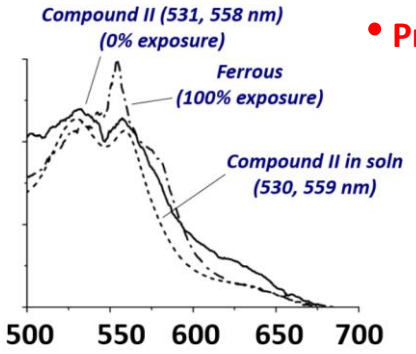


- **Fe-O bond length 1.63 Å**
- **Unprotonated ferryl heme (Fe^{IV}=O)**

Compound II of ascorbate peroxidase



- **Same multicrystal methodolgy as C*c*P CI**
- **Same dose 0.028 MGy**
- **Shorter wavelength radiation (λ = 0.6 Å)**
- **Fe-O bond length 1.84 Å (cf CCPI 1.63 Å)**
- **ESU: Fe 0.015 Å, O 0.088 Å**
- **Protonated ferryl heme (Fe^{IV}-OH)**



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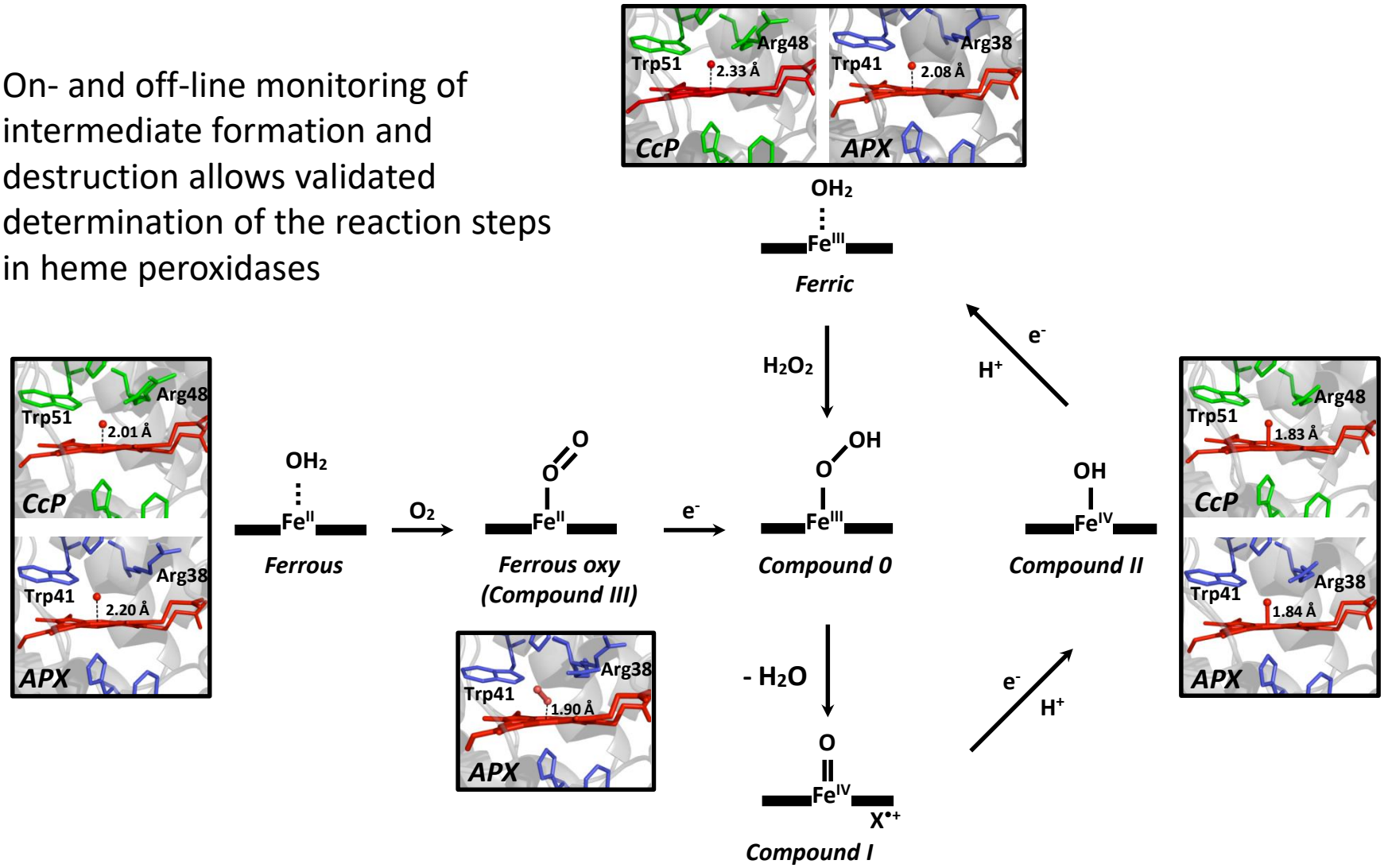
On- and off-line monitoring of intermediate formation and destruction allows validated determination of the reaction steps in heme peroxidases



Gumiero *et al*. (2010).
The nature of the ferryl heme in compounds I and II
J. Biol. Chem. doi:10.1074/jbc.M110.183483

BUT - 1

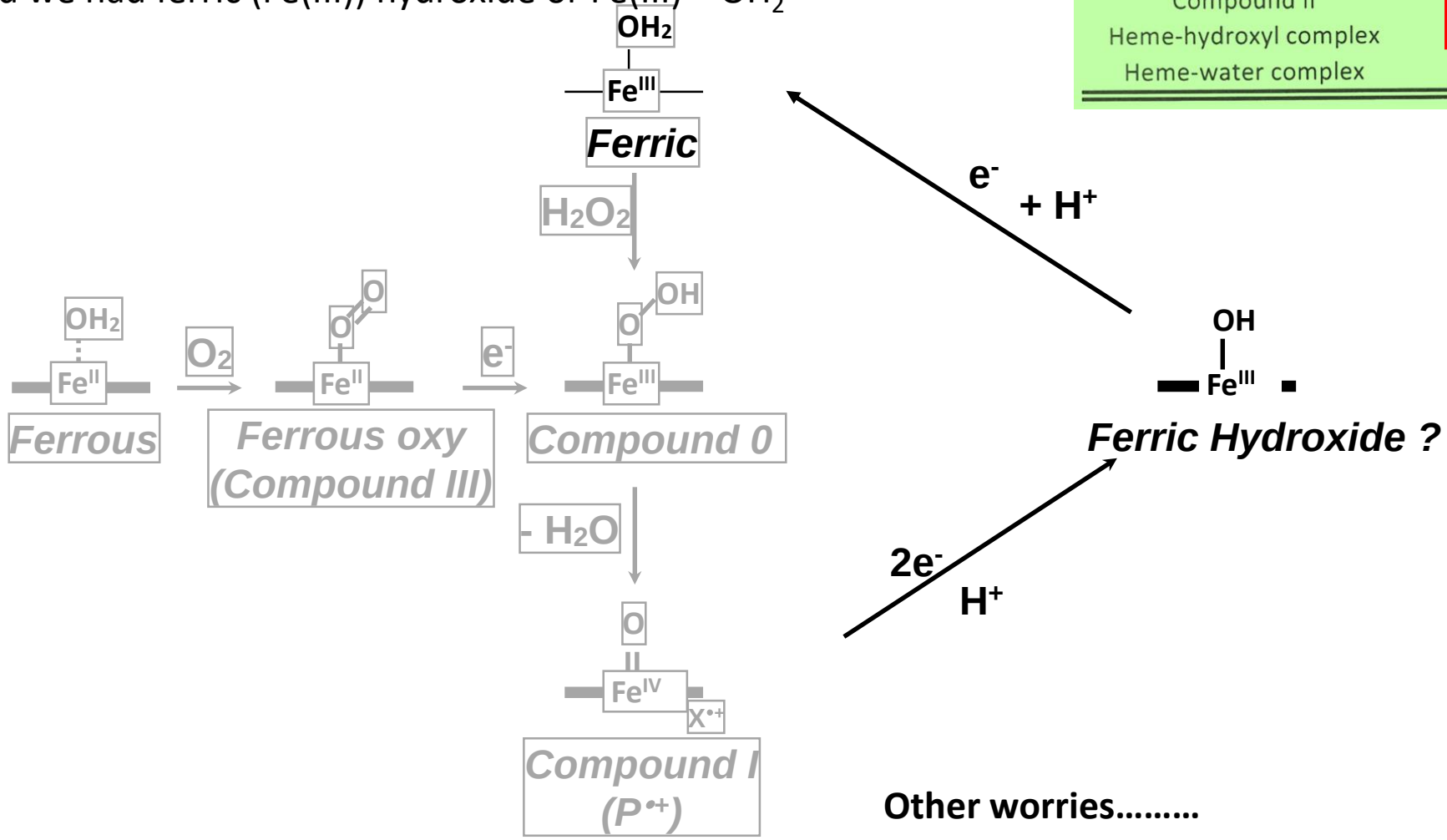
Did not show protonation states and….

Not universally believed <u>especially</u> the CII structure

It was suggested we had ferric (Fe(III)) hydroxide or Fe(III)---OH_2

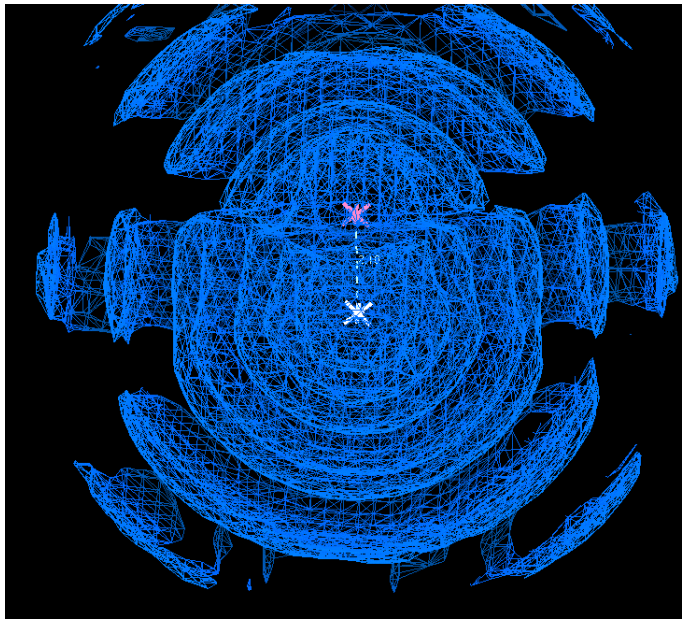
.. not real CII

Species	Fe-O distance (Å)
Compound I	1.65
Compound II	1.80-1.85
Heme-hydroxyl complex	1.95
Heme-water complex	2.30



Other worries……

Series Termination Errors



Map calculated using calculated data 30-1.5Å with isolated Fe & O atoms

Ripples in electron density

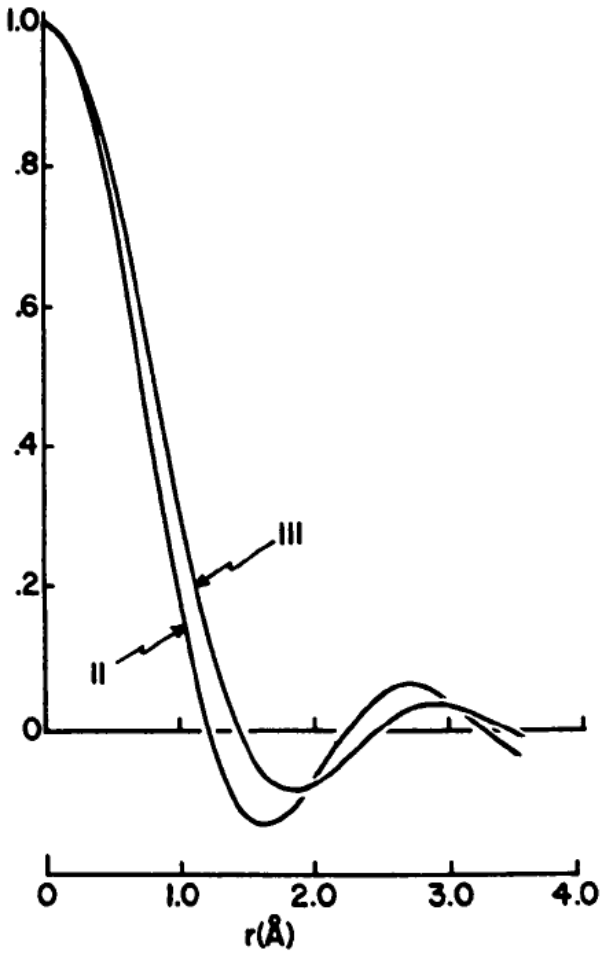


Fig. 2. Relative electron density, $\rho(r)$, for point atoms as a function of r for X-ray data with $d_{min} = 2$ Å. II, the two-dimensional function: III, the three-dimensional function.

Acta Cryst. (1984). A40, 251-254
Resolution Revisited: Limit of Detail in Electron Density Maps
RONALD E. STENKAMP & LYLE H. JENSEN

Positional Errors?

e.g. "As shown for the high-resolution structure of nitrogenase, these effects can be simulated, such that their influence on the metal–ligand distance can be estimated [27]. We find that at a resolution of 1.26 Å, a bond distance of **2.04 Å** is observed for a true ligand distance of **2.25 Å**. ***Mechanistically, this finding may be crucial, because the value of 2.04 Å falls between the values expected for a hydroxo ligand (1.9–2.1 Å) and a coordinated water (2.0–2.3 Å). The two possibilities lead to different mechanistic scenarios: …"***

Fülöp V, Watmough NJ, Ferguson SJ (2000) *Adv. in Inorganic Chemistry* **51** :163–204

[27] Einsle O, Tezcan FA, Andrade SL, Schmid B, Yoshida M, Howard JB, Rees DC (2002) Science 297:1696–1700.

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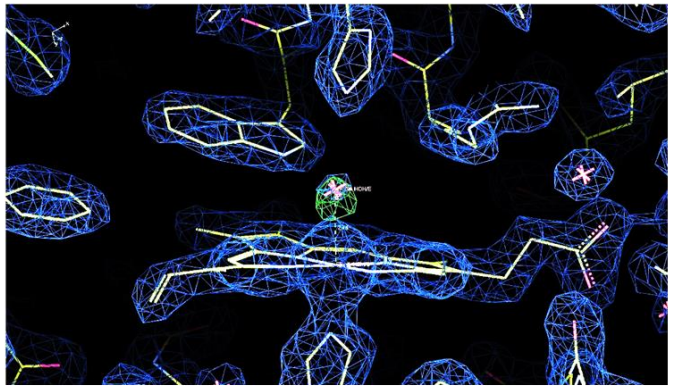
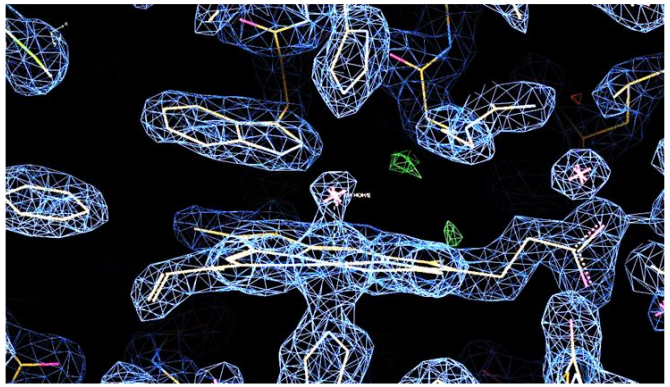
Refinement restraints?

RT Fo-Fc real-space Fe-O 2.45Å

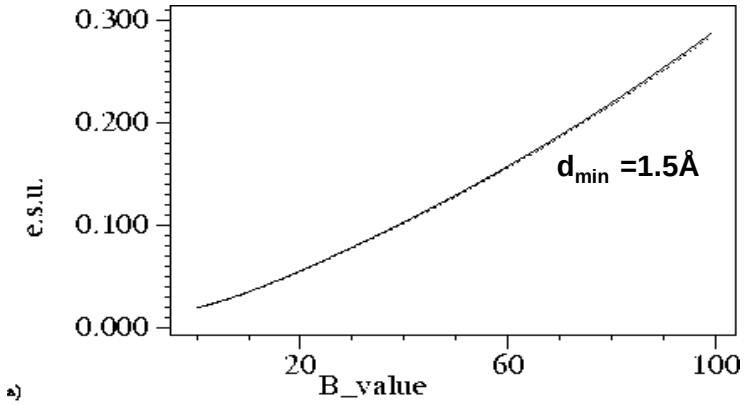


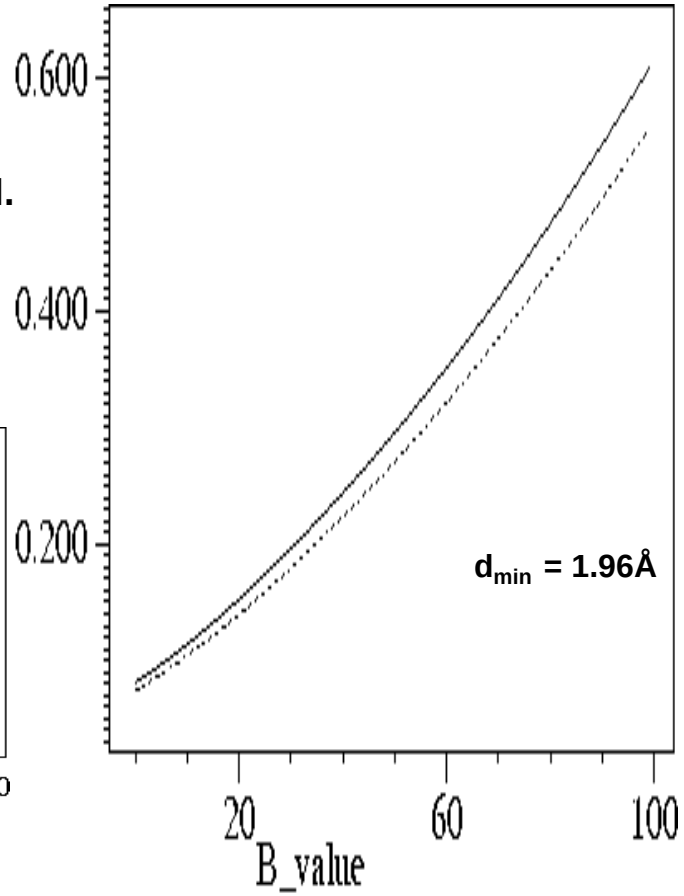
RT Refmac Fe-O 2.19Å



Limited data?

There is dependence of e.s.u. on B-values
There is dependence of e.s.u. on resolution
There is dependence of e.s.u. on completeness of data,
There is dependence of e.s.u. on completeness of model.
There is dependence of e.s.u. on the quality of data





B-value dependence of e.s.u. for positional parameters

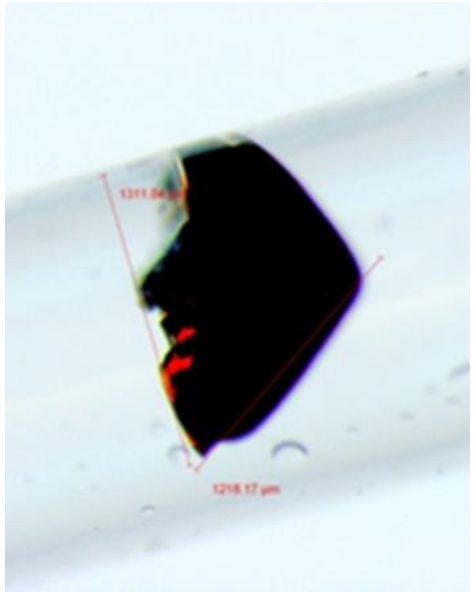
Dashed lines correspond e.s.u. derived using agreement of `free' reflections, solid lines show e.s.u. derived using agreement of reflections included in refinement.

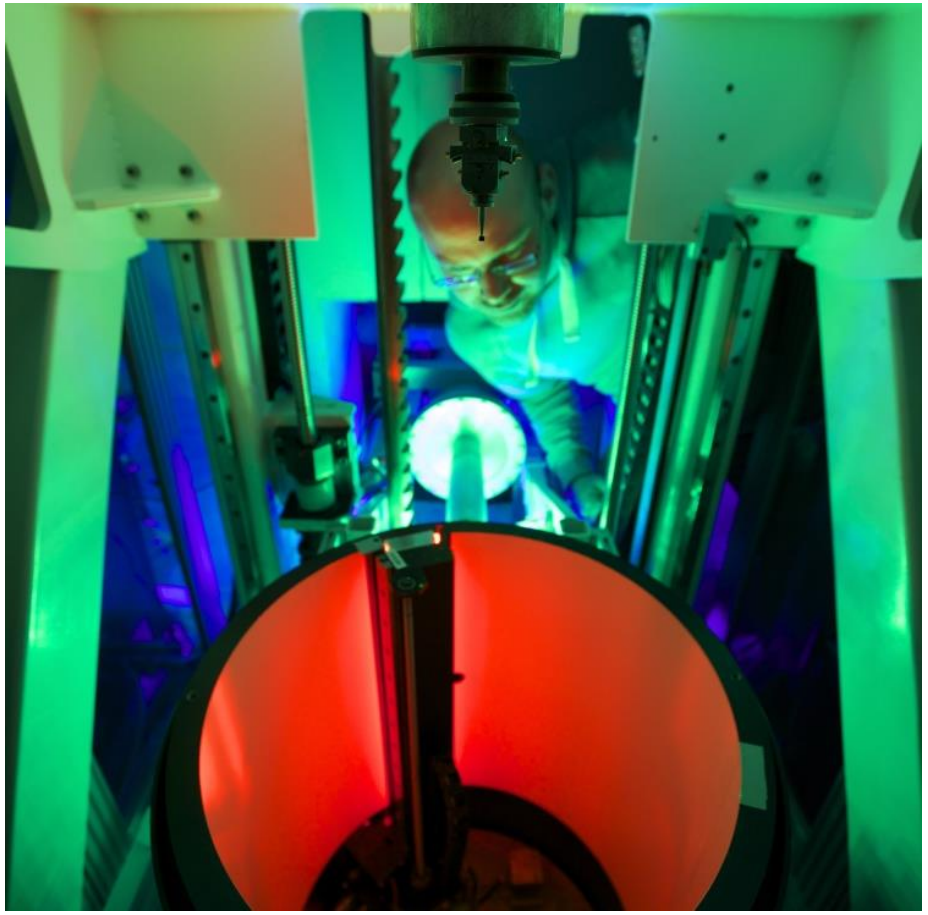
Simplified error estimation *a la* Cruickshank in macromolecular crystallography
Garib N. Murshudov and Eleanor J. Dodson *CCP4 Newsletter - January 1997*

Positional Errors?

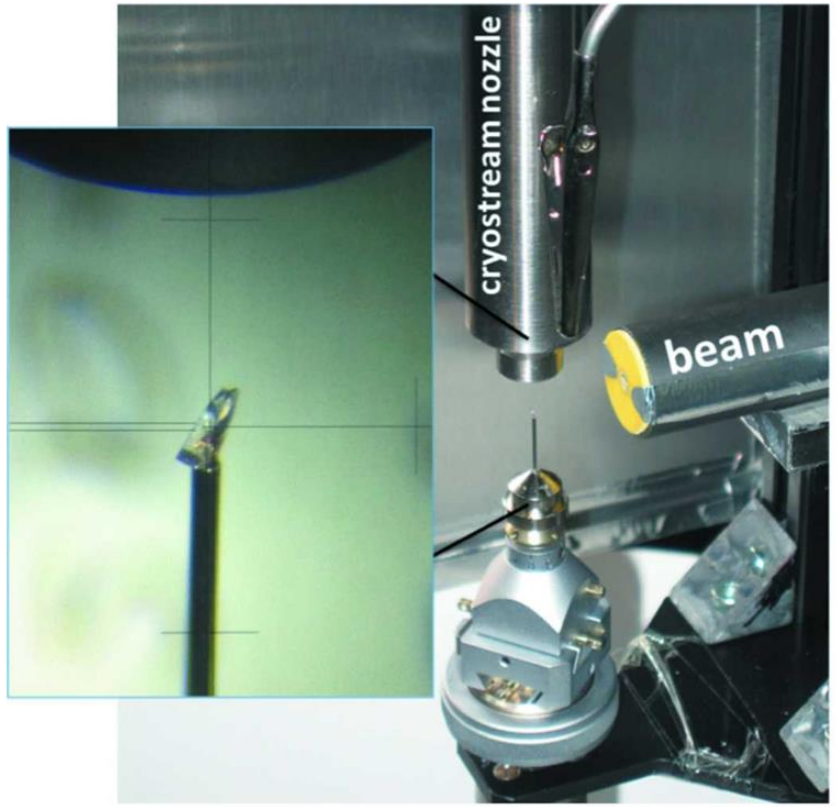
e.s.u. = estimated standard uncertainty (of position)

Need to directly show protonation states
– Need neutron crystallography





LADI-III



Coates *et al.* *(2014) J. Appl. Cryst*. 47 (4) 1431–1434

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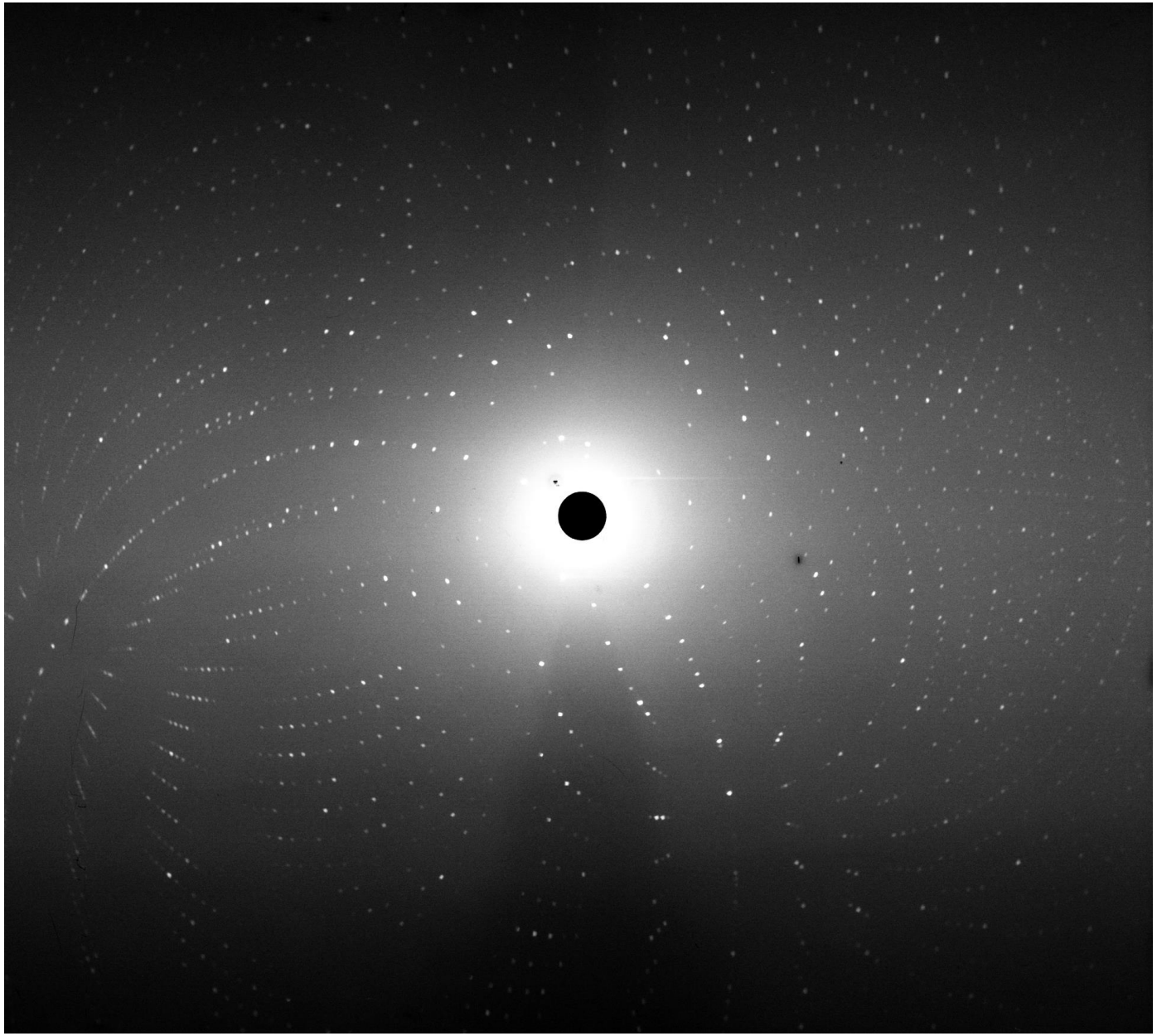
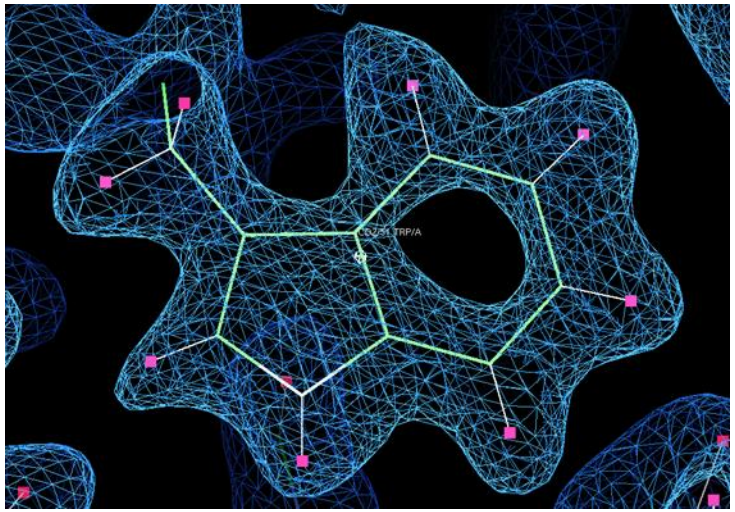
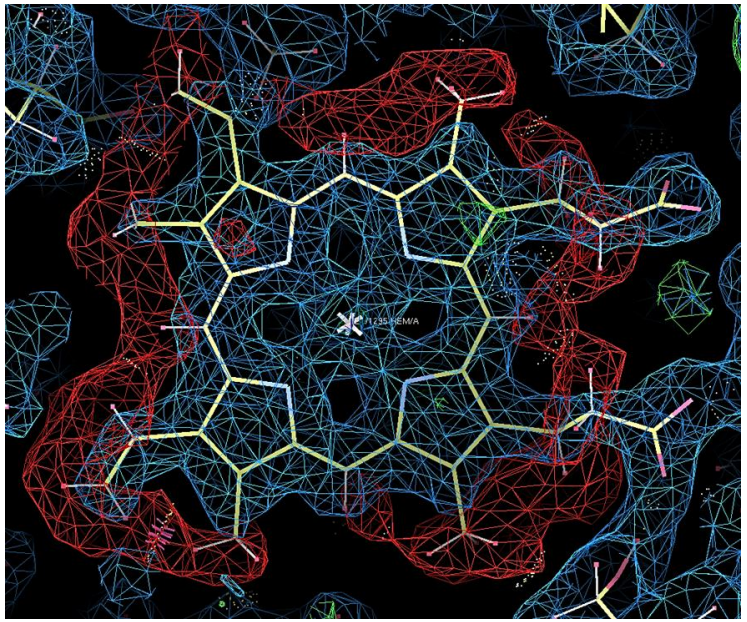
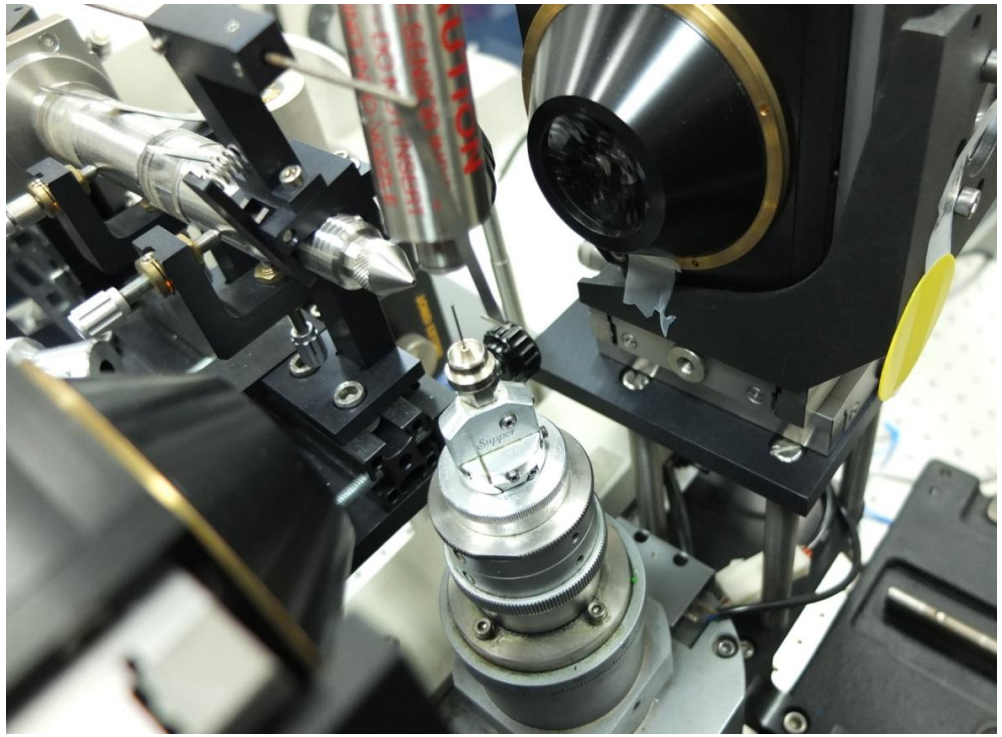
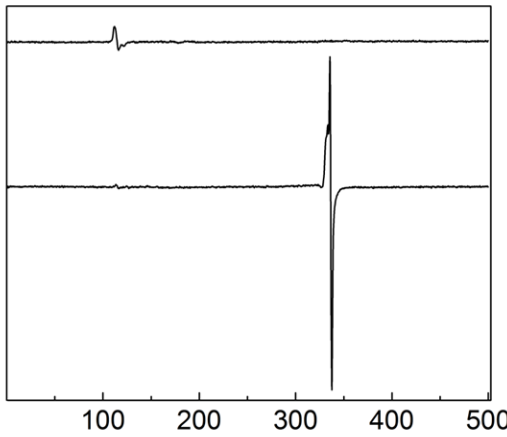




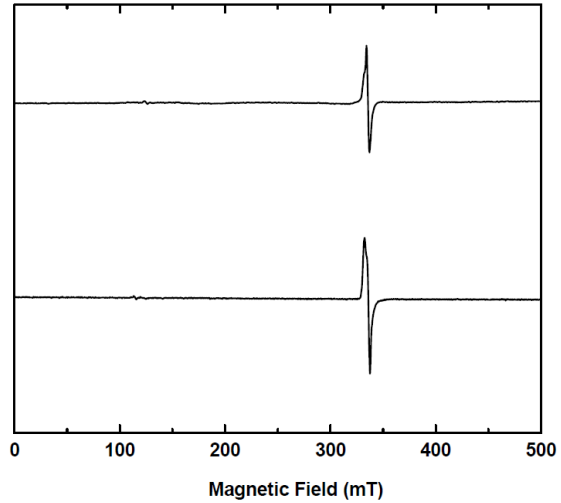


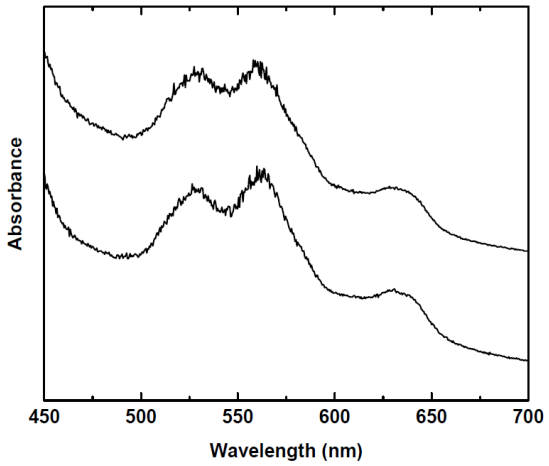
Spectroscopy shows CI made in C*c*P stable at 100 K for weeks





EPR spectra
C*c*P ferric (top)
C*c*P CI crystal (bottom).

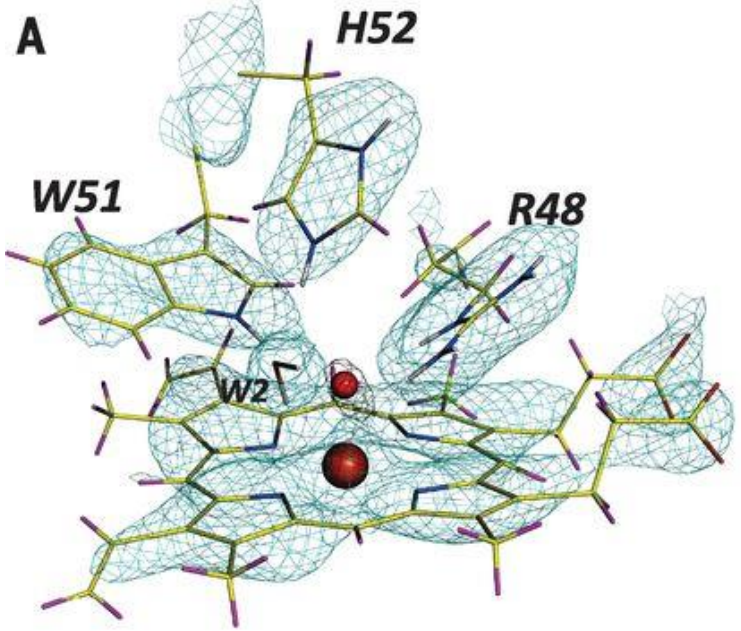




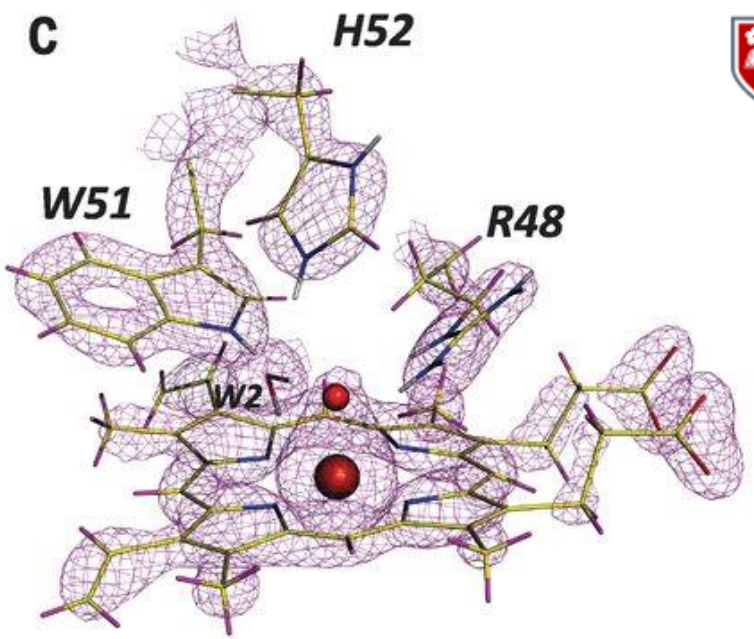
UV-VIS spectra of a D-exchanged single crystal
10 minutes H_2O_2 30 mM at 4˚C. Spectra were collected at 100 K.

The crystal was stored for 20 days at 77 K.
Bottom: Day 0, Top: Day 20.

EPR spectra of a D-exchanged single crystal.
H_2O_2 30 mM at 4˚C for 10 minutes.
The spectra were collected at 5 K.
The crystal was stored for 20 days at 77 K.
Bottom: Day 0, Top: Day 20.

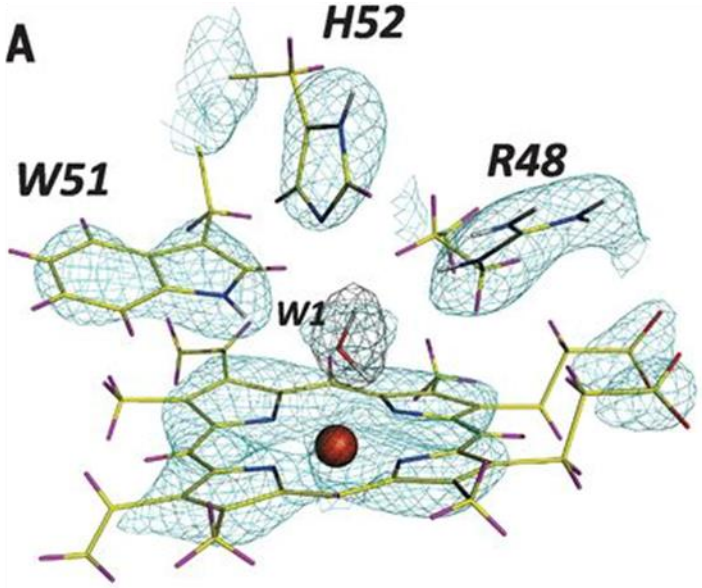


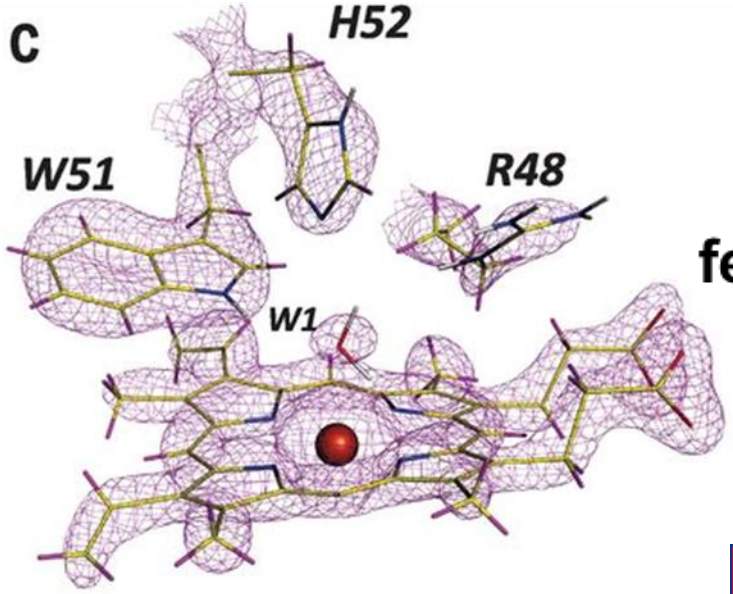
Neutron



X-ray

Cl CcP



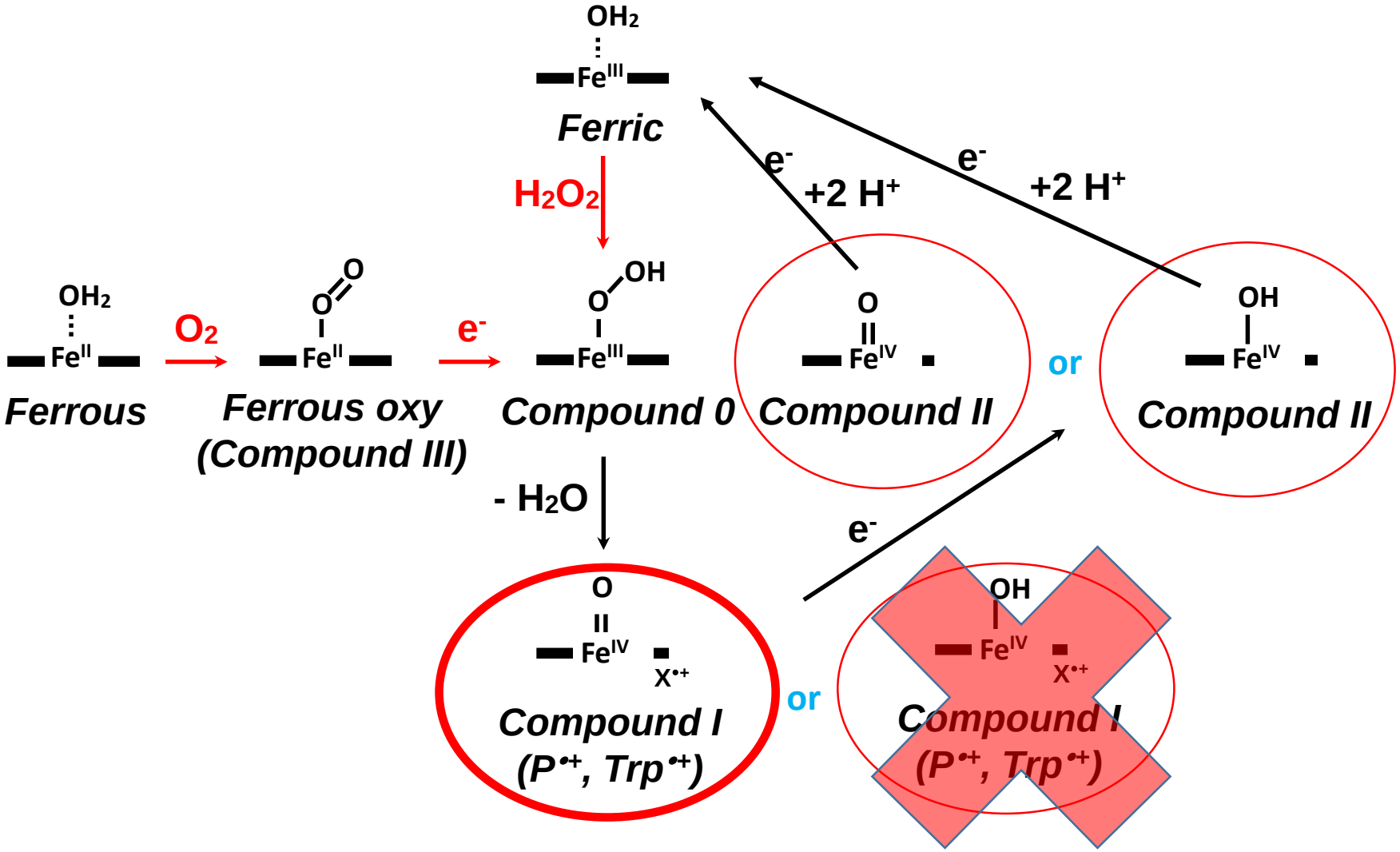


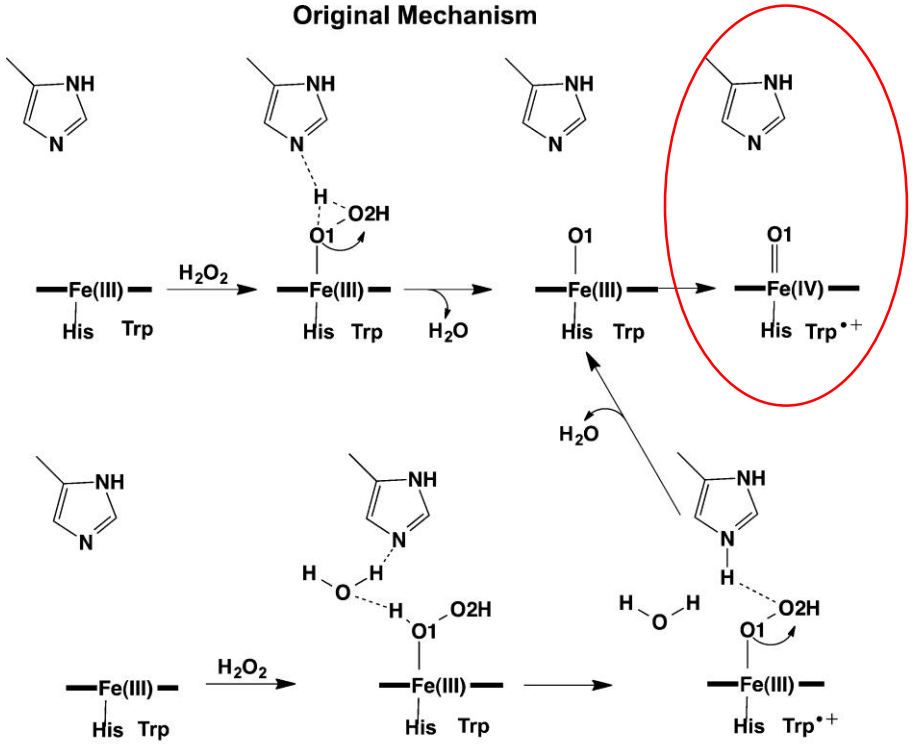
ferric CcP

Published by AAAS

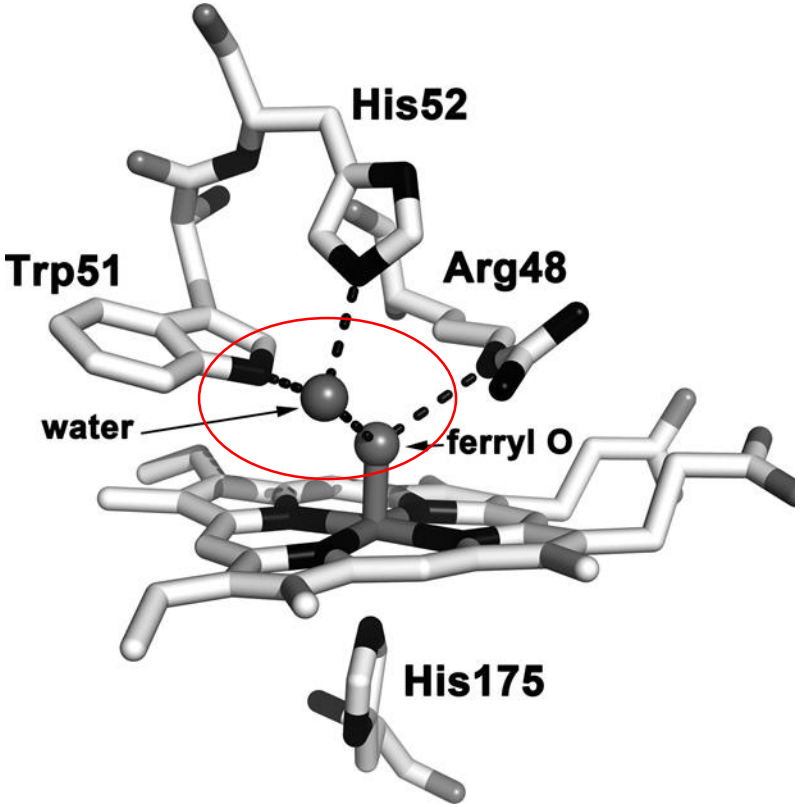
C M Casadei et al. Science 2014;345:193-197

Ferryl heme in heme peroxidases



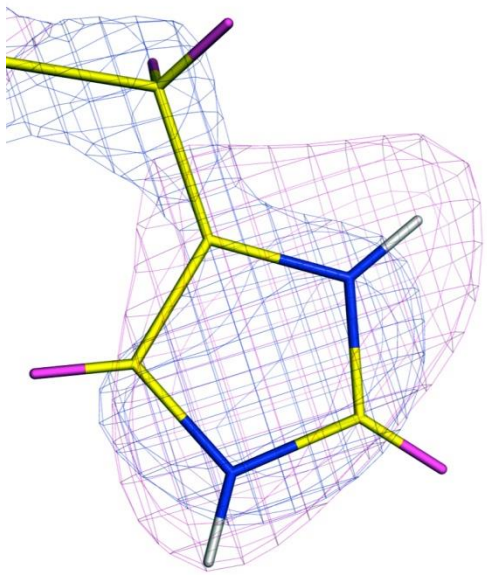


Mechanism of peroxidase compound I formation. In the original mechanism(32) the distal His shuttles the peroxide O1 proton to the O2 oxygen which promotes heterolysis of the O–O bond. However, the distal His is too far from O1 for direct H-bonding, so in the modified mechanism,(39) a water molecule assists in the transfer of the O1 proton to O2.

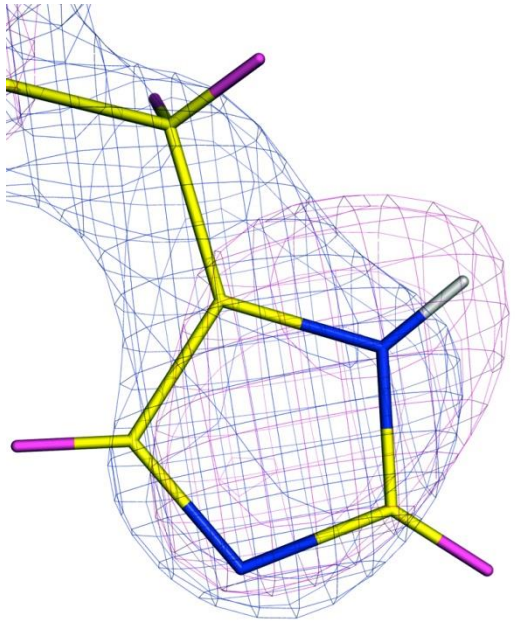


Crystal structure of CCP compound I(38) which is basically the same as the HRP compound I structure.(37) The water molecule H-bonded to the ferryl O atom is ideally positioned to assist His52 in acid–base catalysis as suggested.(39)

Neutron structure of C*c*P compound I shows the water molecule H-bonded His 52 does not hydrogen bond to the ferryl O atom. Trp 51 interacts directly with the ferryl O.

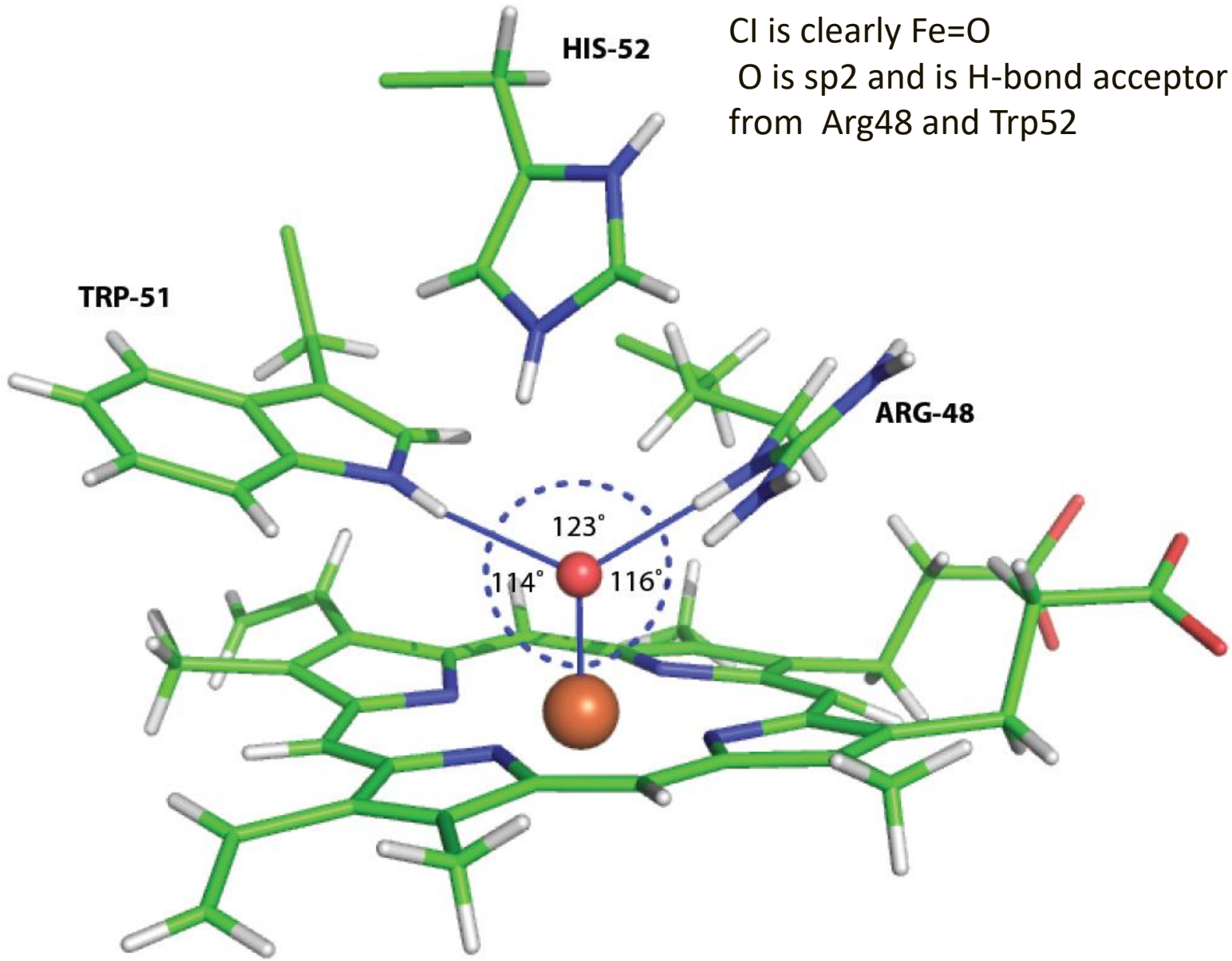


His 52 Cl

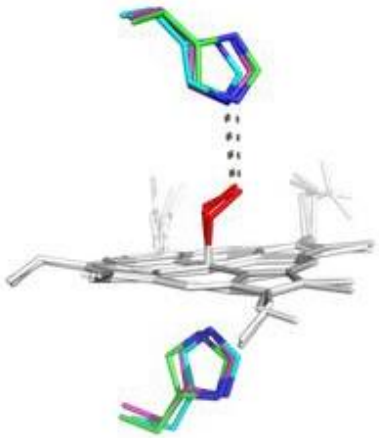


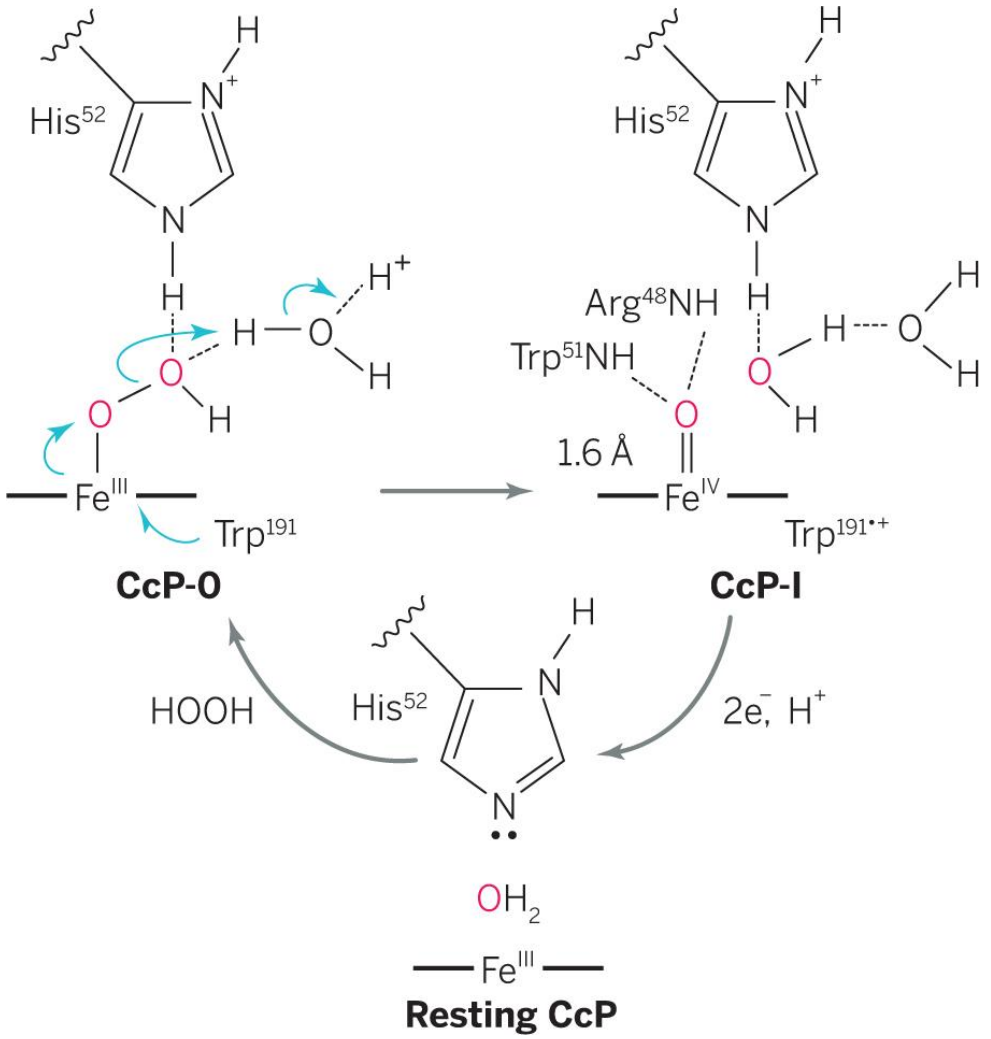
His 52 ferric

CI is clearly Fe=O
O is sp2 and is H-bond acceptor from Arg48 and Trp52



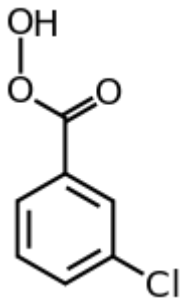
Proton-mediated mechanism. Reaction of ferric CcP with H_2O_2 first gives CcP-0, followed by O-O bond scission driven by external protonation to afford CcP-I.

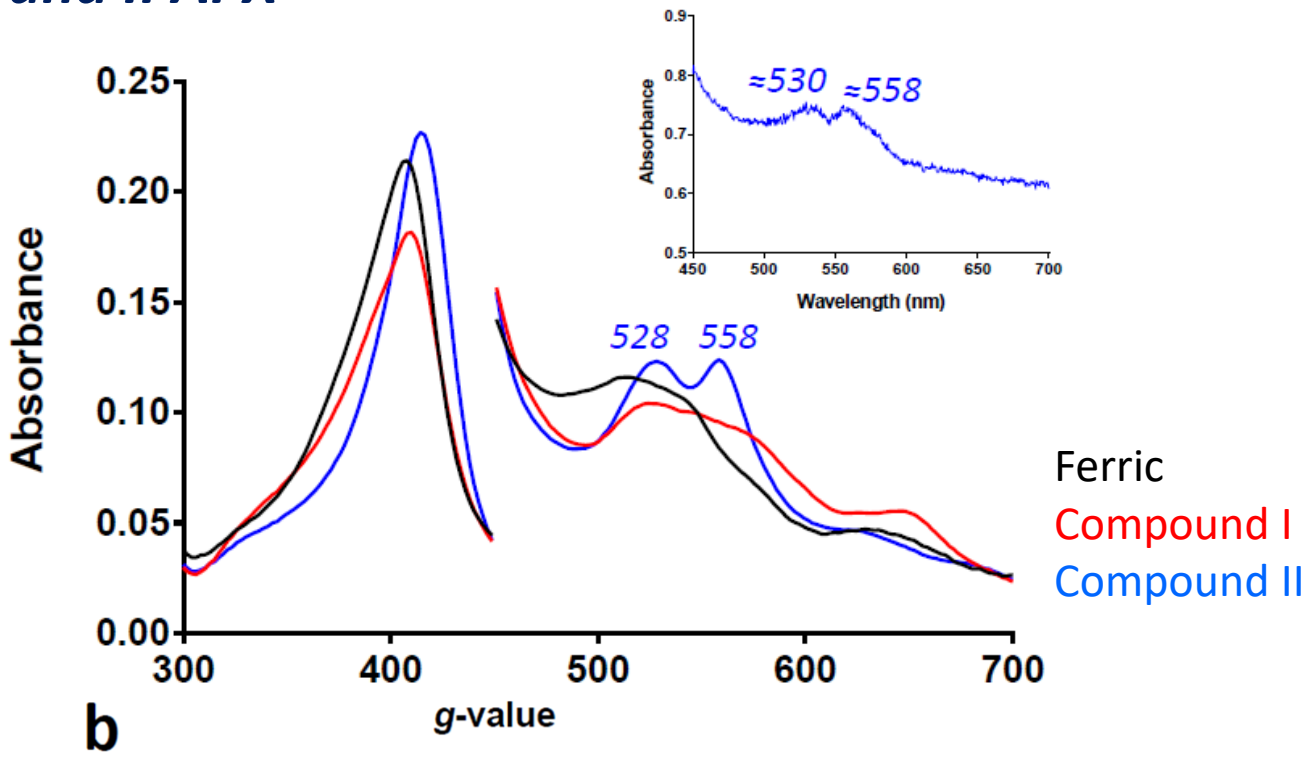




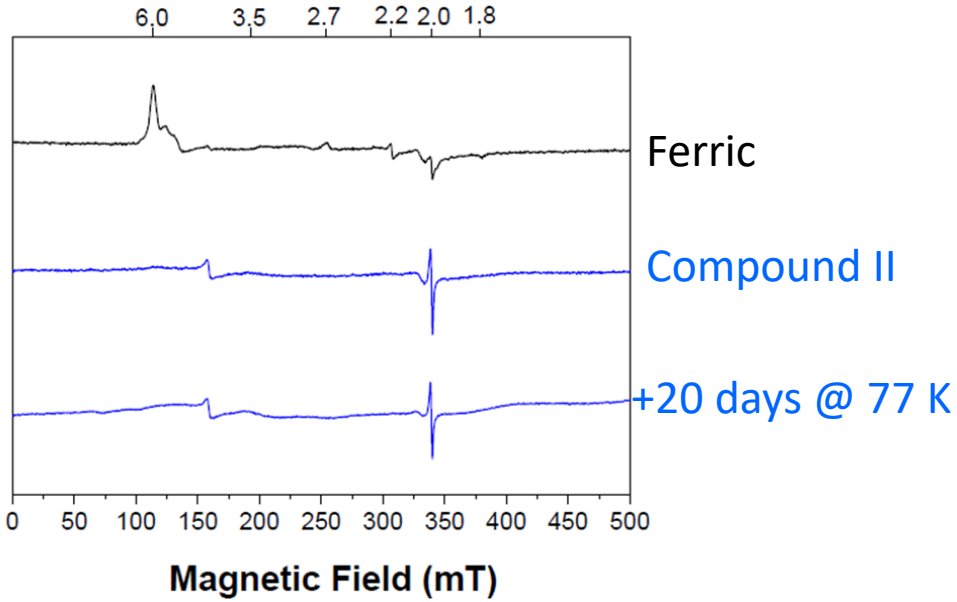
C M Casadei et al. Science 2014;345:193-197
J T Groves, and N C Boaz Science 2014;345:142-143

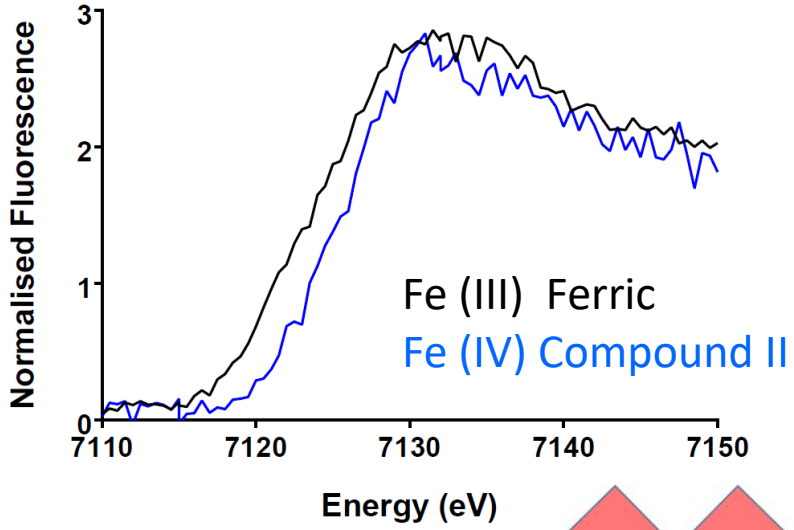
Compound II APX

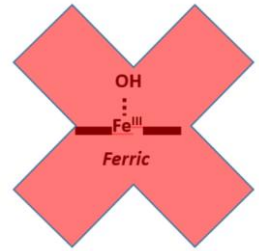




b







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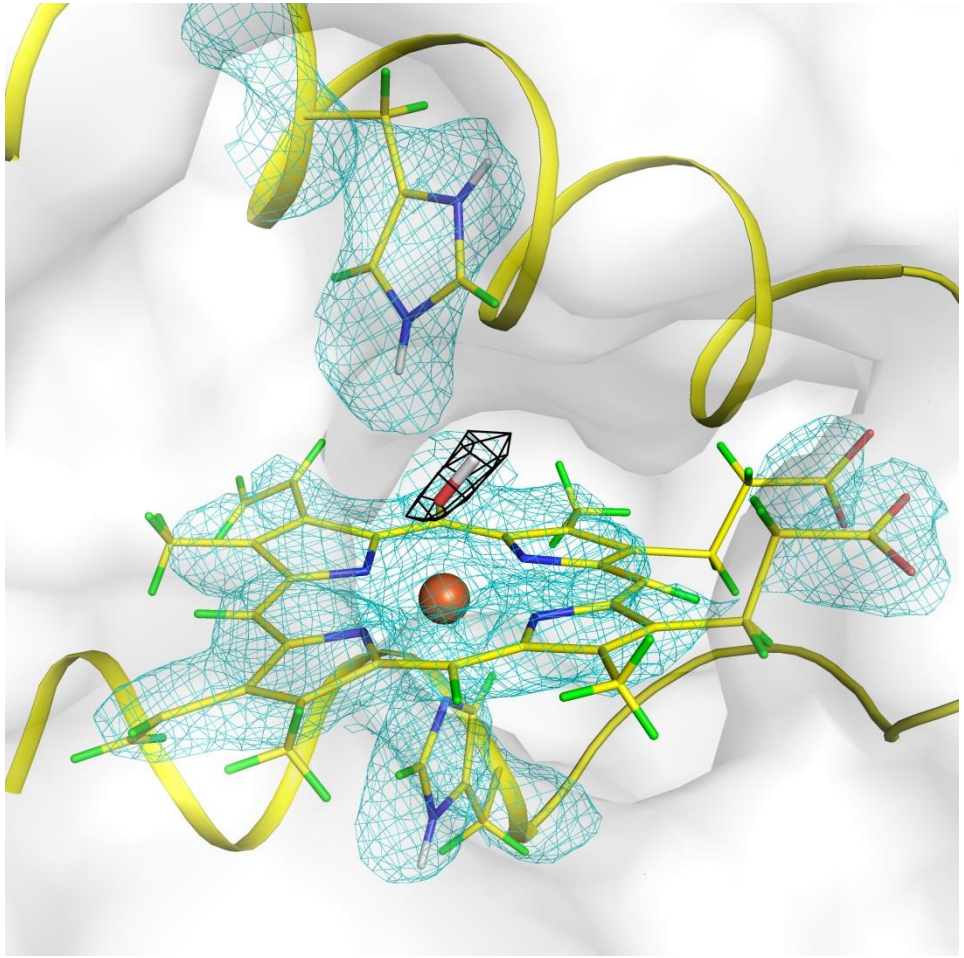
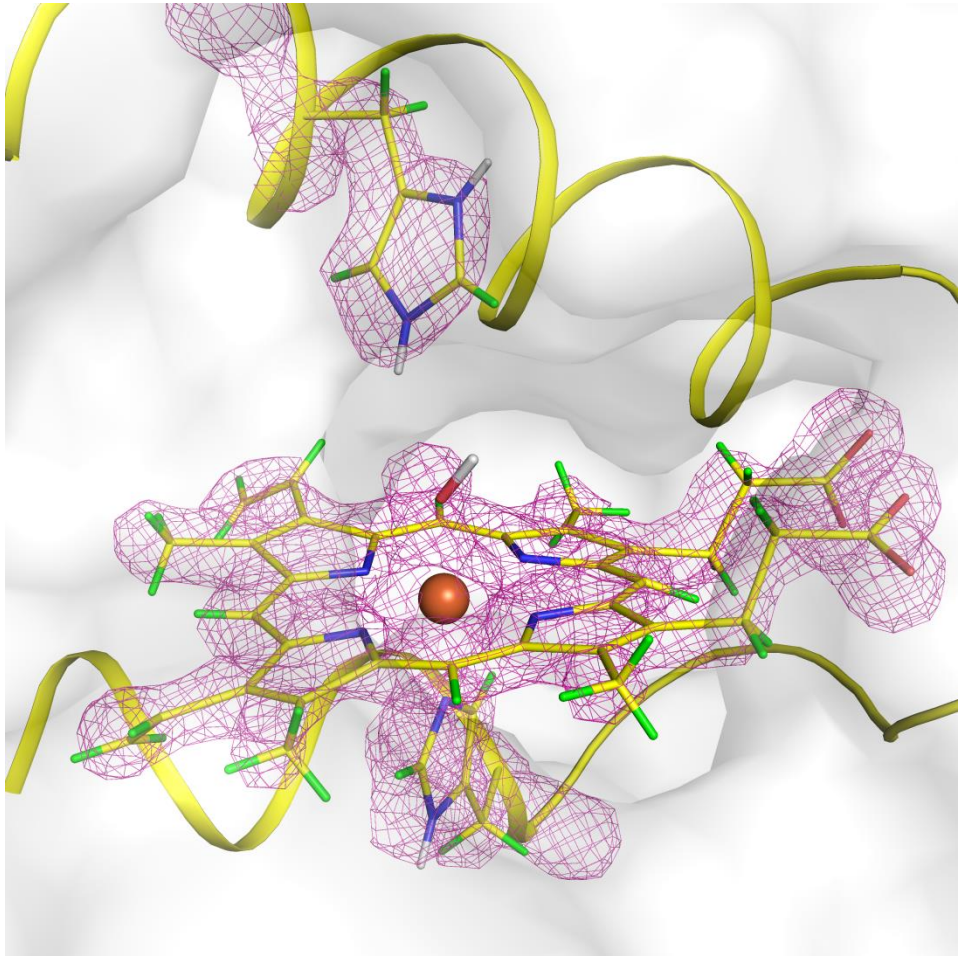


neutron



X-ray

Compound II APX

Kwon et al. (2016) Direct visualisation of a Fe(IV)-OH intermediate in a heme enzyme. Nature Comm. 7; article 13445 (2016) DOI:10.1038/ncomms13445

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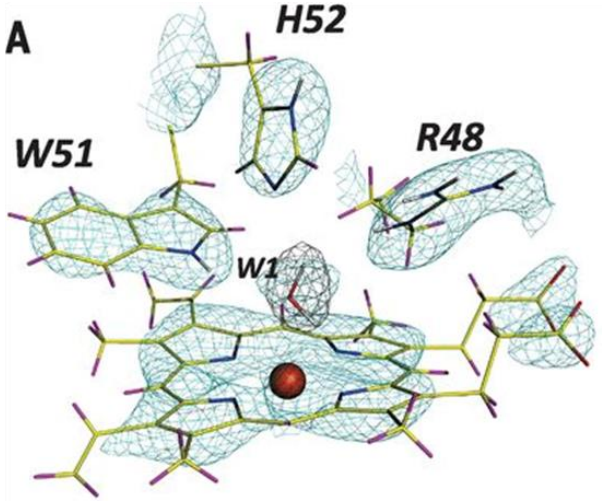
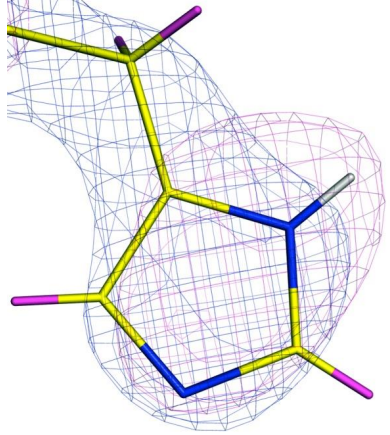
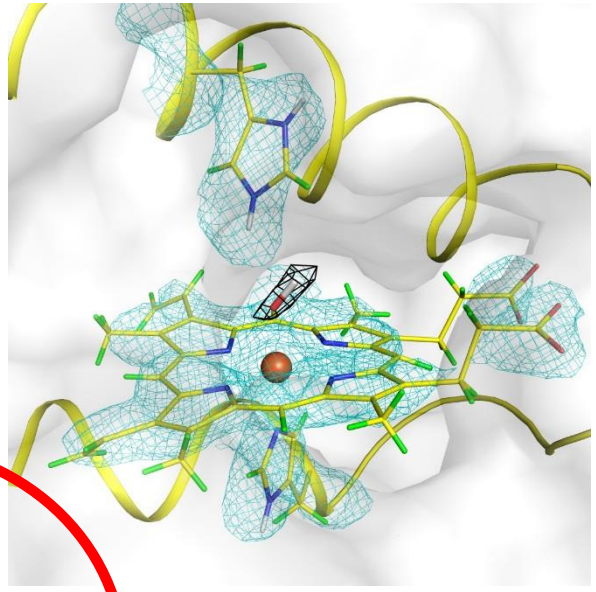






Ferric

H_2O_2

Ferrous → (+ O_2) Ferrous oxy (Compound III) → (+ e^-) Compound 0

Compound II

$e^- + H^+$

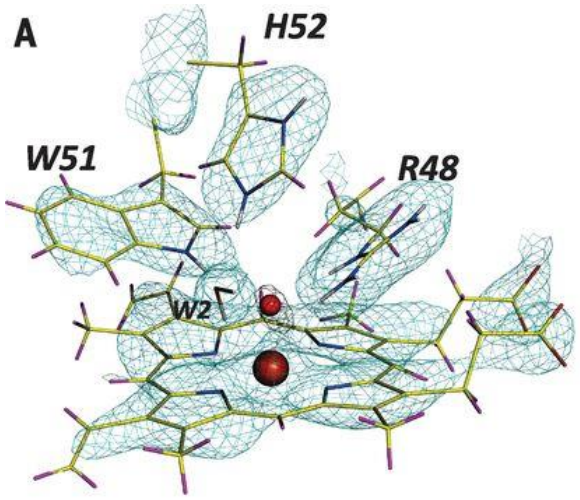
Compound II

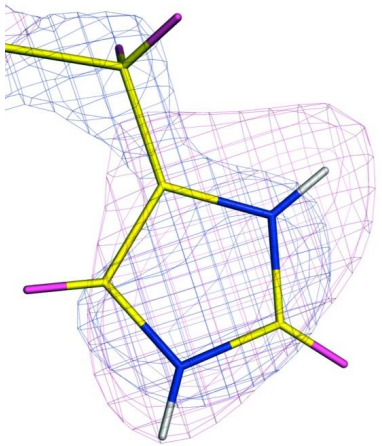
- H_2O

Compound I ($P^{\bullet+}$, $Trp^{\bullet+}$)

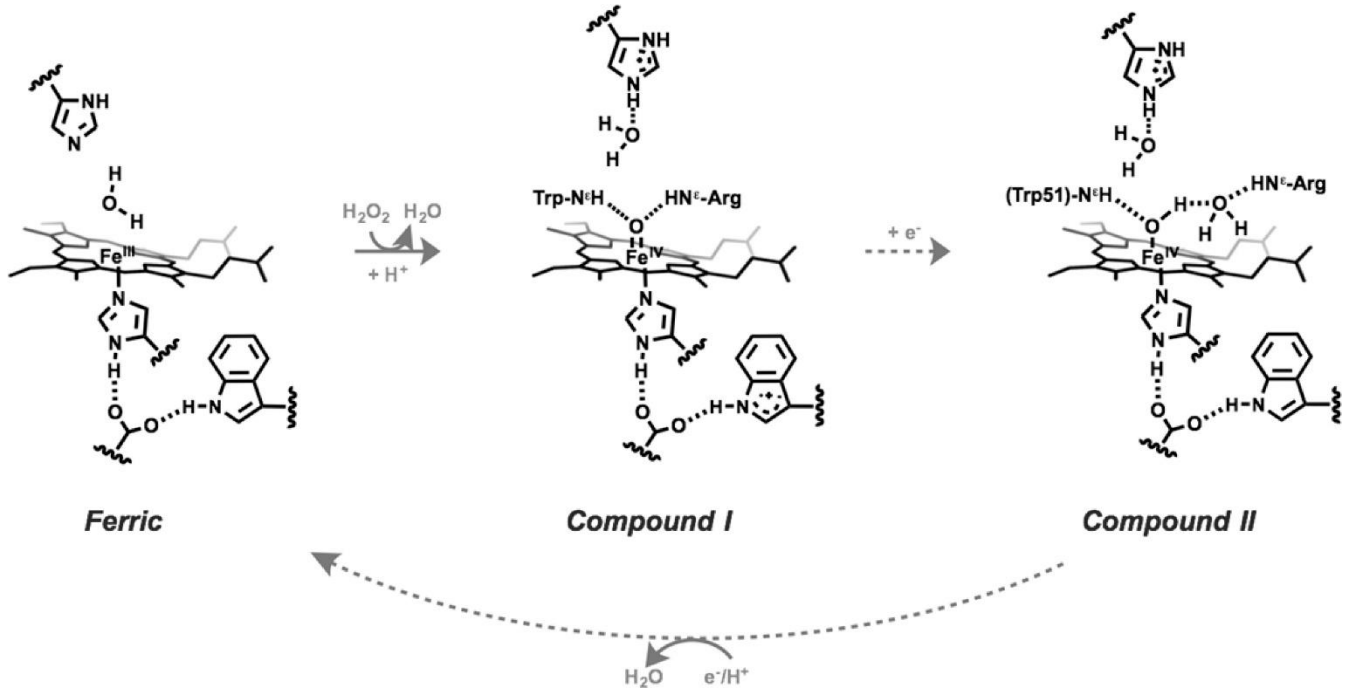
$e^- + H^+$

Compound I ($P^{\bullet+}$, $Trp^{\bullet+}$)



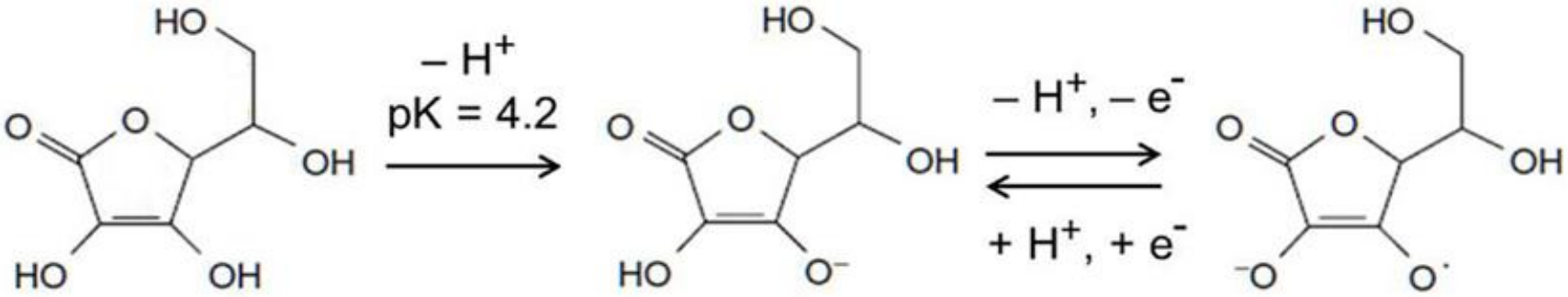


Casadei et al. (2014) Science
Kwon et al. (2016) Nature Communications



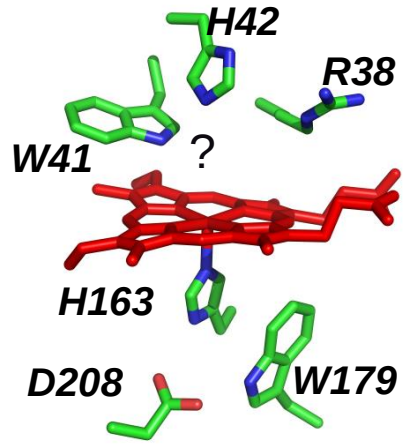
Protonation states of active site residues in C*c*P and APX as identified from the neutron structures of ferric C*c*P (PDB 4CVI), Compound I of C*c*P (PDB 4CVJ), and Compound II of APX (PDB 5JPR).
Note the change in position of the distal Arg side chain between Compound I of C*c*P and Compound II of APX.
Reaction mechanism arrows are shown hypothetically in gray, to indicate that the species are taken from two different enzymes.
There is no neutron structure for Compound II of C*c*P, but X-ray data(59) show that Arg48 has the same orientation as the Compound I neutron structure.

Moody & Raven (2018) *Acc. Chem. Res.*, 51, 2, 427–435 DOI: (10.1021/acs.accounts.7b00463)



Ascorbate peroxidase

- How do we get the H from the Ascorbate to the ferryl centre?



- **H_2O_2 reduced to H_2O and substrate oxidized**

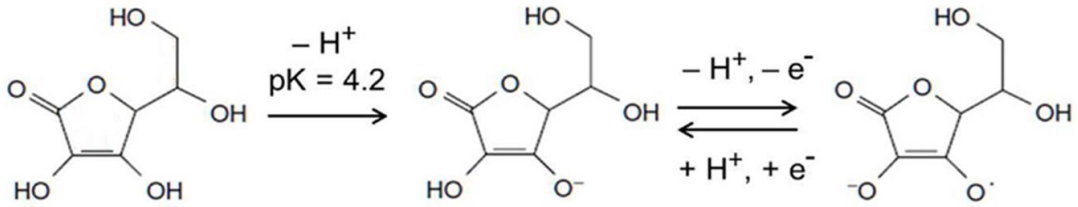
Fe(III) P + H_2O_2 → **Fe(IV)-O P·** + H_2O (1)
(Compound I)

Fe(IV)-O P· + S → **Fe(IV)-O P** + S· (2)
(Compound II)

Fe(IV)-O P + S → Fe(III) P + H_2O + S· (3)

Ascorbate peroxidase

- How do we get the H from the Ascorbate to the ferryl centre?



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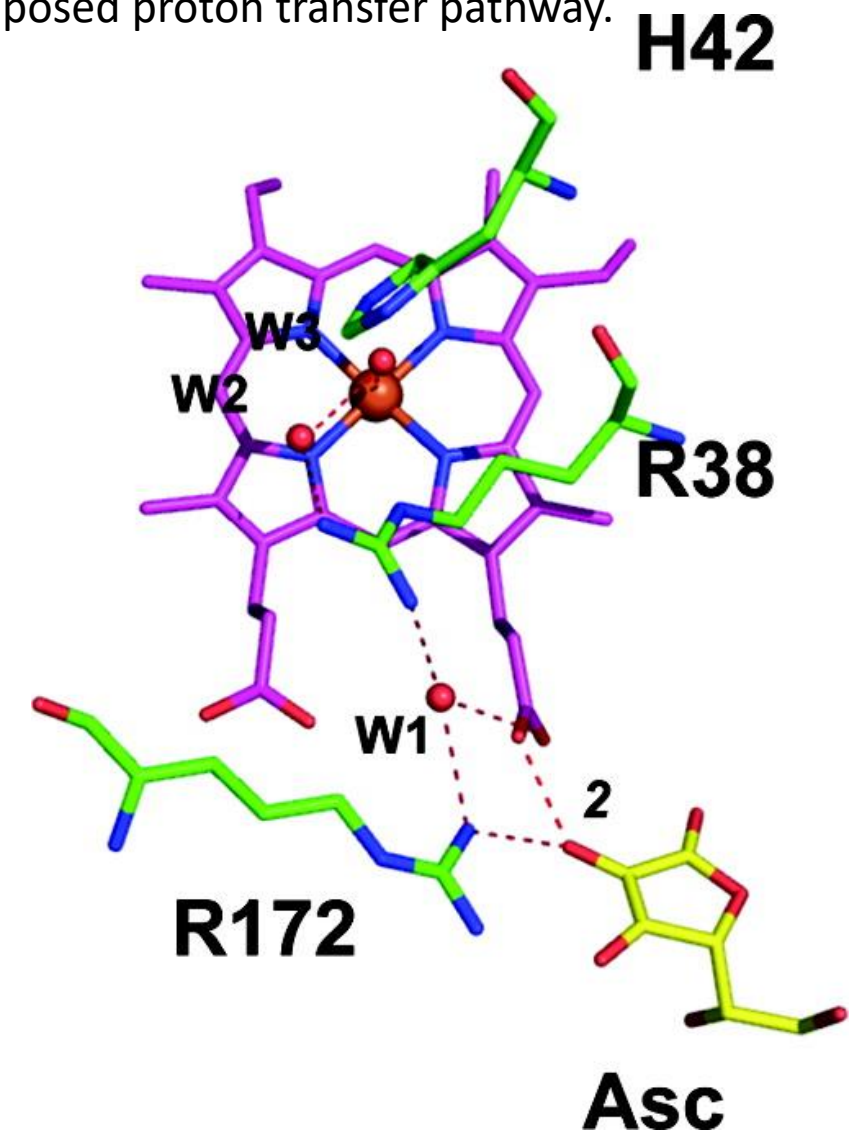
(Compound I)

Fe(IV)-O P· + S → **Fe(IV)-O P** + S· (2)
(Compound II)

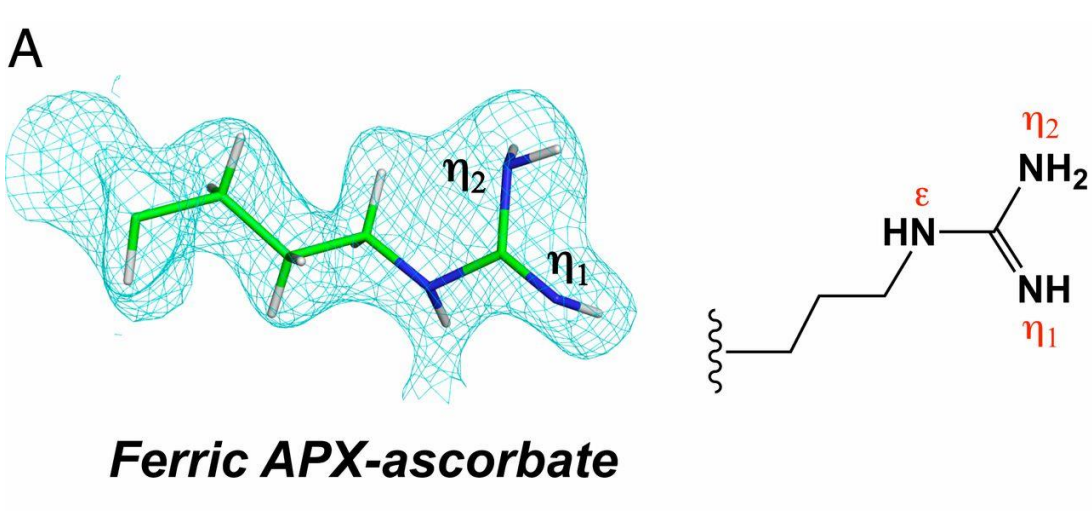
Fe(IV)-O P + S → Fe(III) P + H_2O + S· (3)

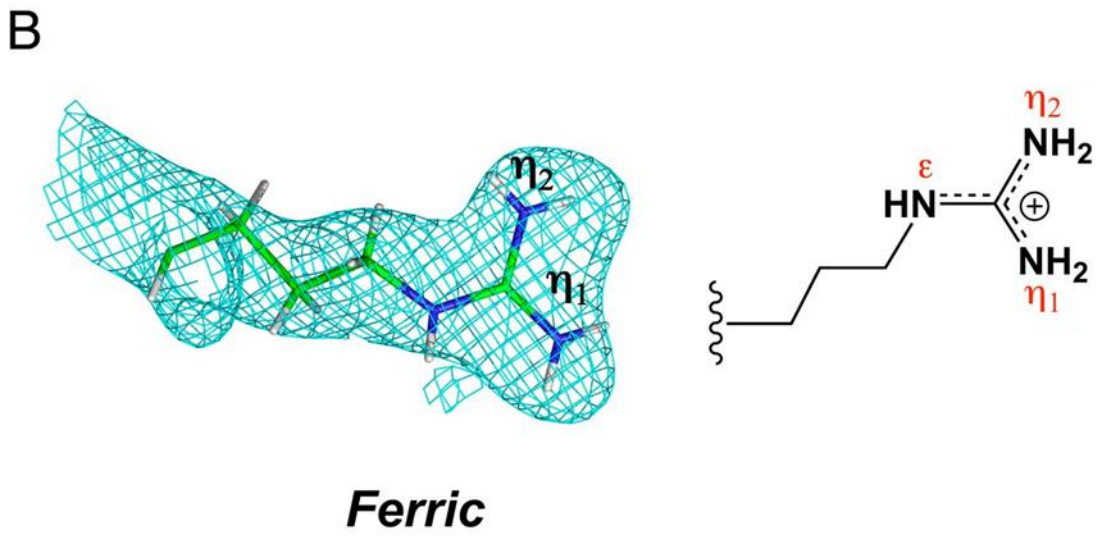
X-ray structure of the ferric APX–ascorbate complex, showing the hydrogen bonds which comprise the proposed proton transfer pathway.



Proton Delivery to Ferryl Heme in a Heme Peroxidase: Enzymatic Use of the Grotthuss Mechanism
Efimov et al; *J. Am. Chem. Soc.* **2011,** 133, 15376-15383.

Nuclear density (2Fo − Fc), shown in cyan and contoured at 1.5σ for Arg38 in (A) the ferric APX–ascorbate complex and (B) ferric APX structure.





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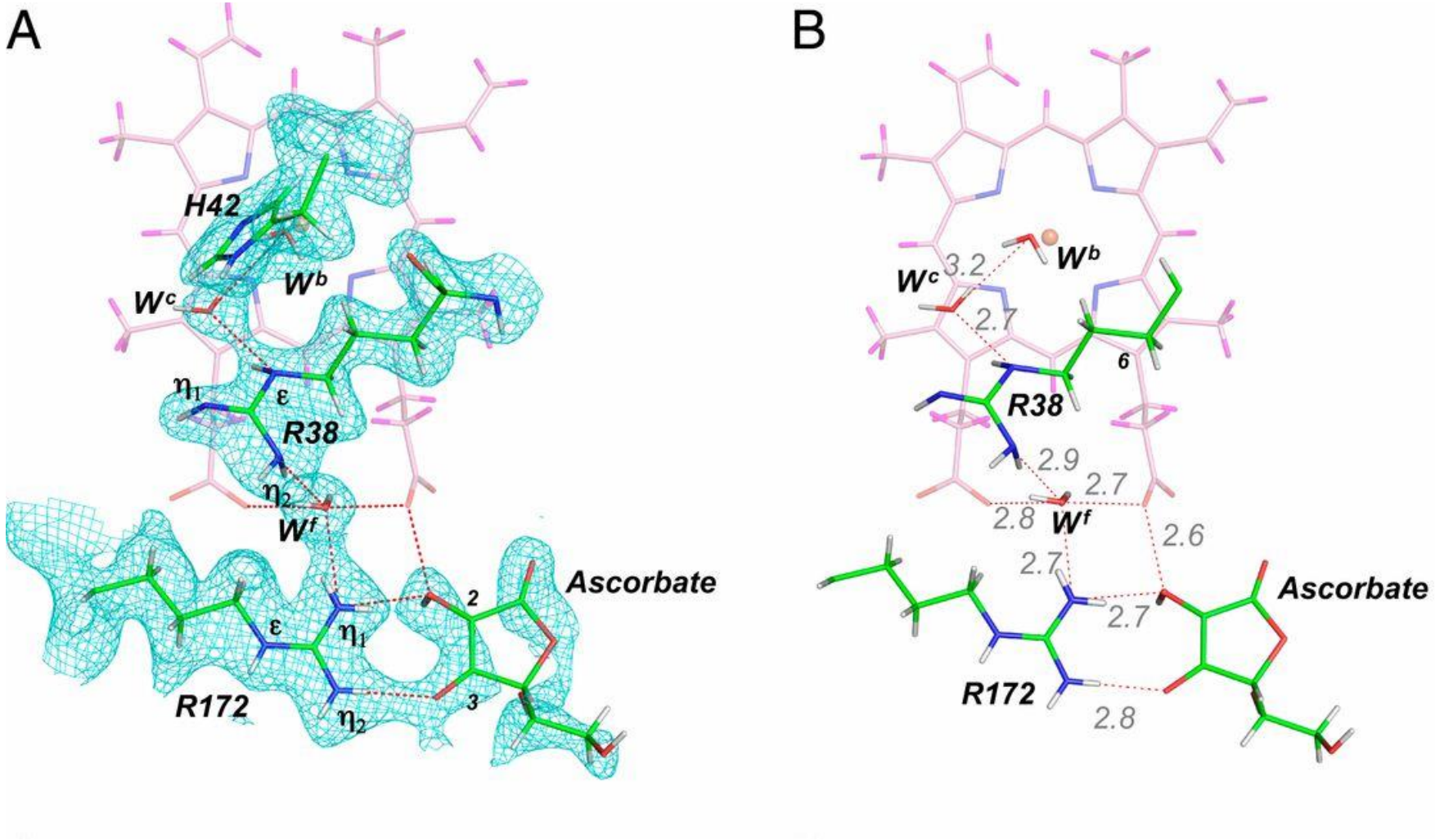
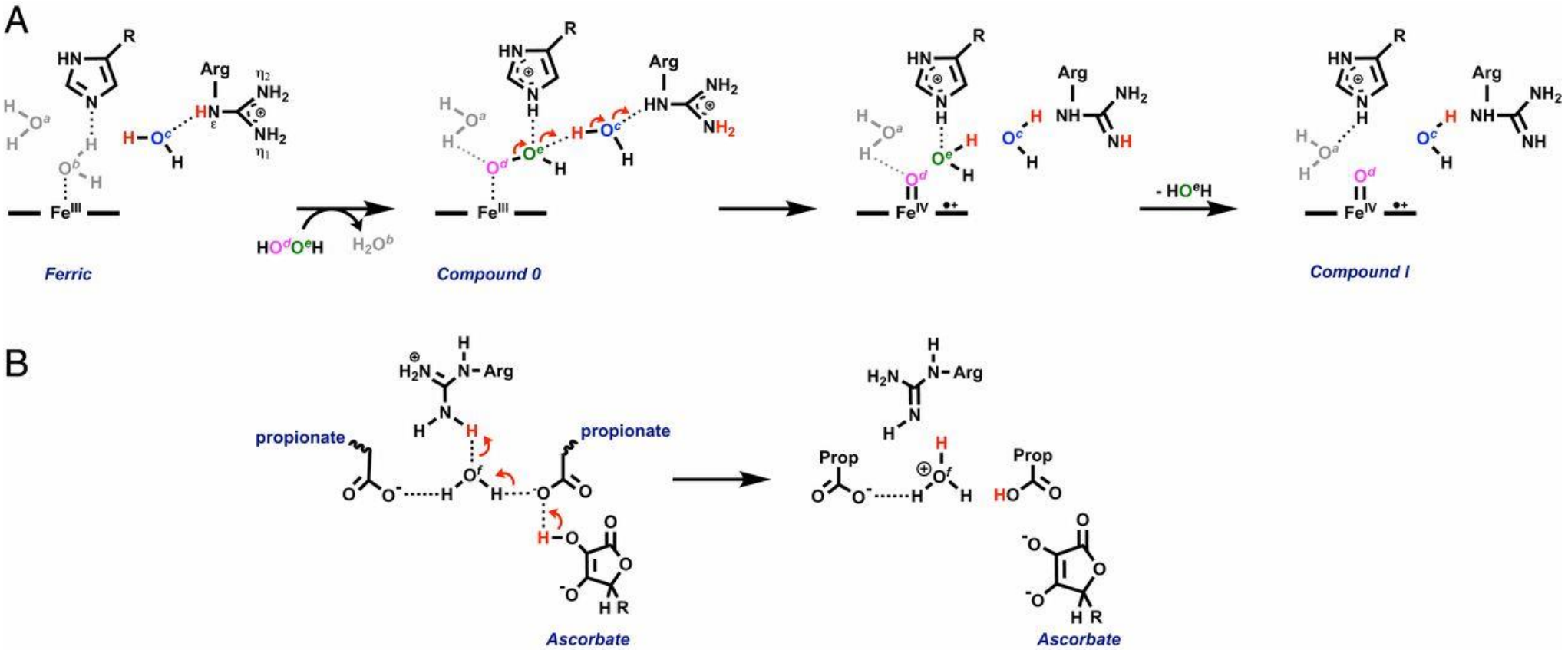
Kwon et al. PNAS 2020;117:12:6484-6490

The neutron structure of the ascorbate peroxidase (APX)–ascorbate electron transfer complex.

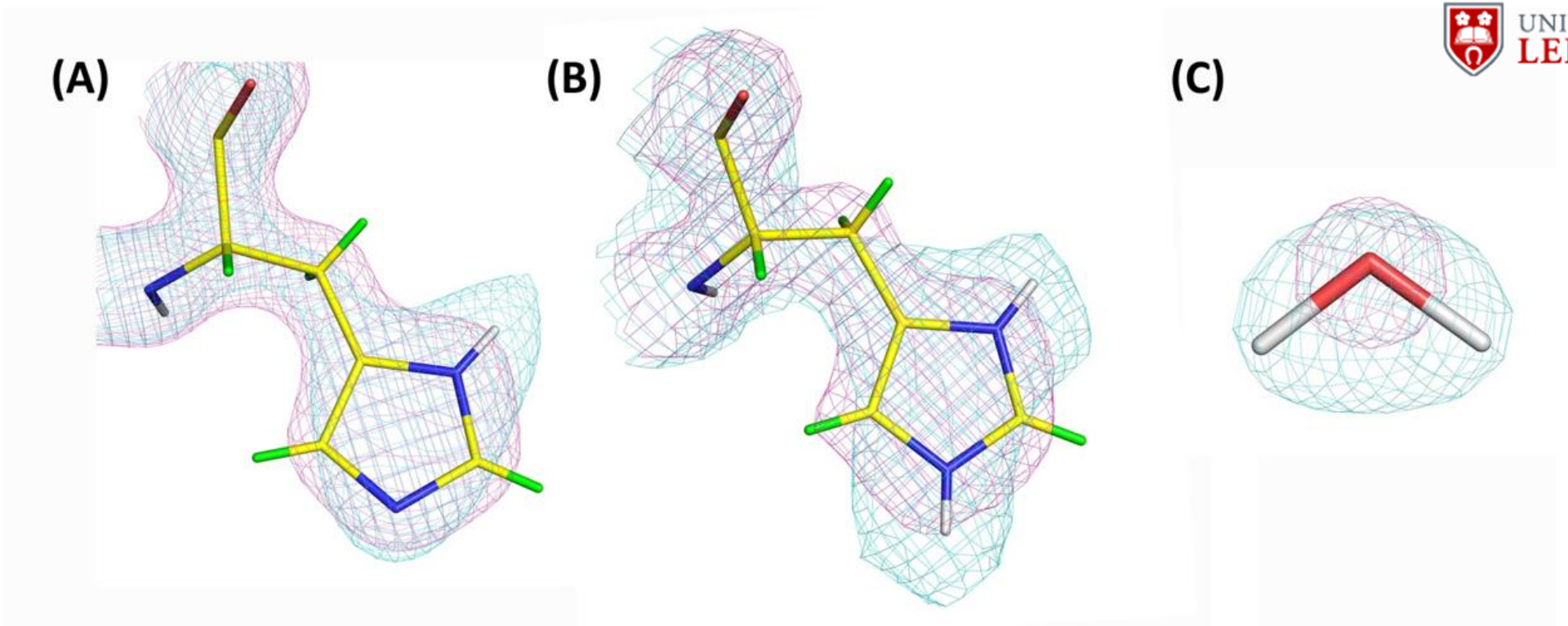


Kwon et al. PNAS 2020;117:12:6484-6490

Exemplars of possible movements of protons involving Arg38, based on an analysis of neutron structures for ferric APX and the ferric APX–ascorbate complex and on the neutron structure of compound II of APX (53).

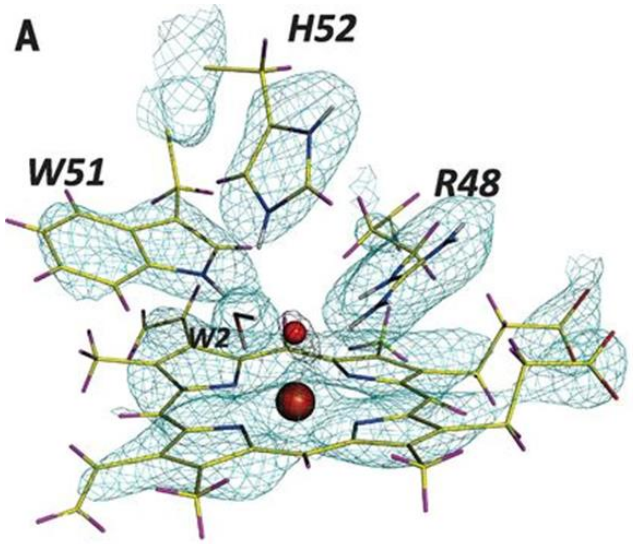


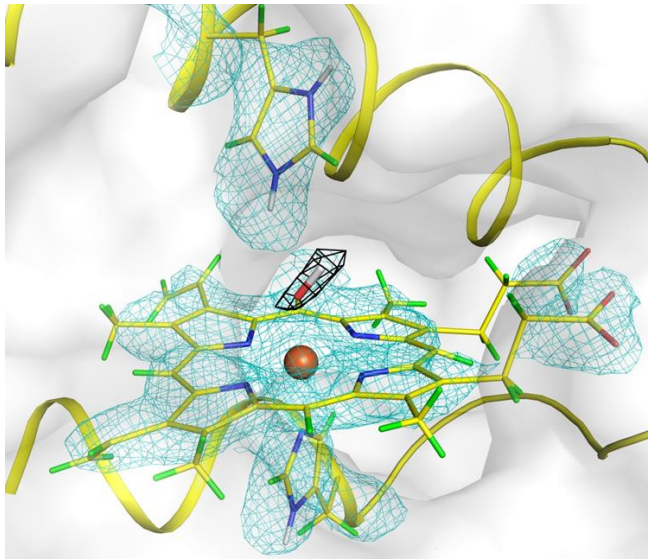
Kwon et al. PNAS 2020;117:12:6484-6490



Summary

Neutrons allow the positions of hydrogen atoms to be seen in conditions free of X-ray induced reduction





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ESRF

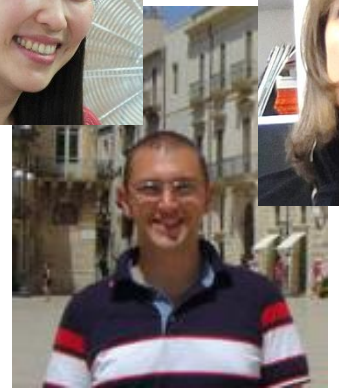
John McGeehan (>Portsmouth)

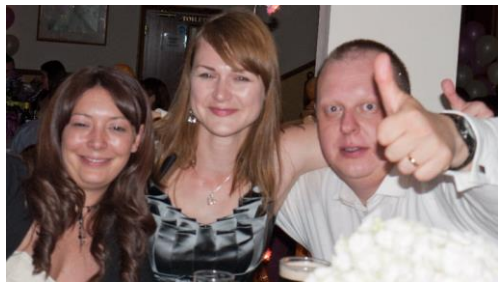
Antoine Royant

Diamond

Pierre Aller







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