Lecture 2:

Cytochromes P450 enzymes (II):

- structure-function
- inhibition

Cytochrome P450:

Structure - function

General overview of P450s

- General reaction catalysed:

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

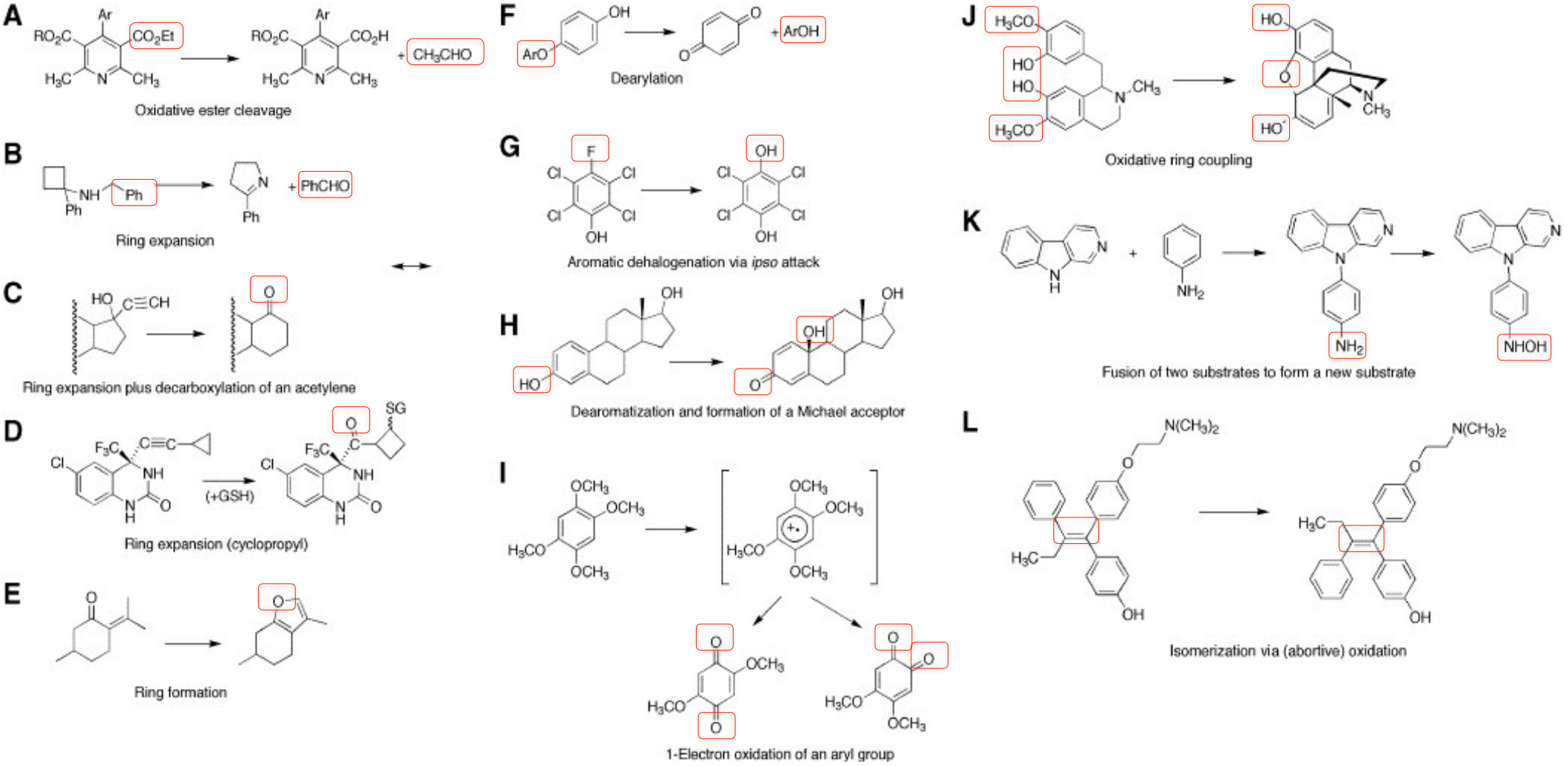
- There are 55 P450s in the human genome:
  - 17 families (>40% sequence identity) **number:** **3**
  - 39 subfamilies (>55% sequence identity) **letter:** **A**
  - member number in the subfamily **number:** **4**

- Most drug metabolism is carried out by families 1, 2 and 3.

- 50kDa size, anchored to smooth ER of liver cells by a transmembrane helix at the N-ter.

- Functions: xenobiotic detoxification, procarcinogen activation, biosynthesis (steroid hormones, prostacyclin, thromboxane, cholesterol, bile acids) and degradation (fatty acids, retinoic acid, steroids).

Some reactions catalysed

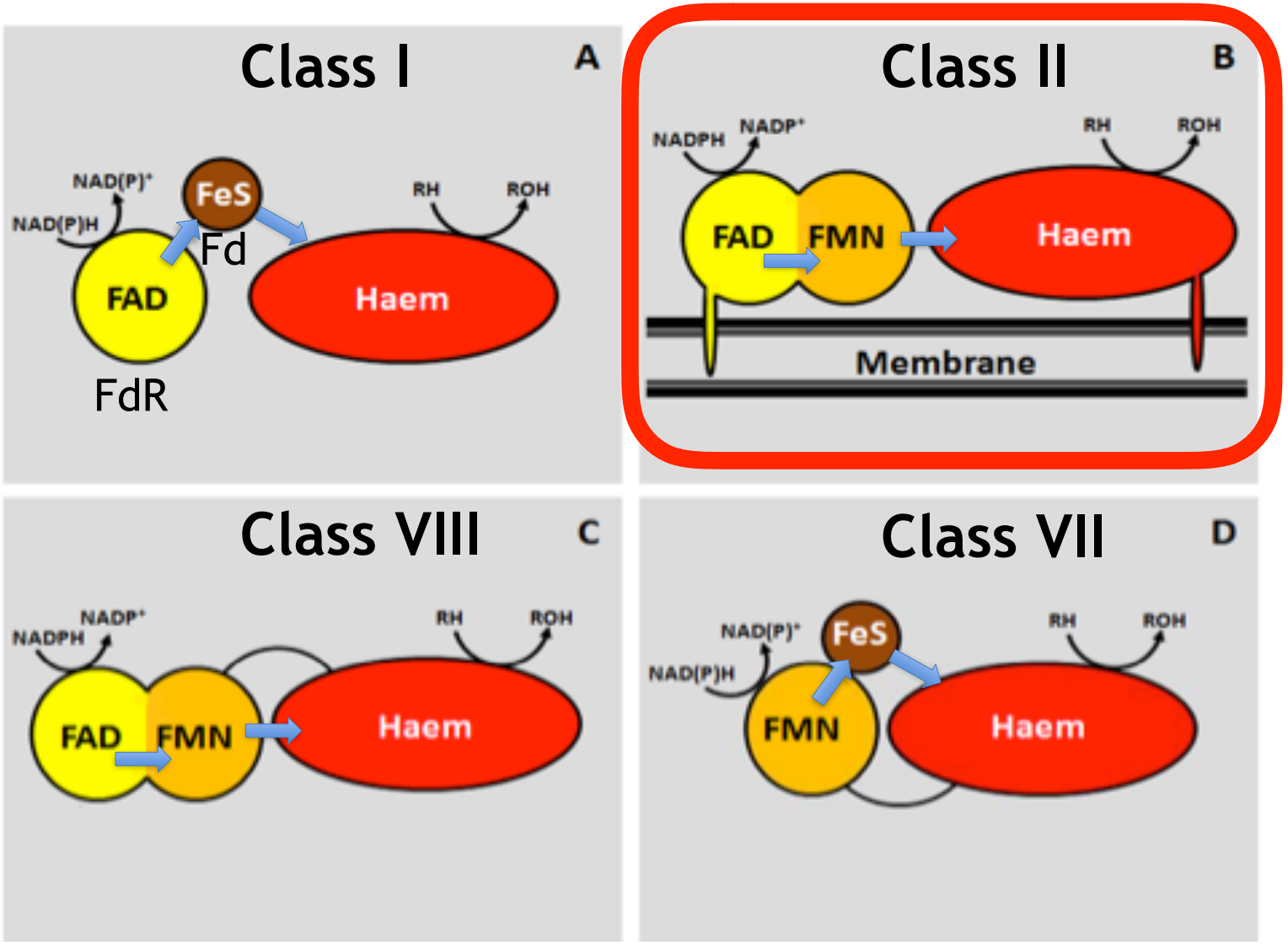


General reaction:

$$R\text{-}H + O_2 + 2e^- + 2H^+ \rightarrow R\text{-}OH + H_2O$$

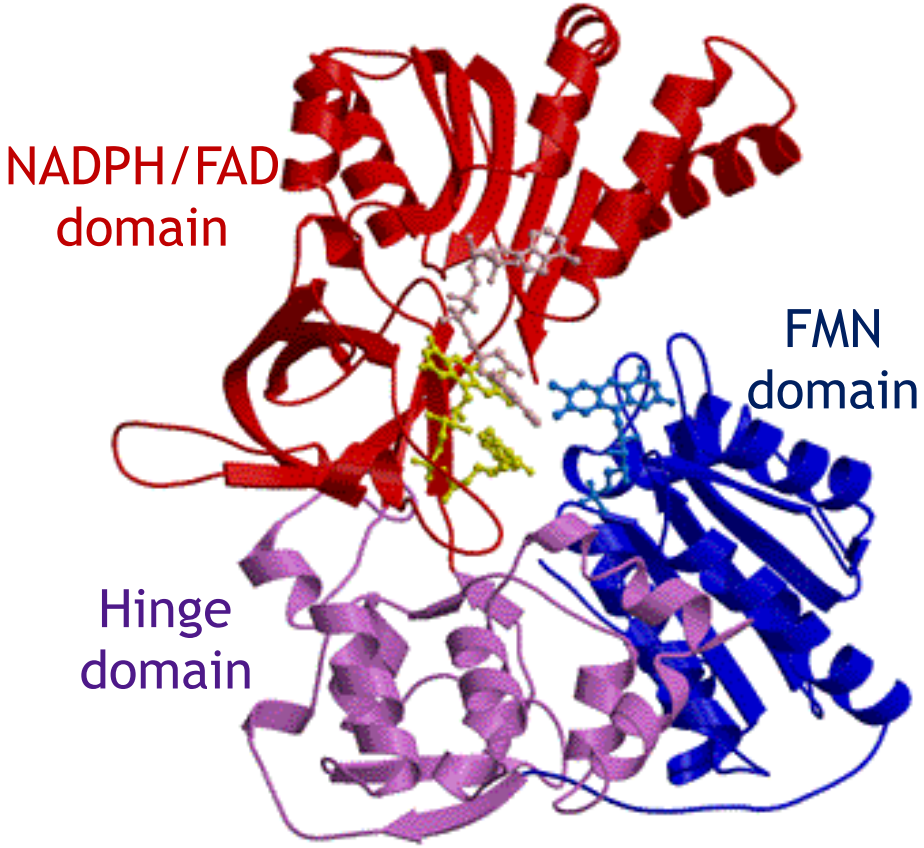
In order to function they need electrons and protons

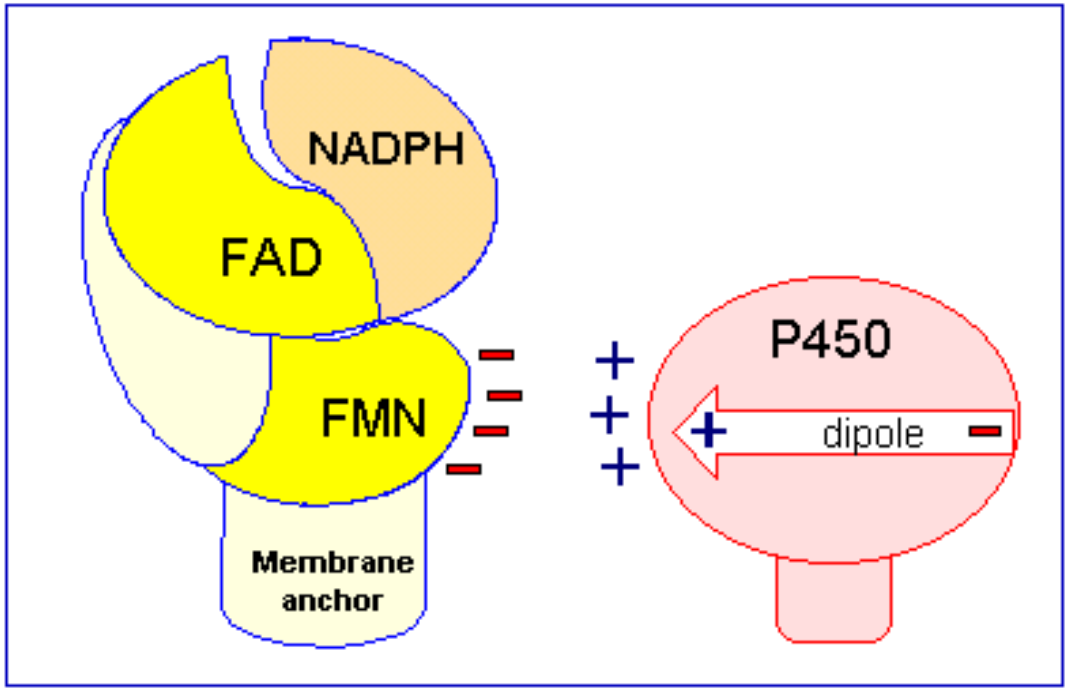
Electron supply to P450s:
Classification based on electron transfer partners

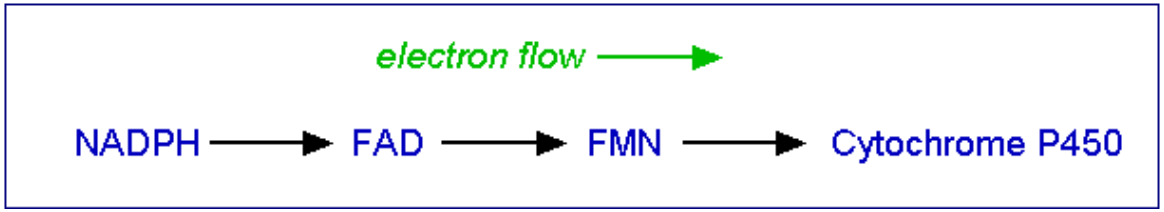


Sadeghi and Gilardi, Biotechnol. Appl. Biochem. 2013, **60**, 102-110.

Mammalian cytochrome P450 reductase





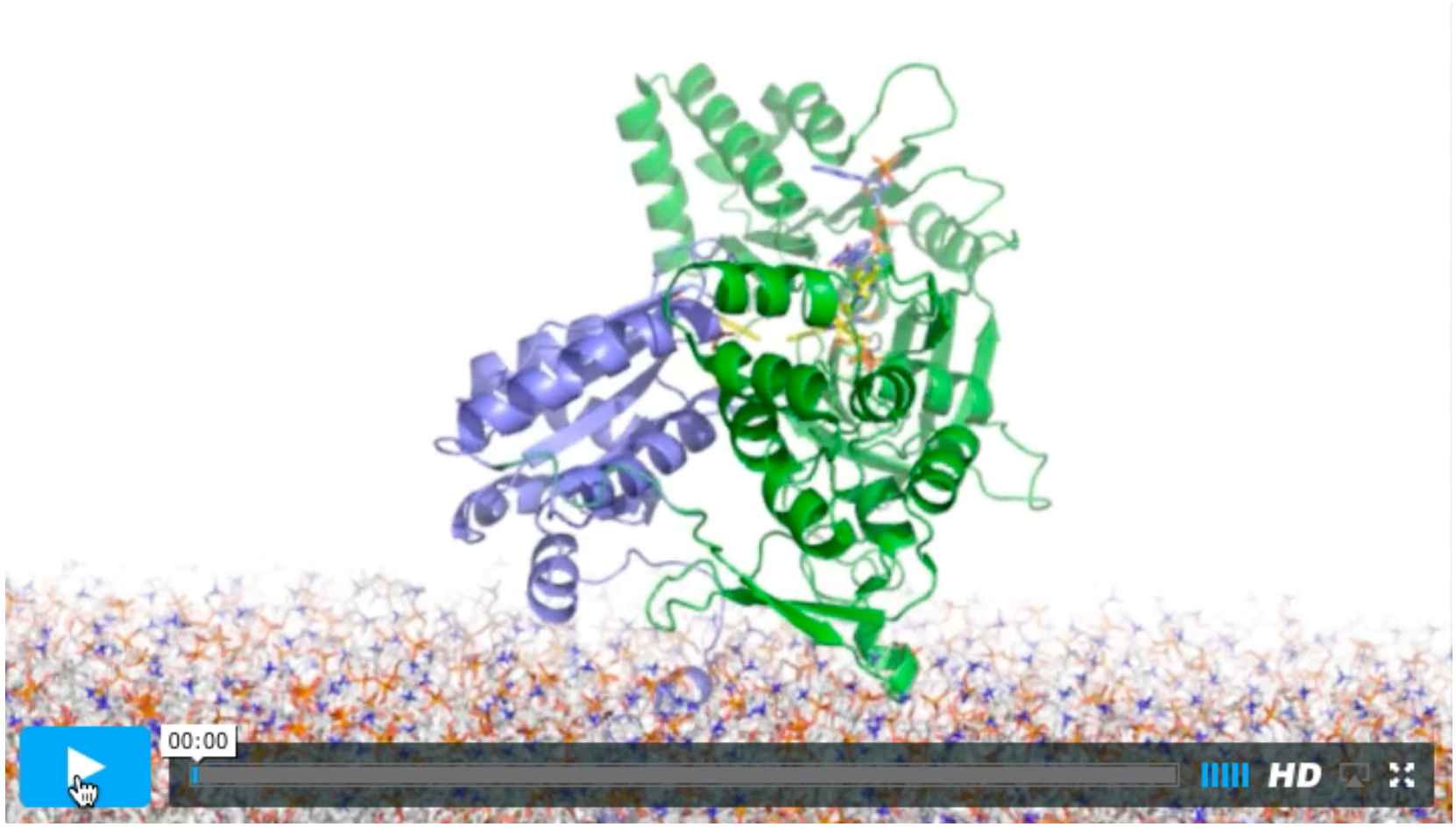


Steric problem for ET

Proteins are dynamic systems

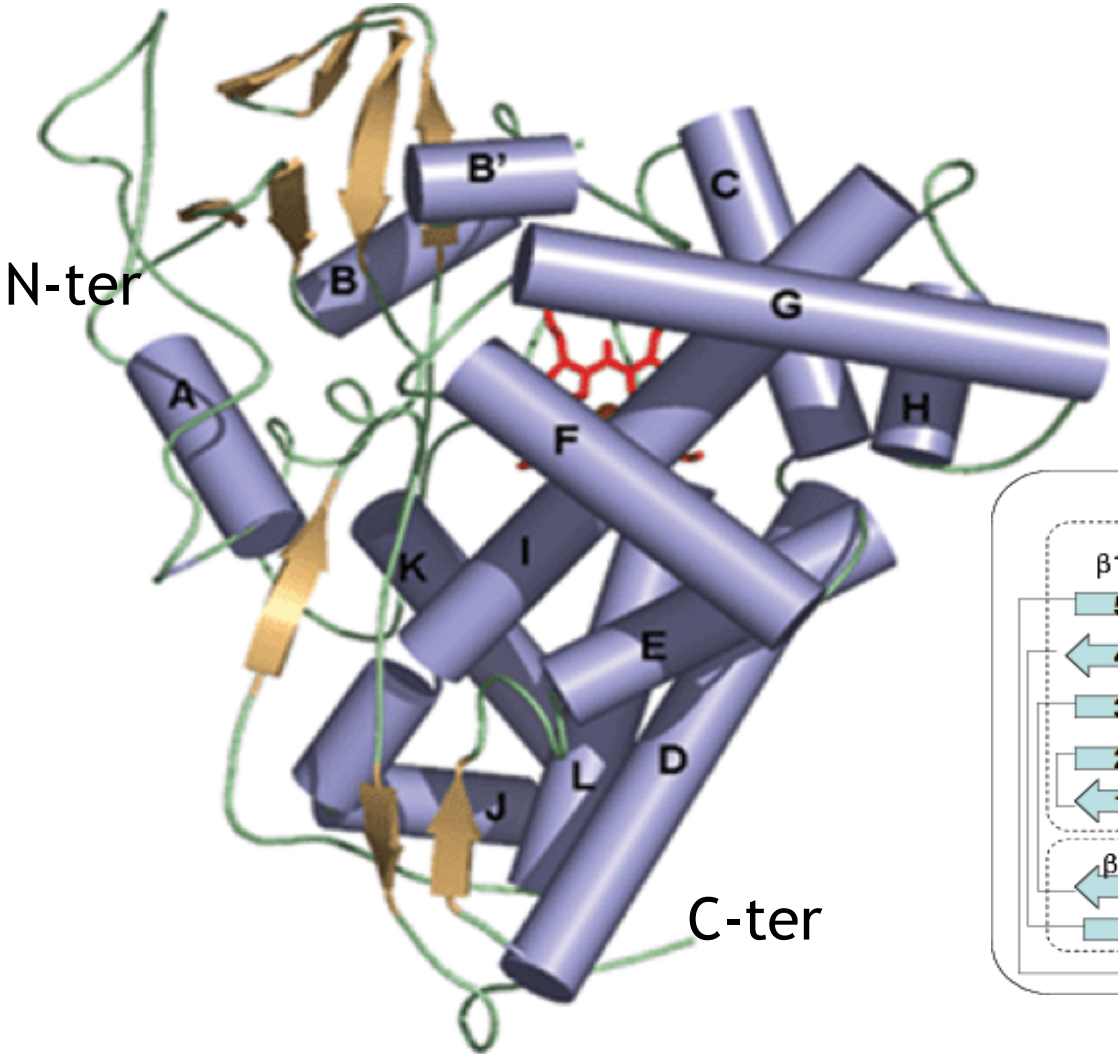
Marcus Theory
on Biological Electron Transfer:

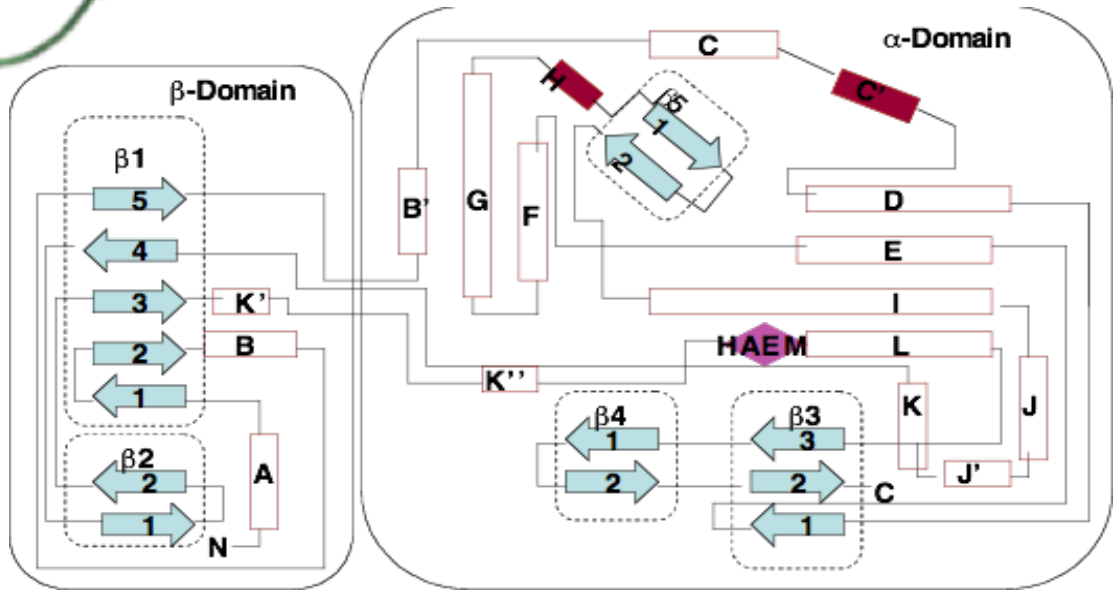
$$k_{et} = \frac{2\pi}{\hbar}|H_{AB}|^2 \frac{1}{\sqrt{4\pi\lambda k_b T}} \exp\left(-\frac{(\lambda + \Delta G^\circ)^2}{4\lambda k_b T}\right)$$

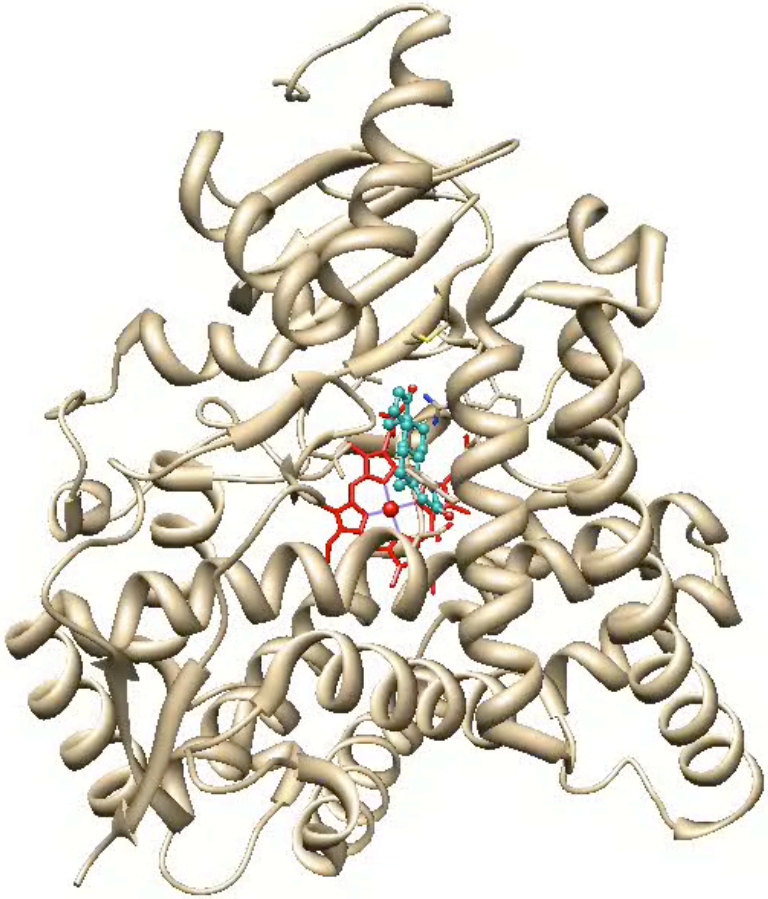


Simulation by Jung-Ja Kim, Medical College of Wisconsin, US

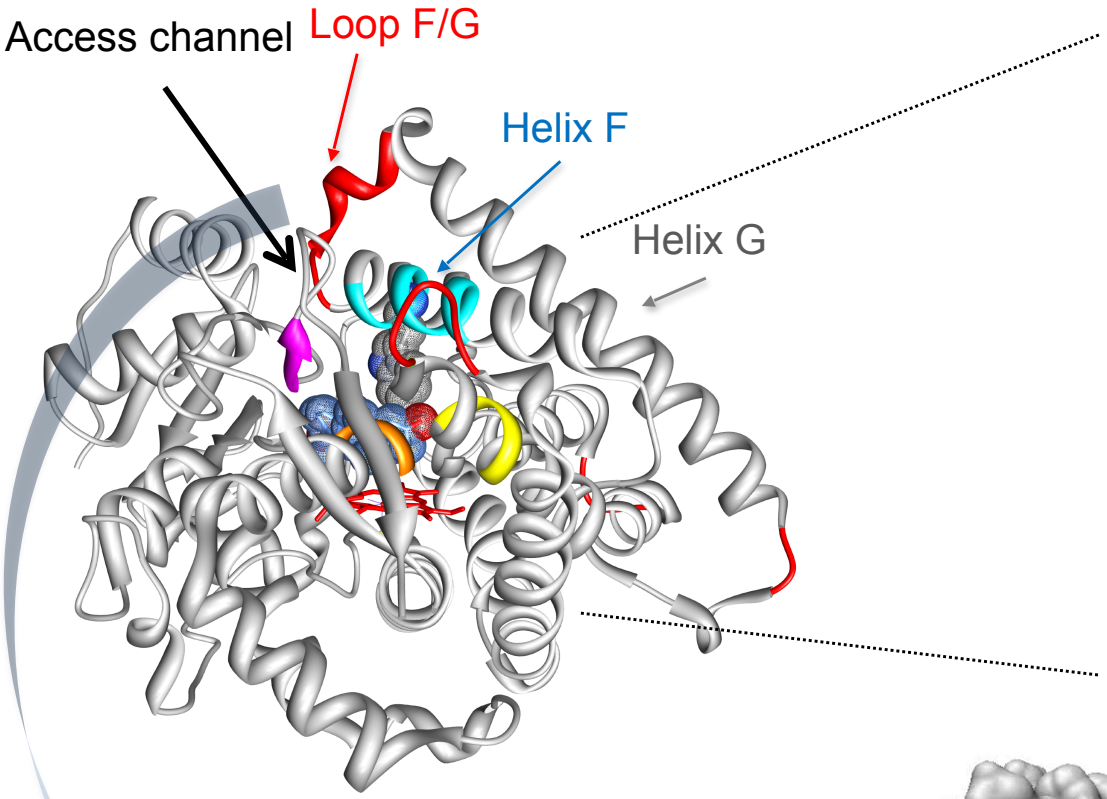
The P450 general fold: details of the catalytic pocket

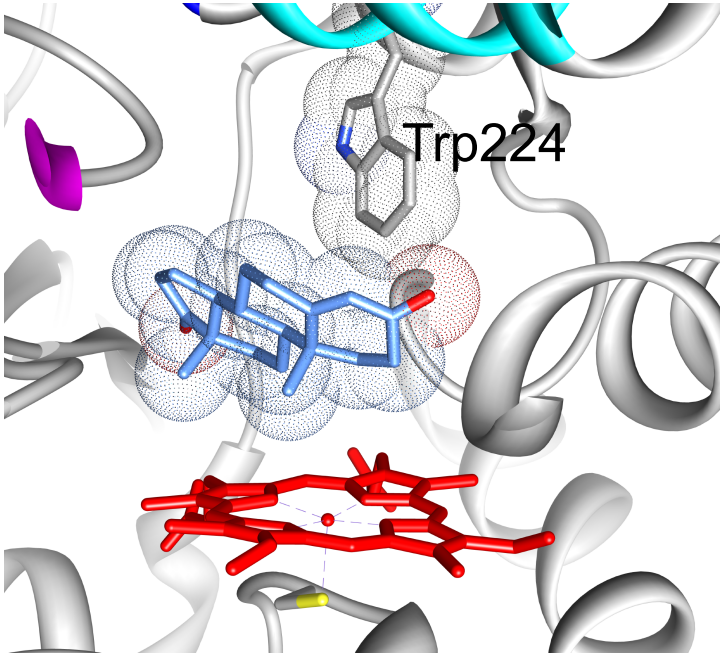


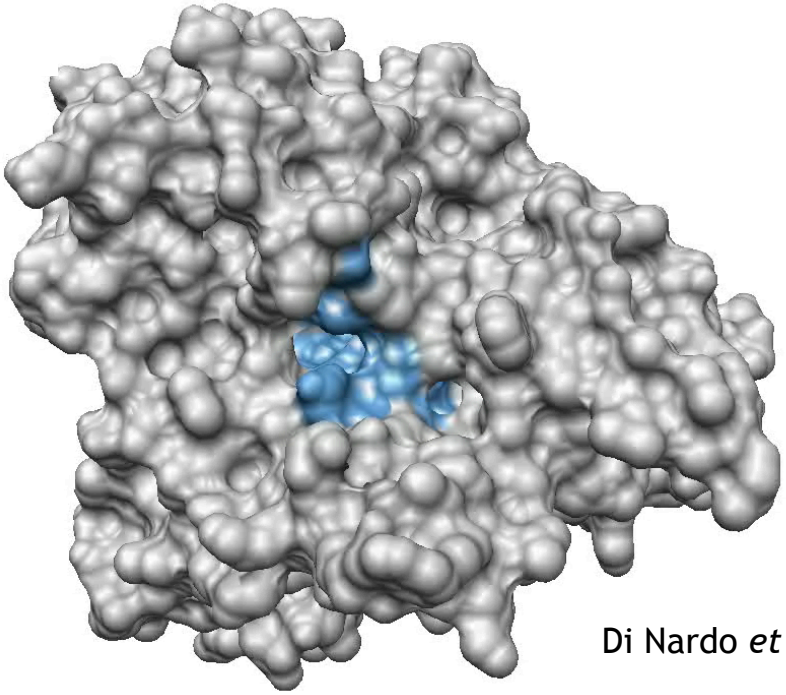




Aromatase complexed with the substrate androstenedione







Di Nardo *et al.*, PlosOne, 2013, **8** (12), e8211

the diameter of the cell body.

Pull-down assay and immunoprecipitation

Purification of recombinant GST fusion proteins from *Escherichia coli*, *in vitro* binding assays, and immunoprecipitation were done as described[11]. We loaded recombinant GST–RhoG with guanine nucleotides by incubating the protein with 100 μM GTP-γS or GDP in loading buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol and 5 mM EDTA) at 30 °C for 10 min. The reaction was stopped by adding $MgCl_2$ to final concentration of 10 mM. Activation of Rac1 in cells was determined by using the GST-fused CRIB domain of Pak1 as described[9].

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Crystal structure of human cytochrome P450 2C9 with bound warfarin

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Cytochrome P450 proteins (CYP450s) are membrane-associated haem proteins that metabolize physiologically important compounds in many species of microorganisms, plants and animals. Mammalian CYP450s recognize and metabolize diverse xenobiotics such as drug molecules, environmental compounds and pollutants[1]. Human CYP450 proteins CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 are the major drug-metabolizing isoforms, and contribute to the oxidative metabolism of more than 90% of the drugs in current clinical use[2]. Polymorphic variants have also been reported for some CYP450 isoforms, which has implications for the efficacy of drugs in individuals, and for the co-administration of drugs. The molecular basis of drug recognition by human CYP450s, however, has remained elusive. Here we describe the crystal structure of a human CYP450, CYP2C9, both unliganded and in complex with the anti-coagulant drug warfarin. The structure defines unanticipated interactions between CYP2C9 and warfarin, and reveals a new binding pocket. The binding mode of warfarin suggests that CYP2C9 may undergo an allosteric mechanism during its function. The newly discovered binding pocket also suggests that CYP2C9 may simultaneously accommodate multiple ligands

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Human 2C9 structure

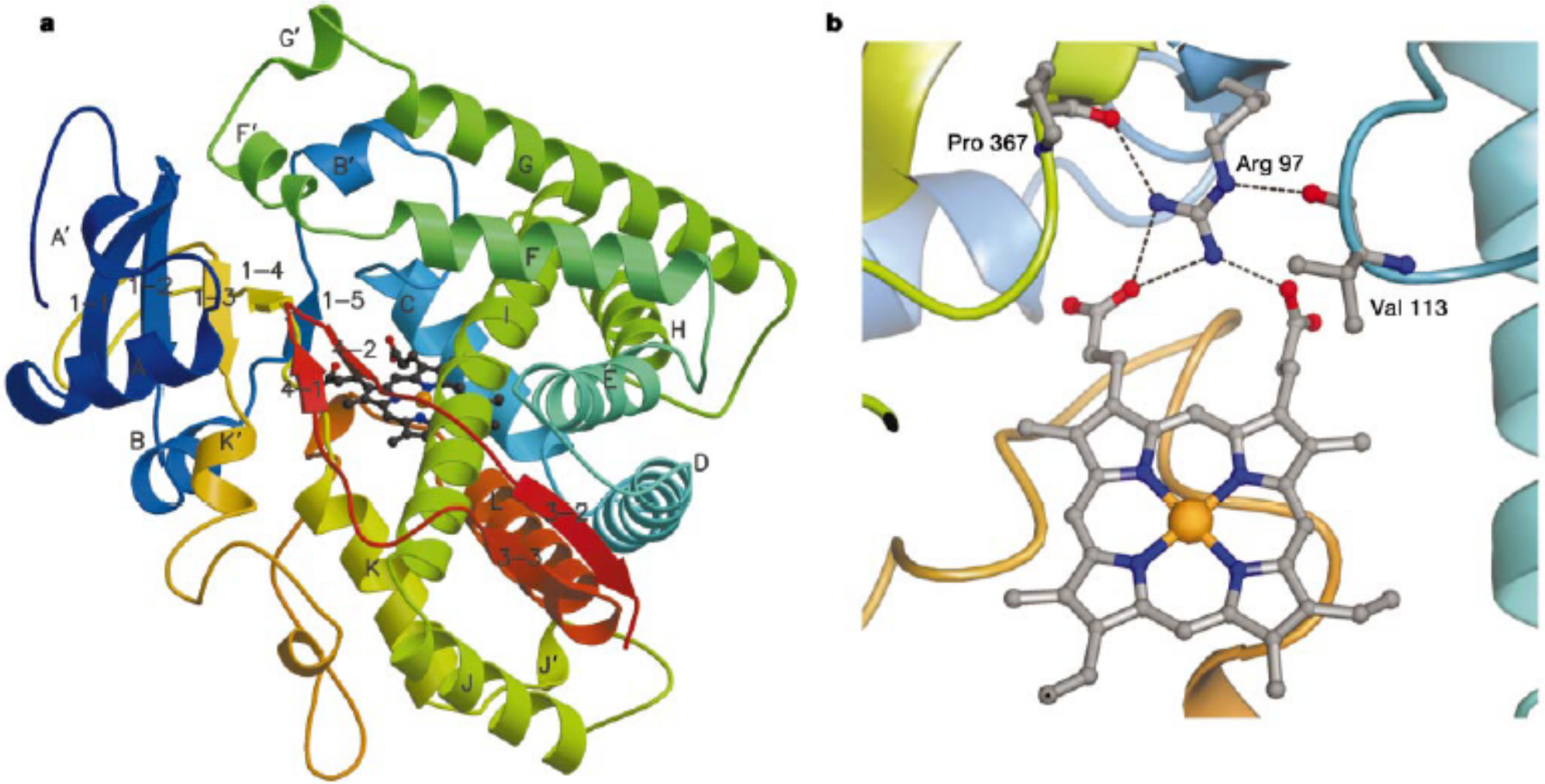


Figure 1 Structure of P450 CYP2C9. **a**, Overall fold of CYP2C9, coloured from blue at the N terminus to red at the C terminus. The haem group is depicted as a ball-and-stick model in the centre of the molecule, flanked by helices I and L. There is a slight distortion in helix I, close to the haem. The substrate access channel is widely acknowledged to involve the loops between helices B and C, and helices F and G. The figure was produced using Molscript (http://www.avatar.se/molscript). **b**, View of Arg 97 and the haem group (shown at the bottom). Arg 97 is held in position by hydrogen bonds (indicated by dashed lines) to the haem propionates and to the carbonyl oxygen atoms of Val 113 and Pro 367. Figures 1b–4b were produced using Aesop 2.5 (M. Noble, unpublished work).

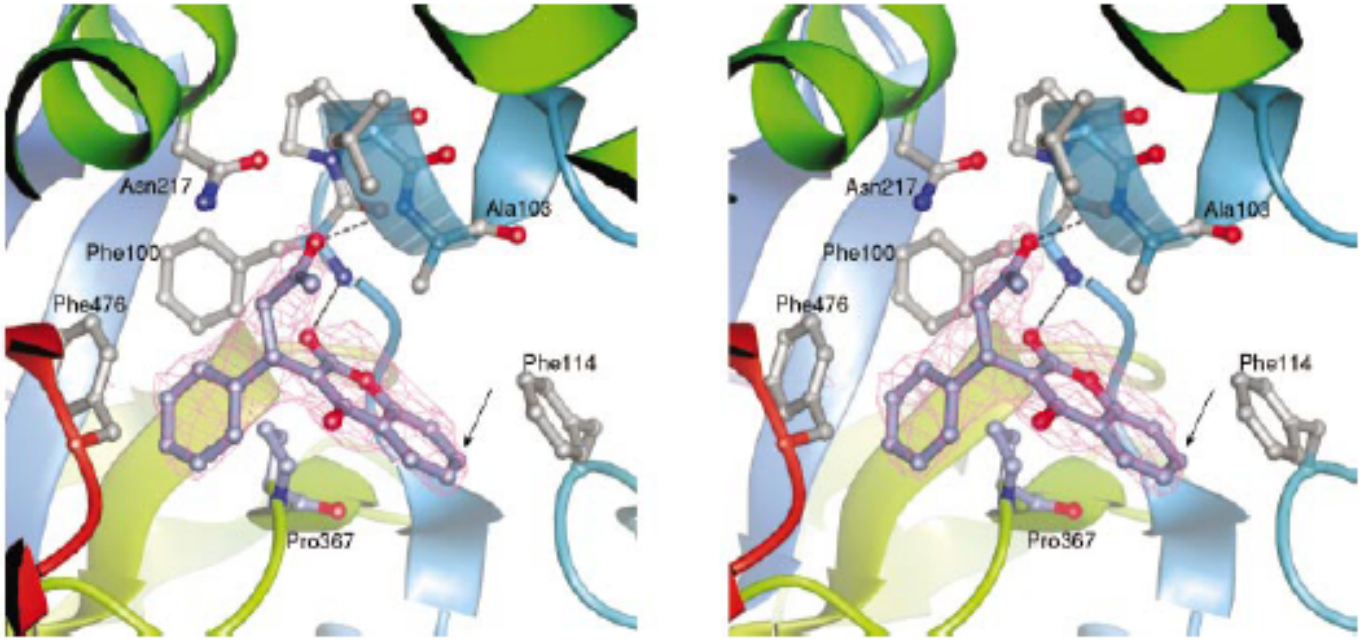


Figure 2 Stereo view of the binding site of *S*-warfarin in CYP2C9. The initial sigma-weighted $F_o - F_c$ electron density map is shown in red at the 2.5 sigma level. The hydrogen bonds between *S*-warfarin and the backbone nitrogen amide atoms of Phe 100 and Ala 103 are depicted as dashed lines. The pi–pi stacking interactions between the phenyl group of *S*-warfarin and Phe 476 is shown, as is the van der Waals contact between the bicyclic template of *S*-warfarin and residues Ala 103, Phe 114 and Pro 367. An arrow indicates the site of the 7-hydroxylation of *S*-warfarin catalysed by CYP2C9.

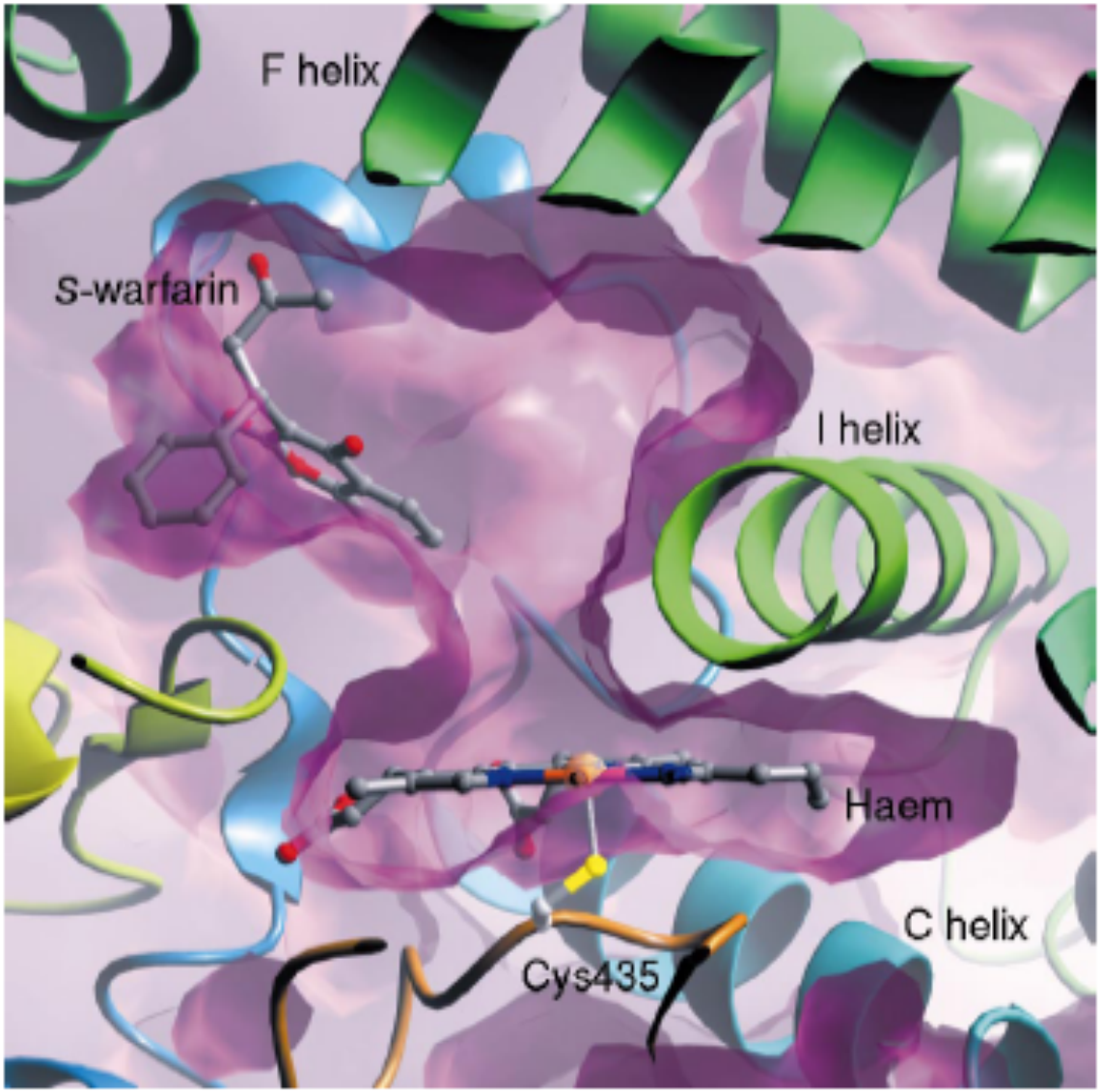


Figure 3 The surface of the active site of CYP2C9. The haem is shown at the bottom, and the *S*-warfarin molecule above and to the left. The active site of CYP2C9 is large and could potentially accommodate either compounds larger than *S*-warfarin or multiple ligands of comparable size to *S*-warfarin without substantial conformational movement. With *S*-warfarin bound in this location the haem group remains available to metabolize additional substrate molecules.

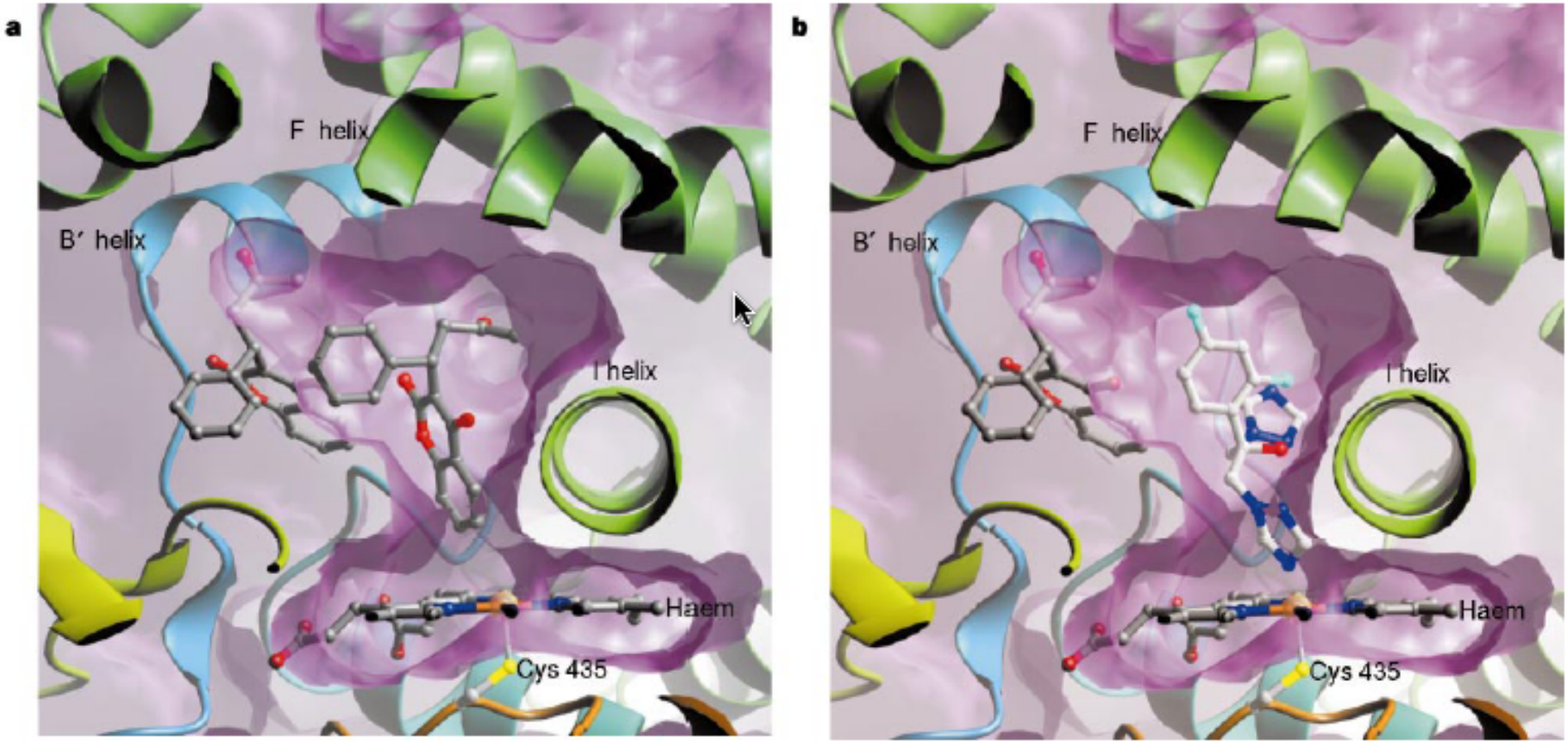
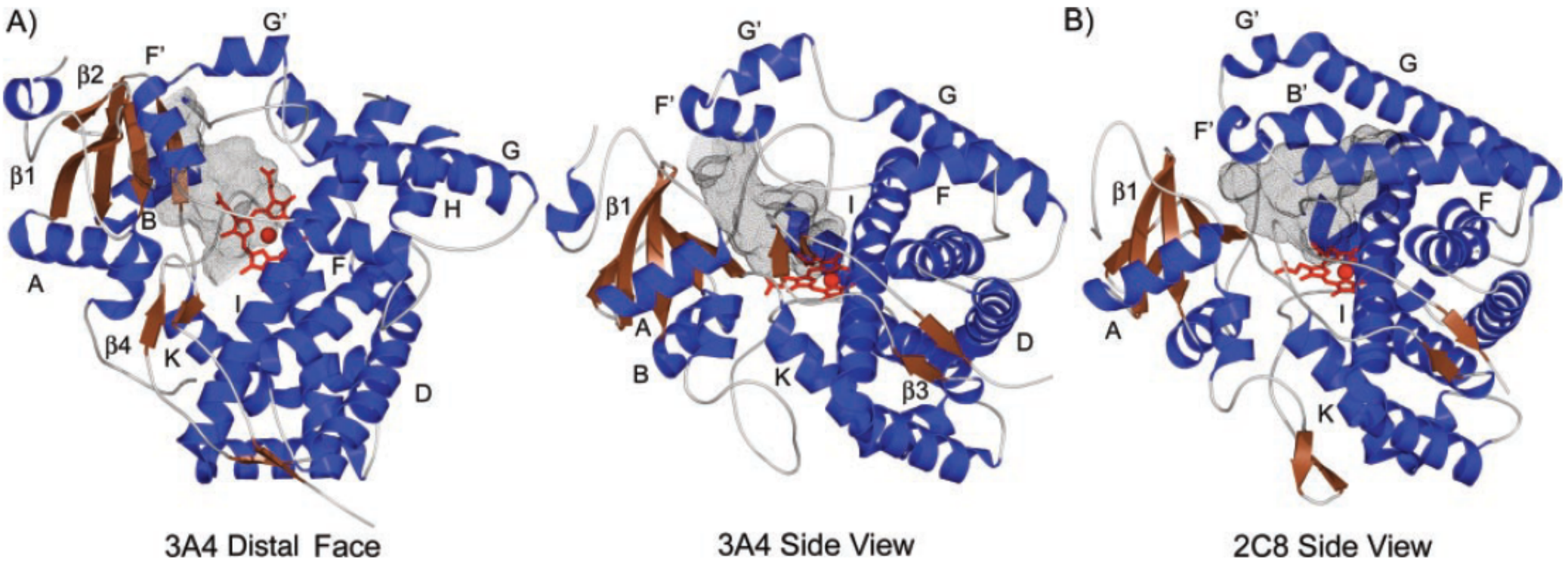
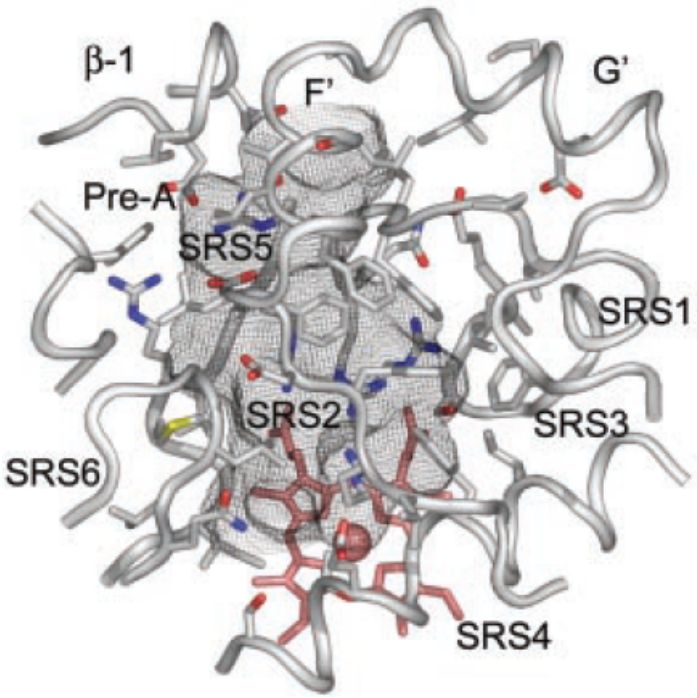


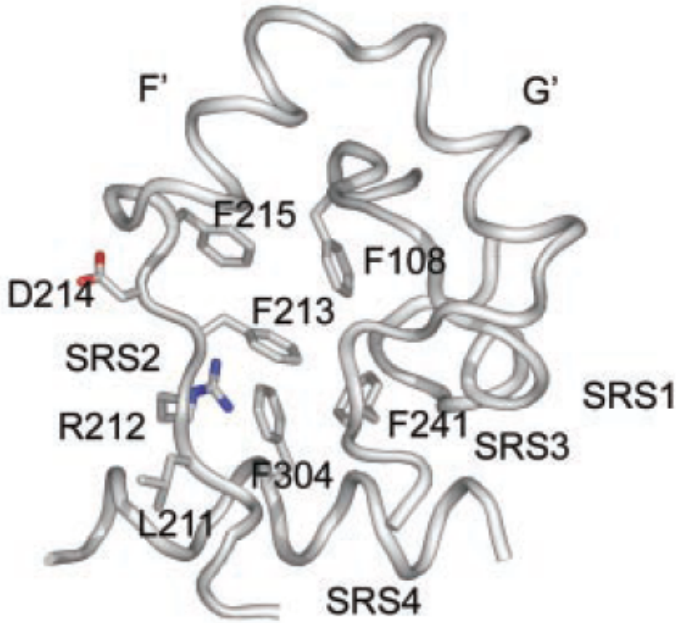
Figure 4 View of the region of the active site of CYP2C9 that remains available to accommodate additional ligand(s) after *S*-warfarin. The bound *S*-warfarin molecule is shown as in Fig. 3. **a**, A second molecule of *S*-warfarin has been modelled into the active with the site of hydroxylation closest to the haem iron. **b**, A known haem binder fluconazole has been modelled into the cavity in a similar conformation to that observed in the complex of CYP51 with fluconazole (Protein Data Bank code 1EA1).

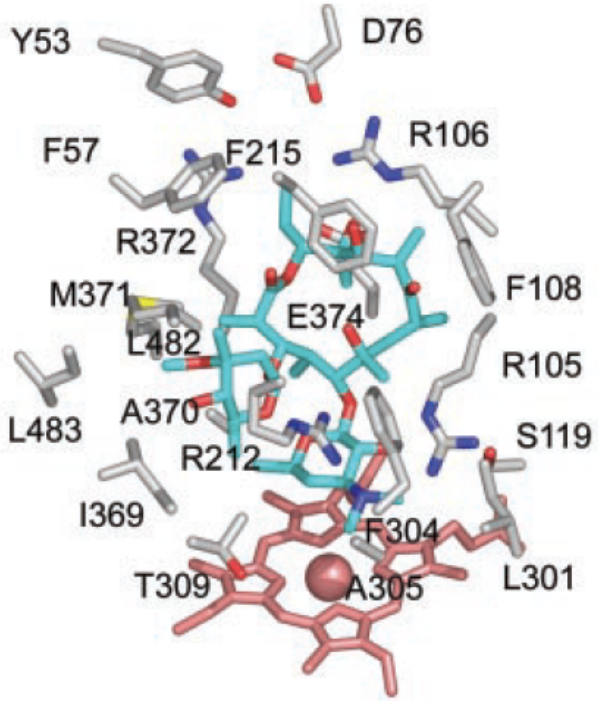
Structure of 3A4

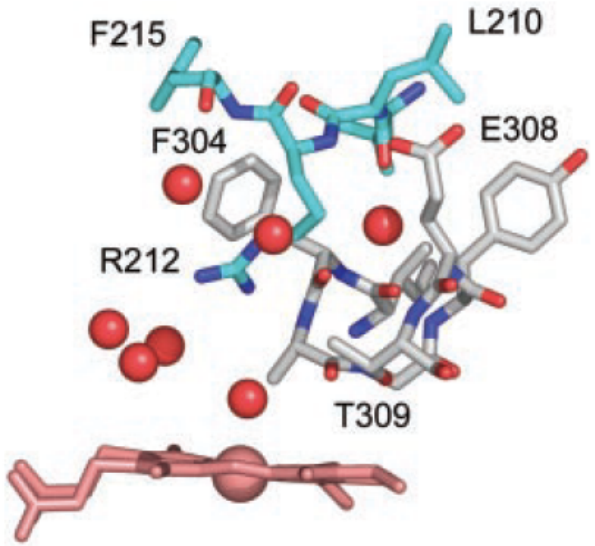


3A4 substrate recognition sites (SRS) …..



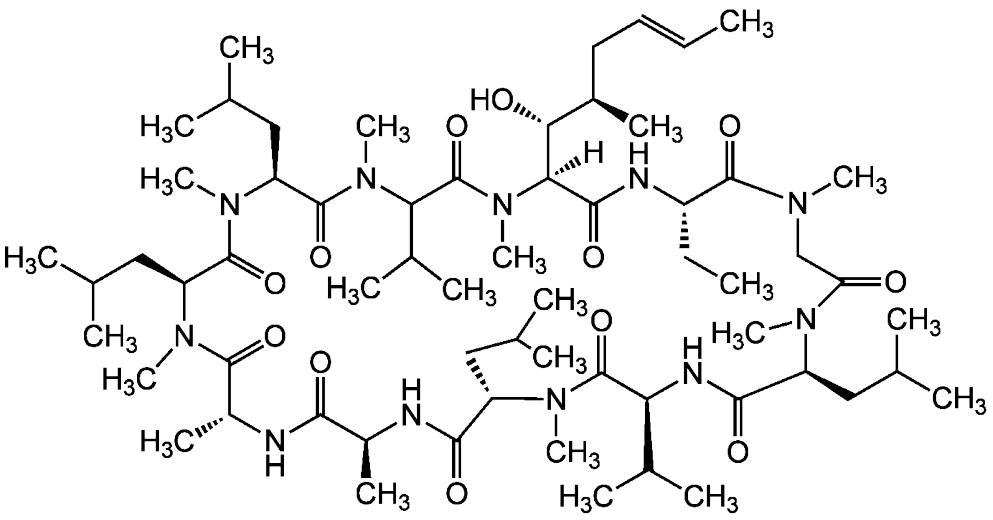




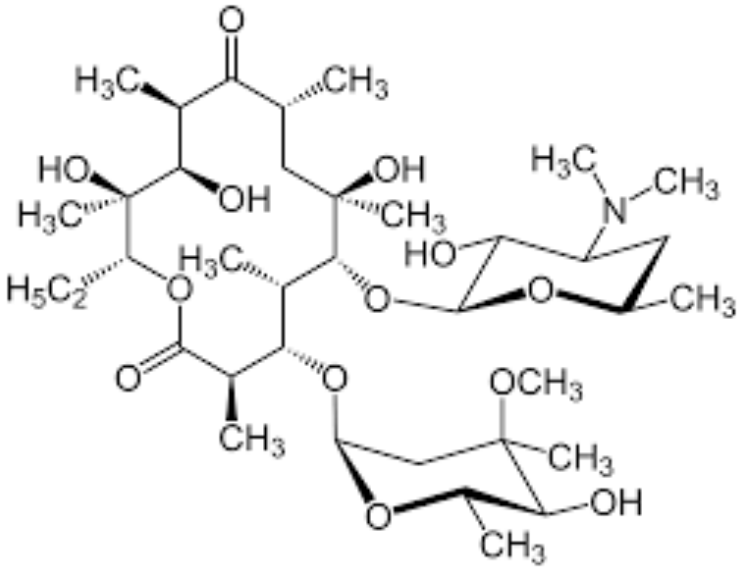


….. and active site

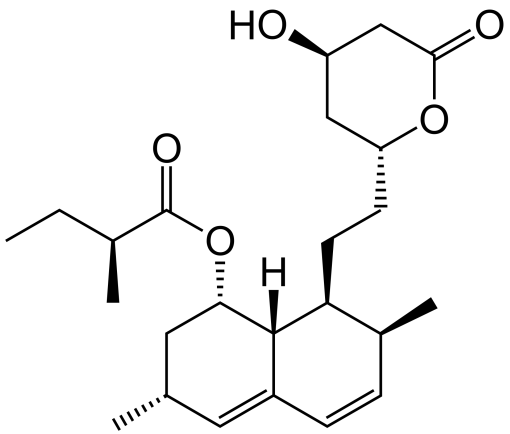
Substrates recognised by 3A4



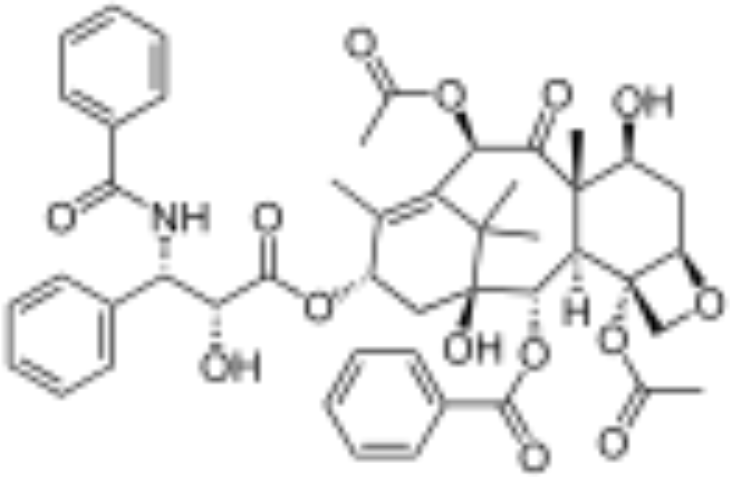
cyclosporine



erythromycin

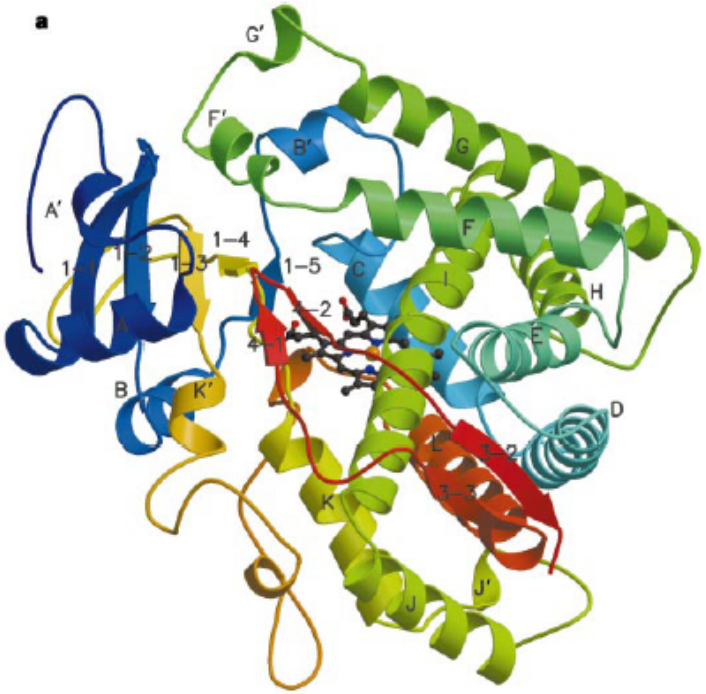


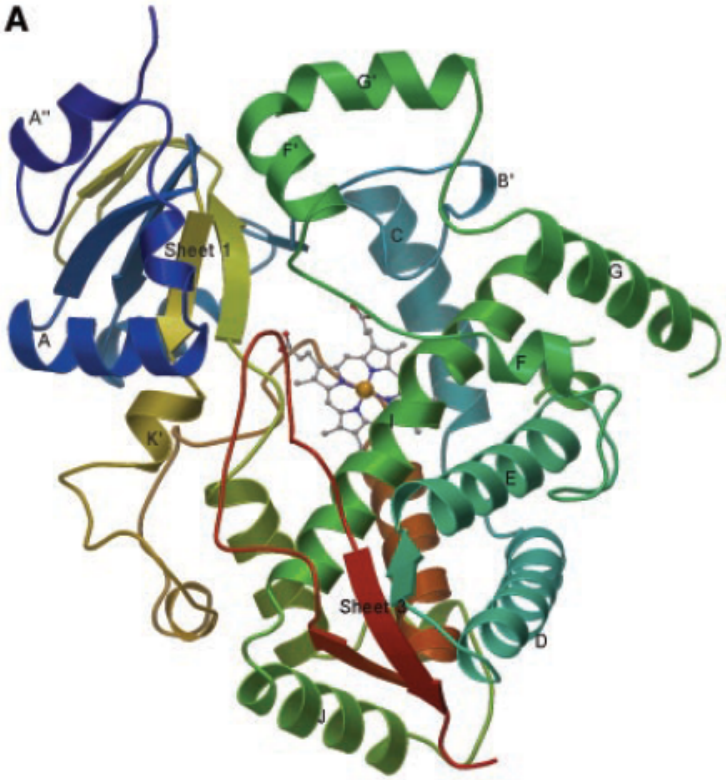
statins (lovastatin)

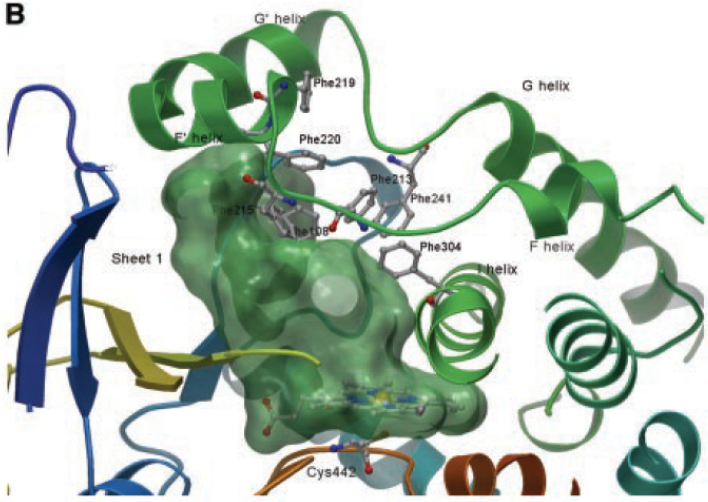


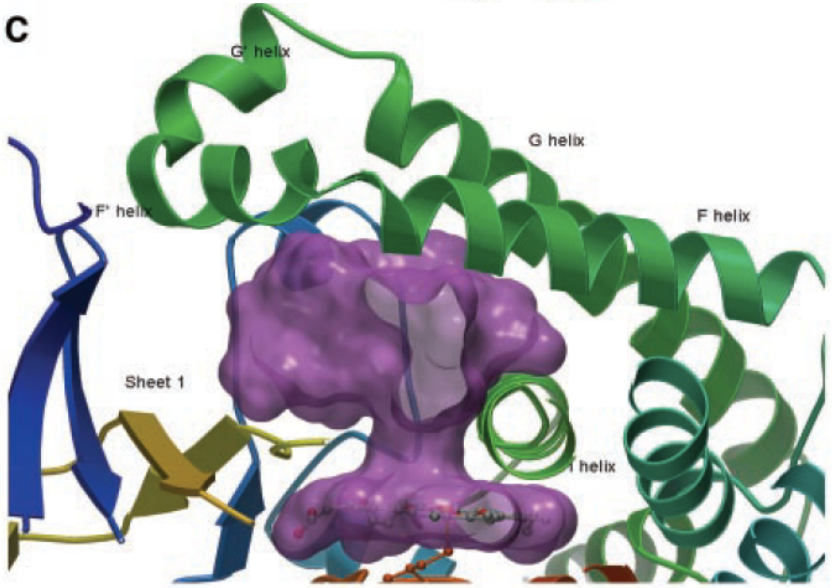
taxanes (paclitaxel)

Comparison of binding pockets





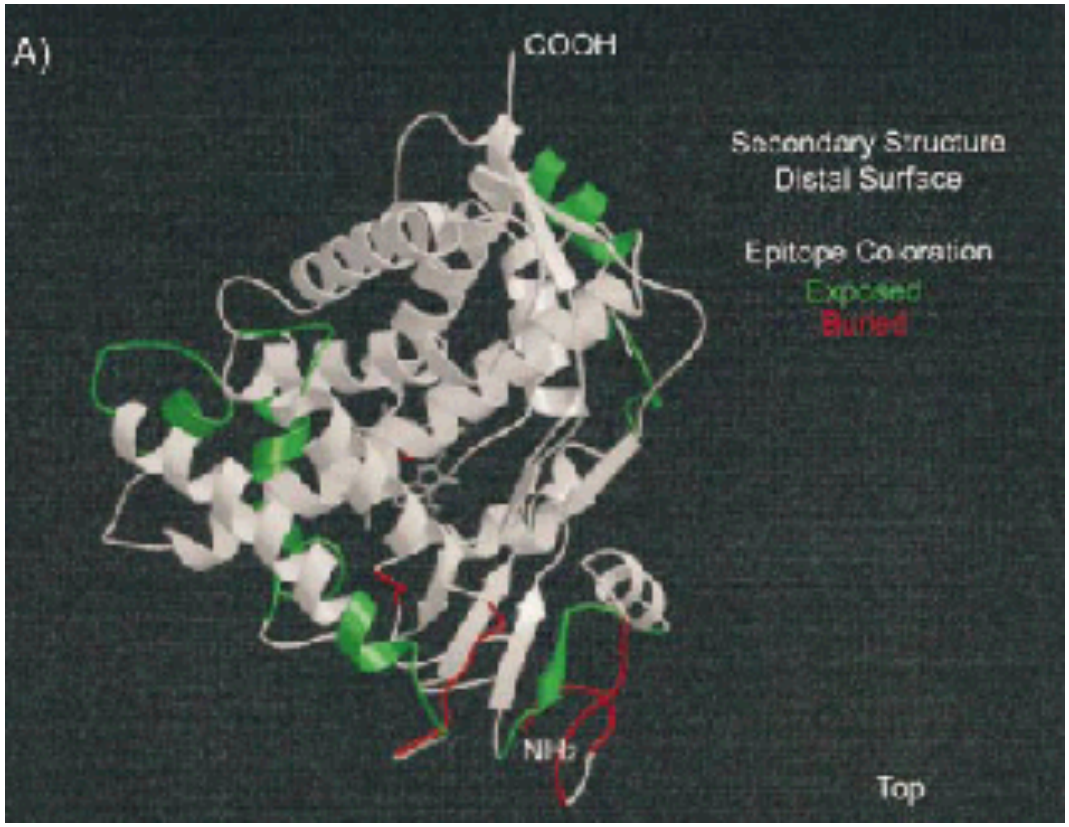


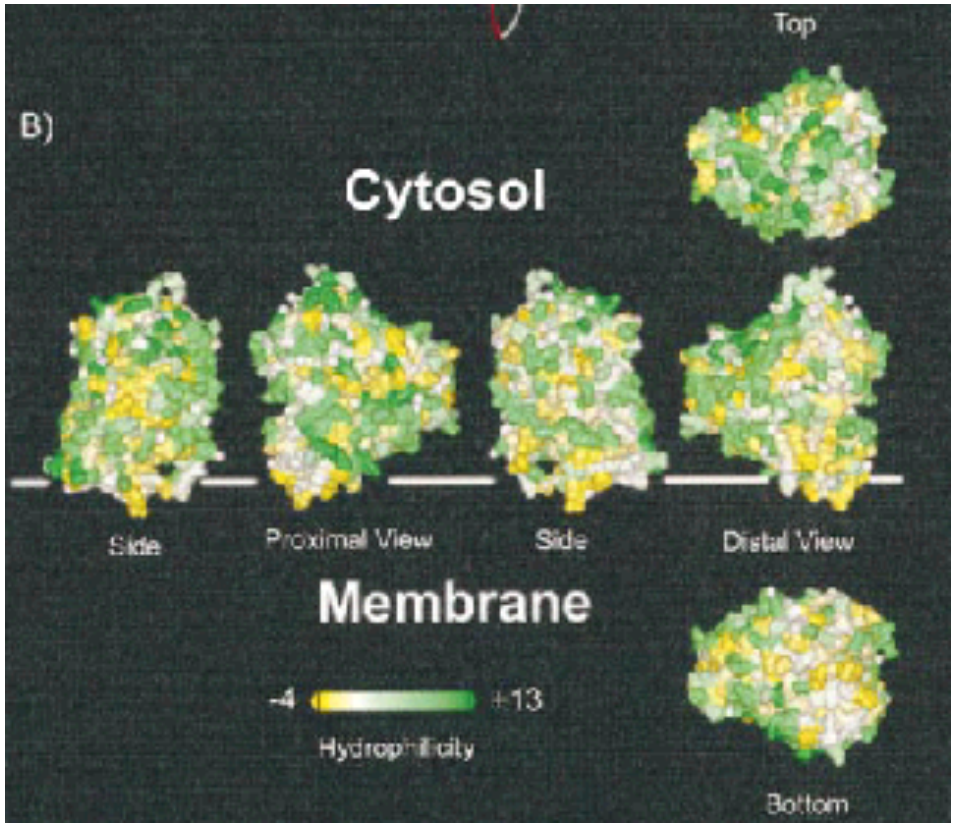


3A4

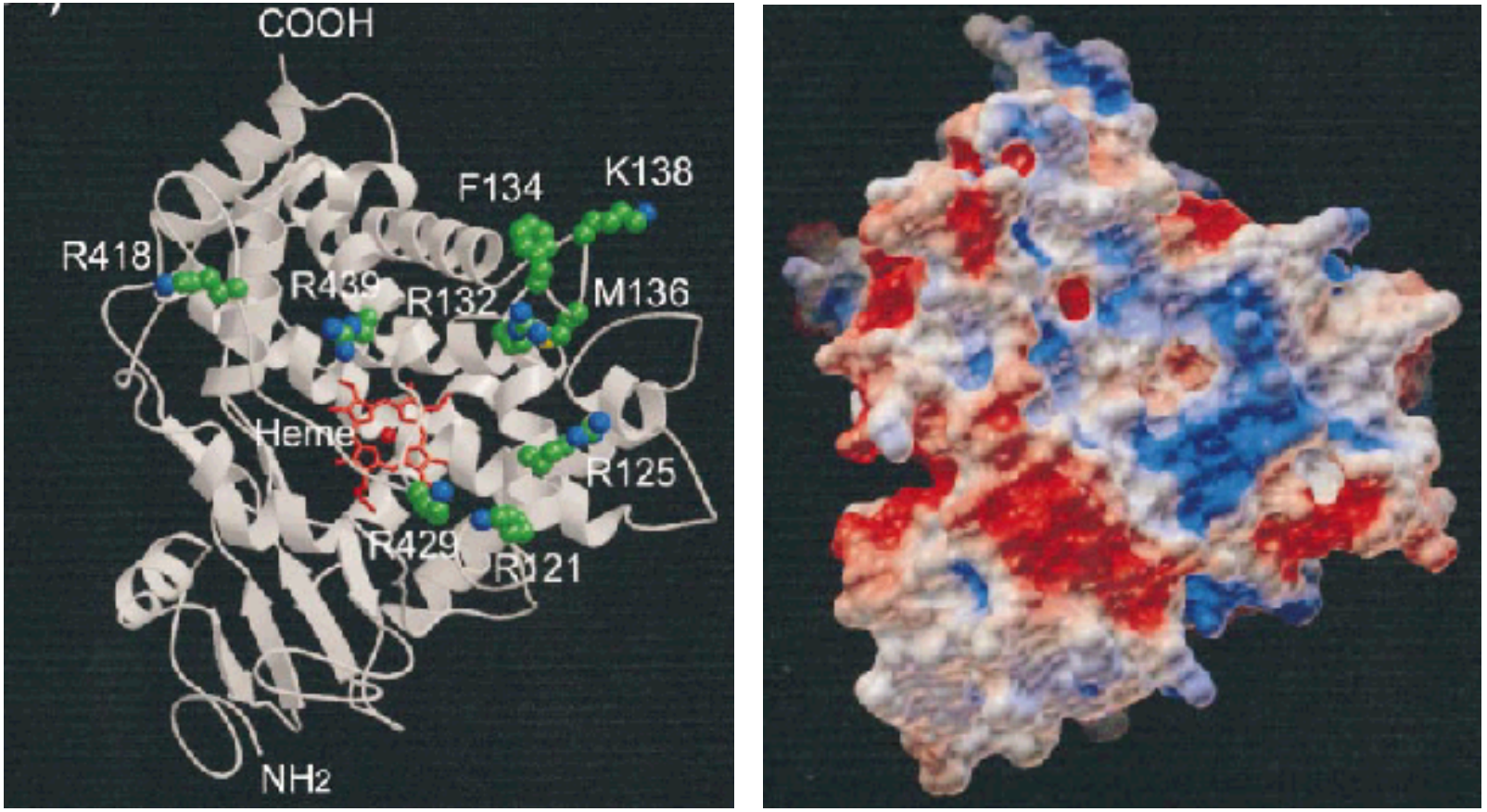
2C9

Membrane binding surface

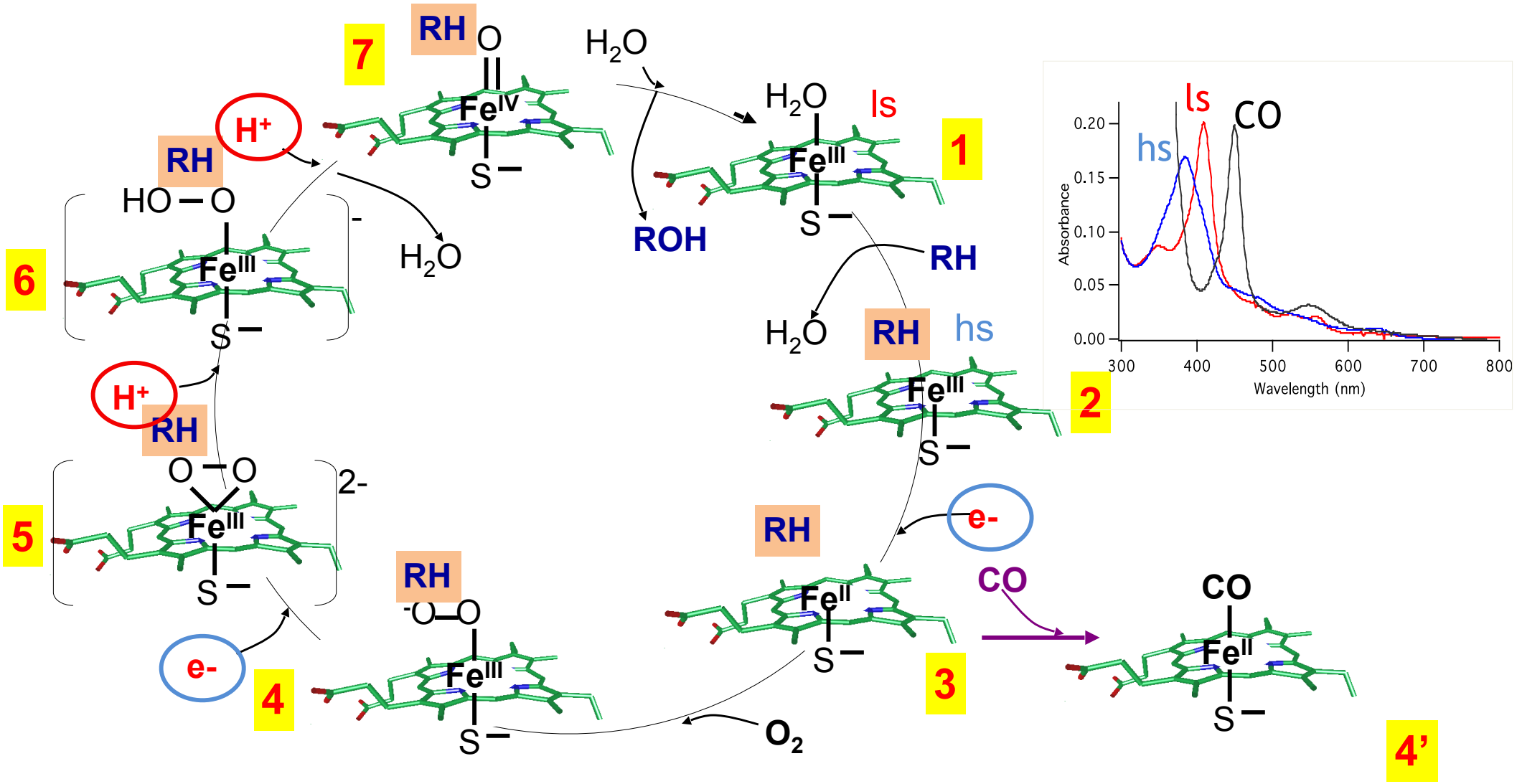




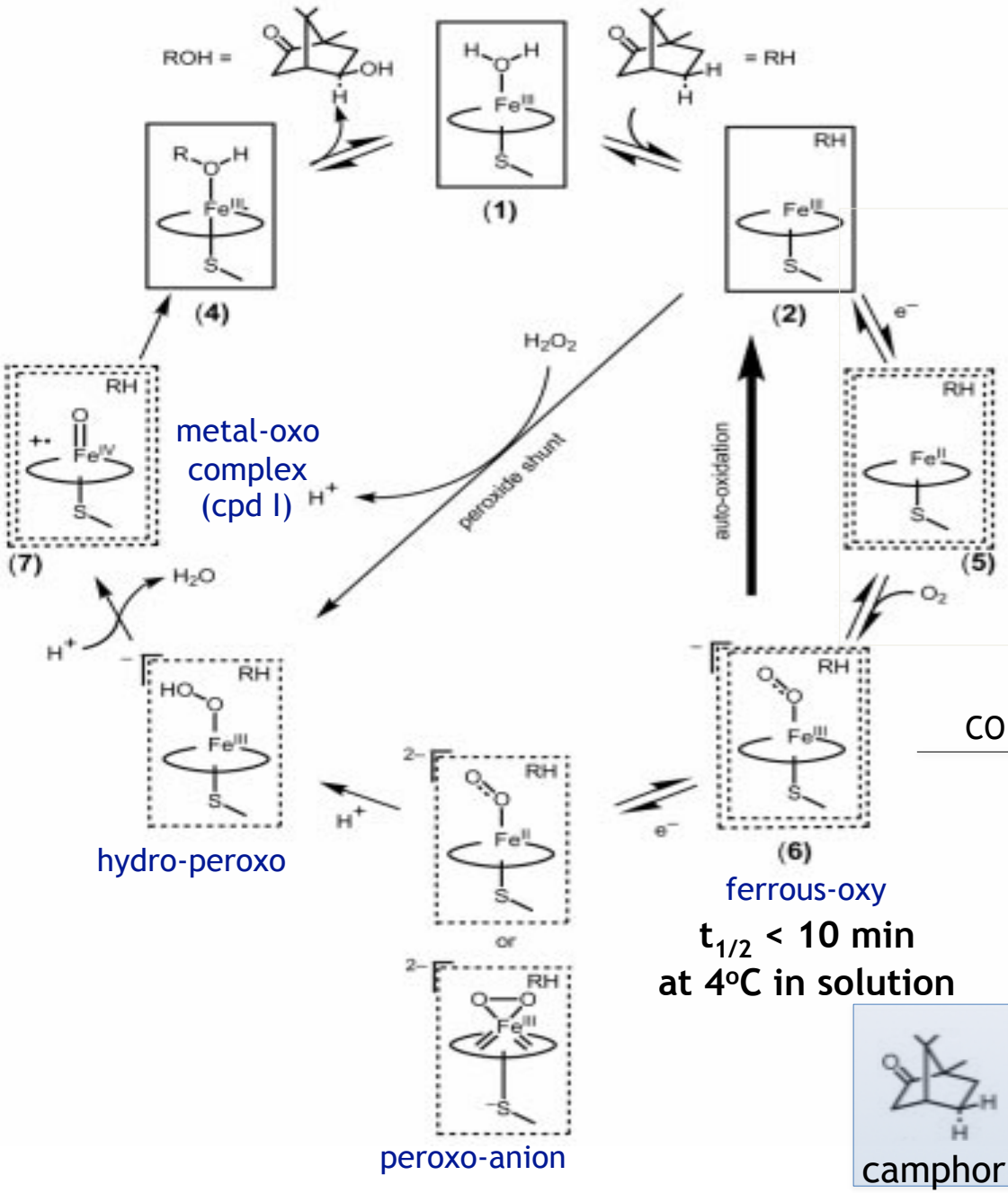
CPR interaction site



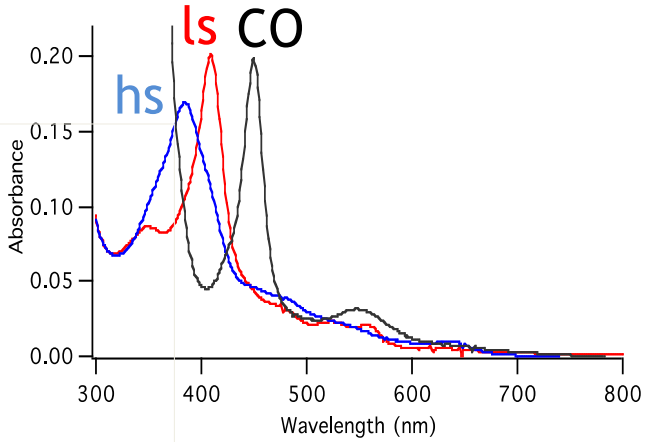
P450 catalytic cycle



What happens at the heme: the P450cam paradigm



$t_{1/2}$ < 10 min at 4°C in solution

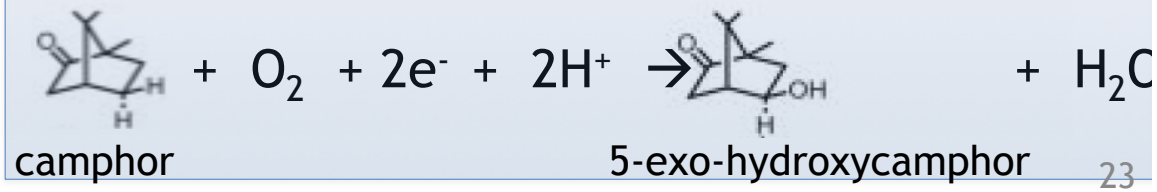


Previously determined crystal structures:

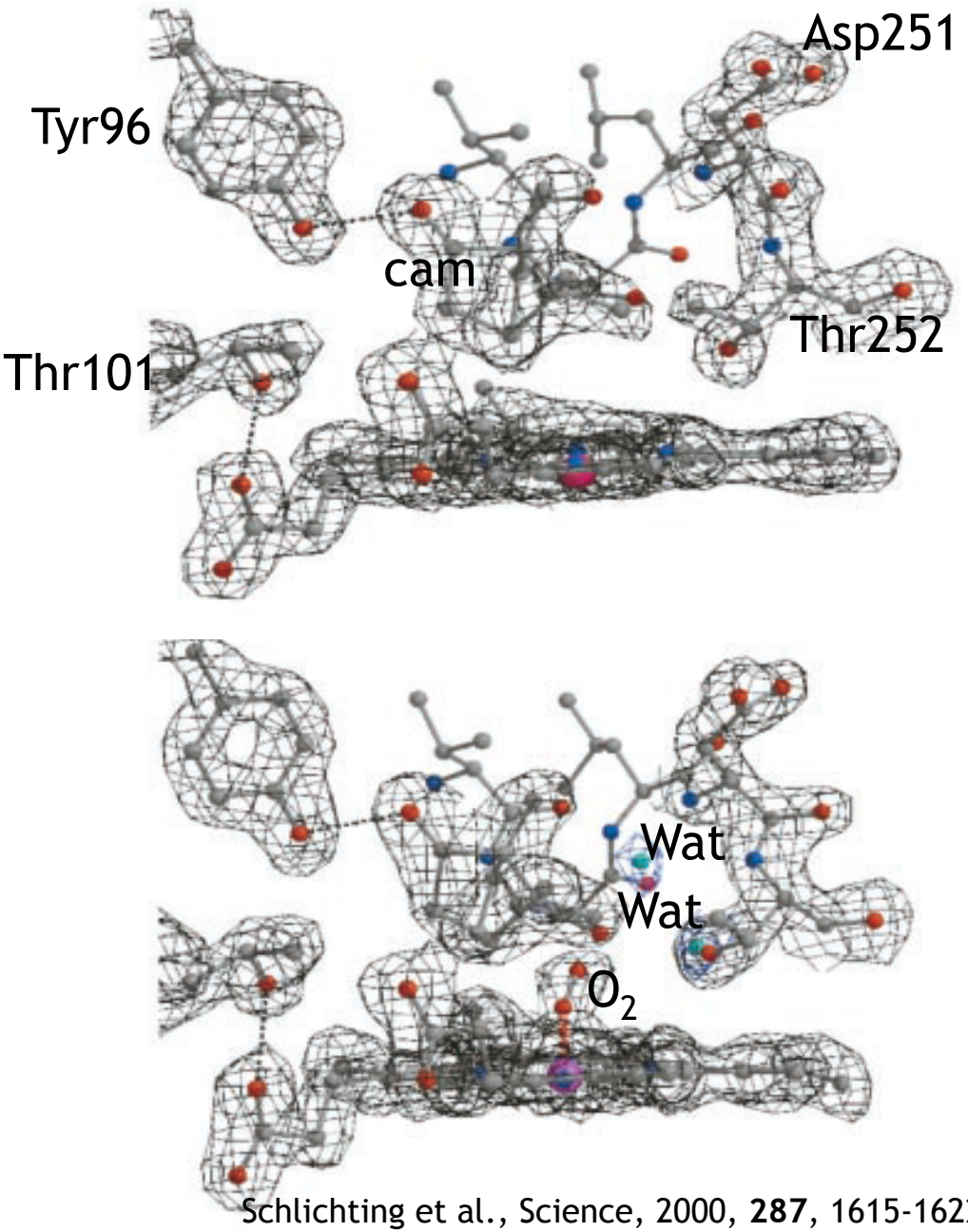
(1) P450 . aquo Fe^{III}
(2) P450 . camphor Fe^{III}
(3) P450 . camphor . CO Fe^{II}
(4) P450 . product Fe^{III}

Intermediates described in the text

(5) P450 . camphor Fe^{II}
(6) P450 . camphor . O_2^- Fe^{III}
(7) P450 . activated oxygen $Fe^{?}$



Structure of the intermediates

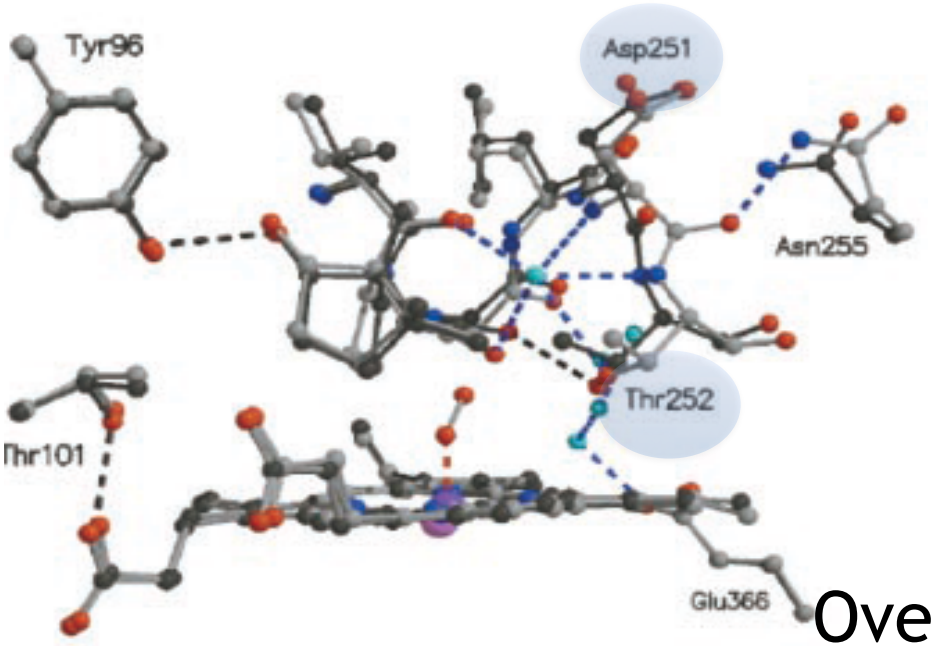


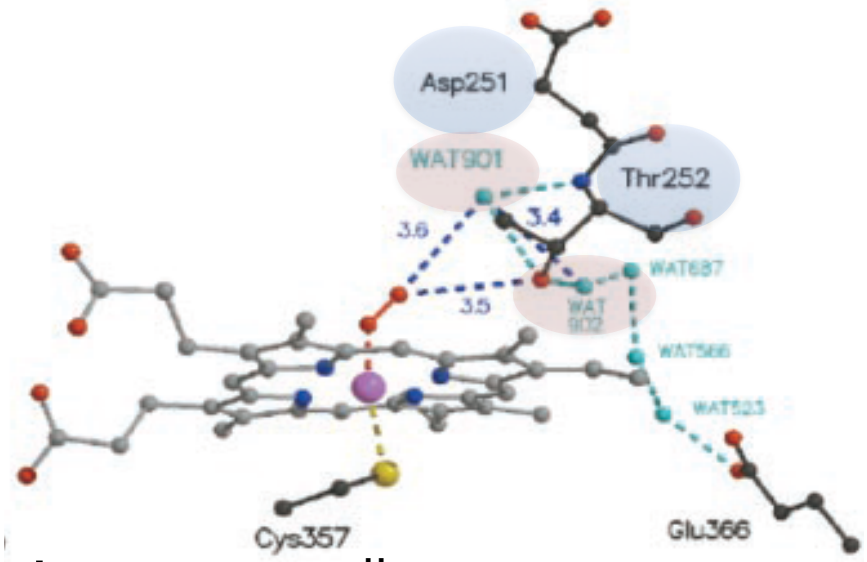
1. Crystalline enzyme
2. Initiation of the reaction in the crystal
3. Generation of intermediates accumulated to high occupancy
4. Cryogenic temperatures (88-100K)
5. Rapid data collection = series of time lapsed pictures at atomic resolution

Reduced P450cam
(Fe^{II})

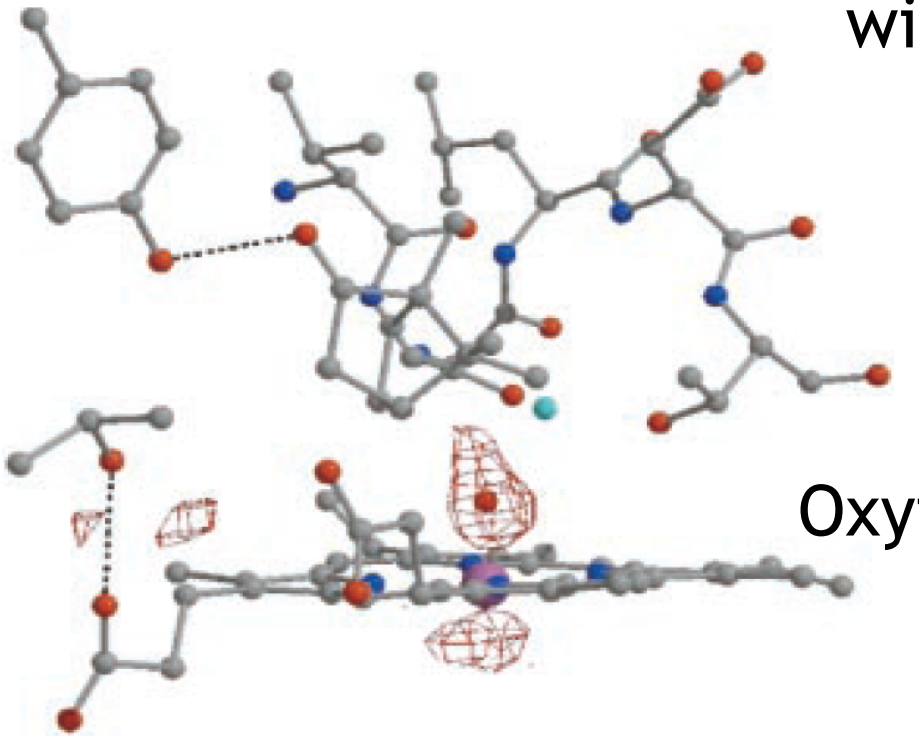
Reduced and O_2-bound P450cam
(Fe^{II}-O_2)

Schlichting et al., Science, 2000, **287**, 1615-1622





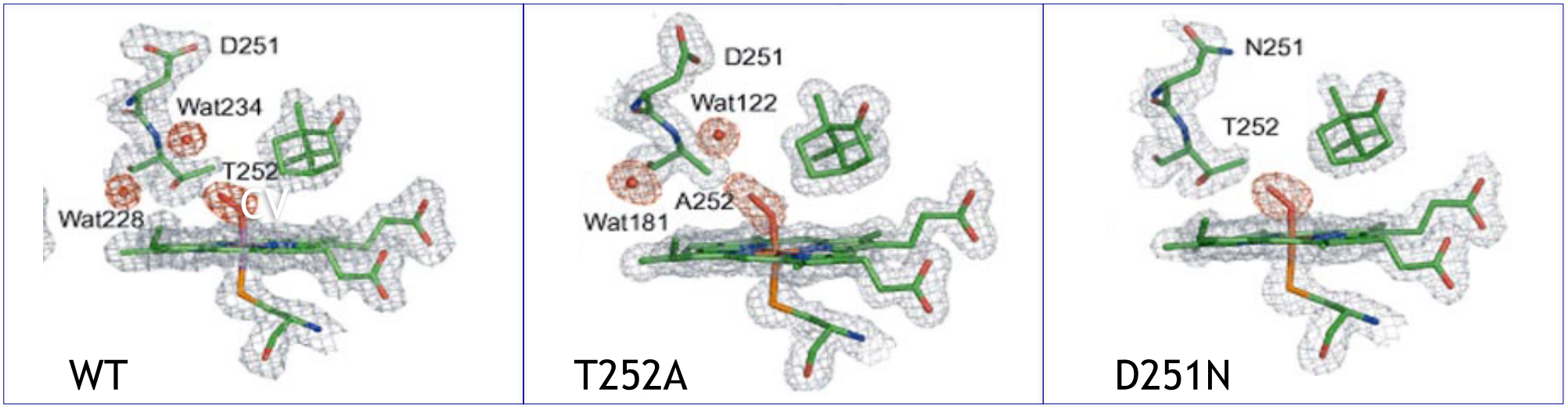
Overlap of the cam-Fe^{II} with cam-Fe^{II}-O_2



Oxyferryl P450cam (Fe^{IV}=O)

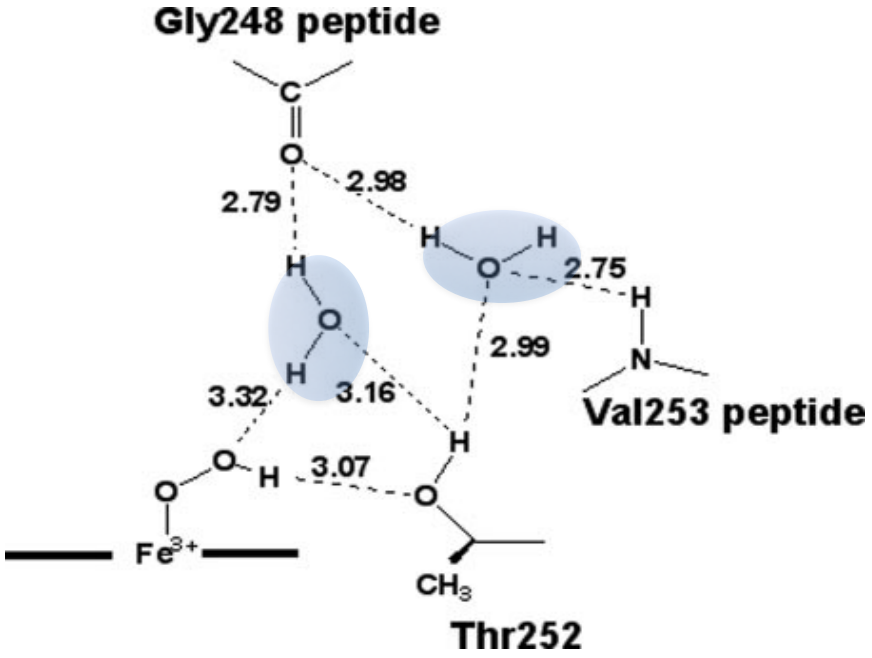
Schlichting et al., Science, 2000, **287**, 1615-1622

Concerted roles of Thr252 and Asp251



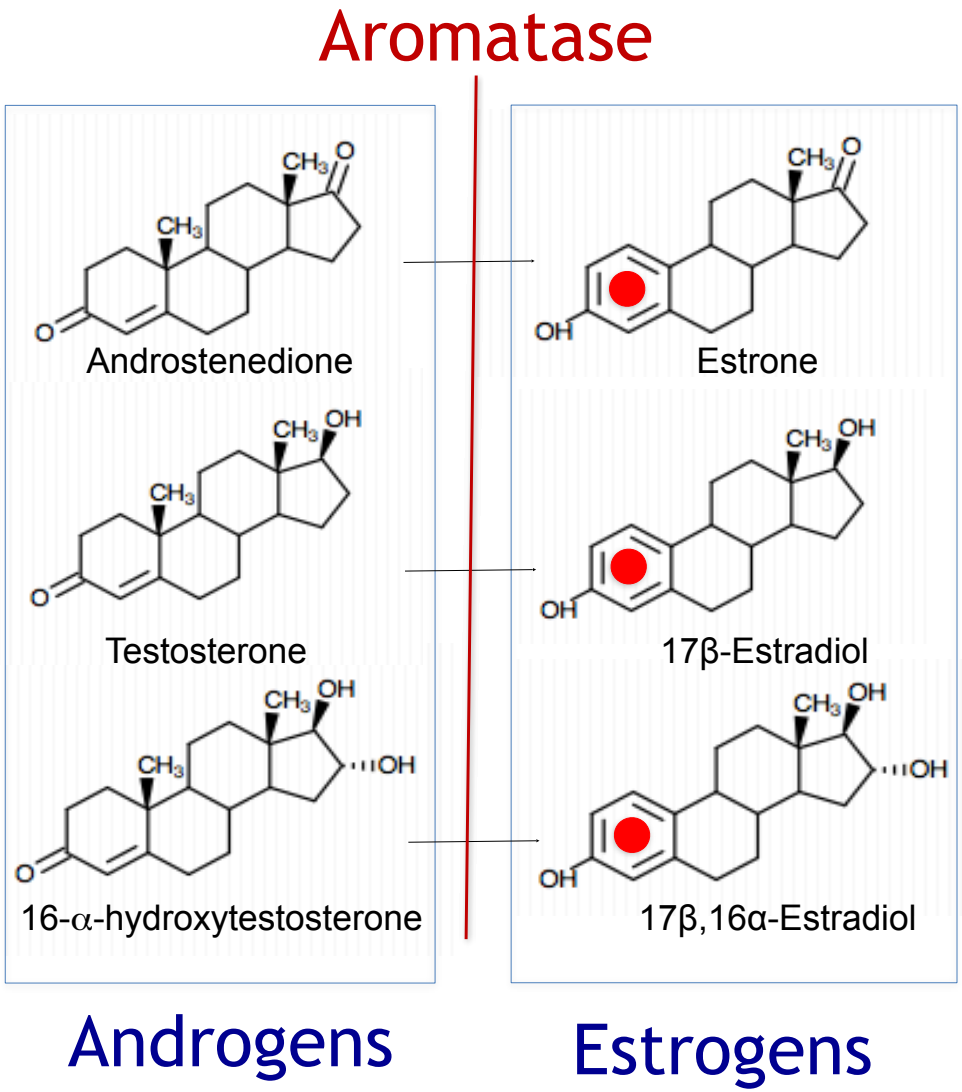
1. Thr252 accepts a H-bond from the hydroperoxy (Fe^{III}-OOH) intermediate promoting the second protonation on the distal oxygen atom, leading to the O-O bond cleavage and formation of compound I (Fe^{IV}=O)

2. Asp251 allows for a movement in the I helix upon O_2 binding that allows the insertion of the two water molecules involved in H-binding responsible for O-O bond cleavage



Nagano and Poulos, J.Biol.Chem., 2005, **280**, 31659-3166

Example of P450 involved in metabolism and drug-target: Human Aromatase (CYP19A1)



Role in brains

Sexual differentiation and dimorphism

Reproduction

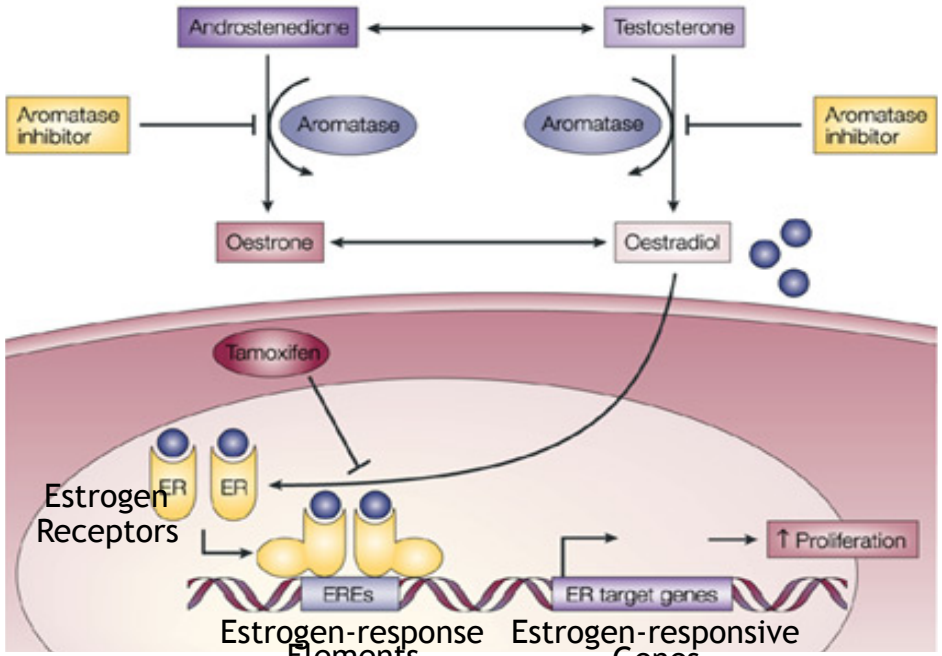
Neuroprotection

Neuroplasticity

Cell growth and migration

Modulation of mood, affective status, aggressive behavior, memory and cognitive functions.

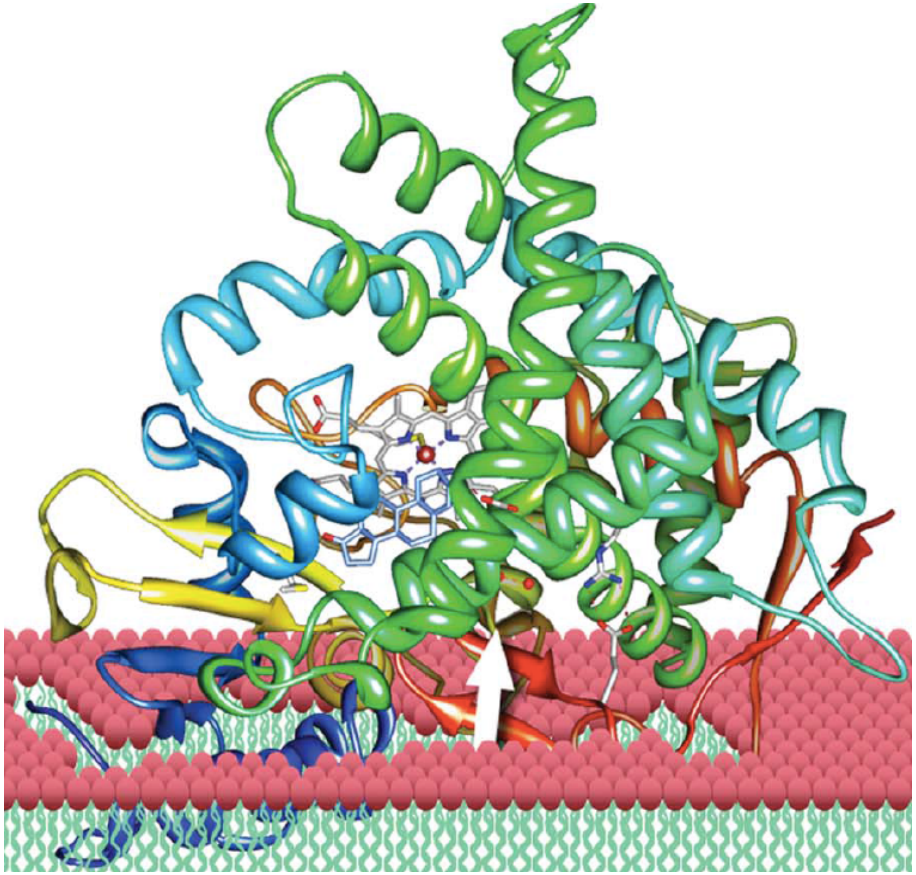
Role in breast cancer



Johnston et al., Nature, 2003

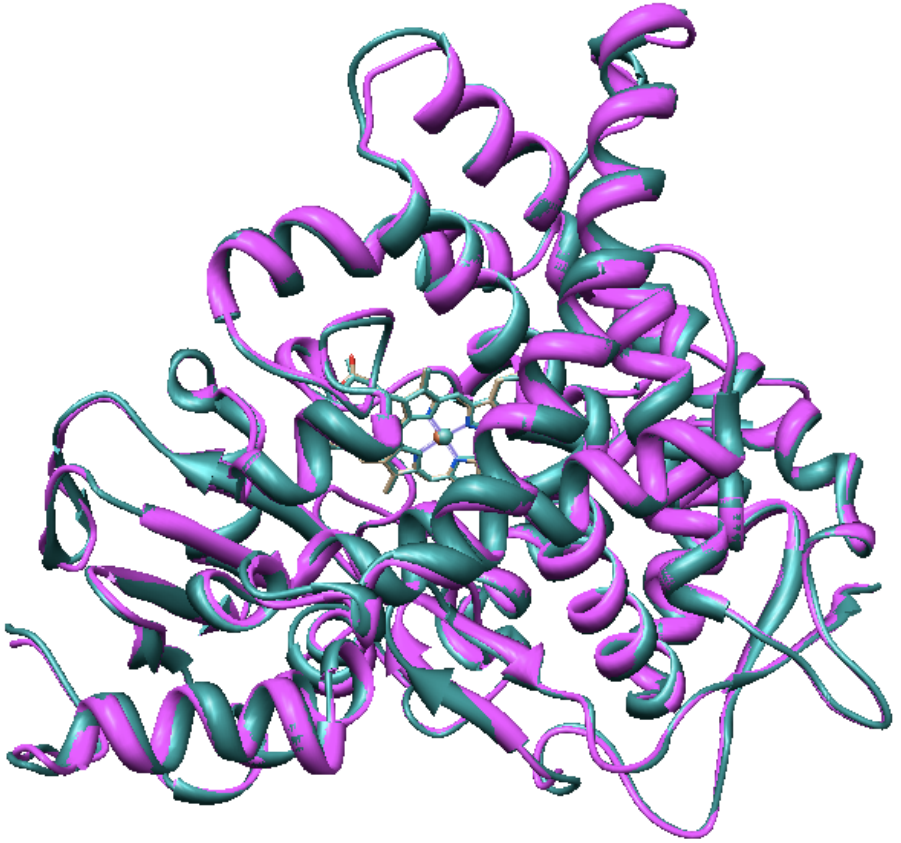
Structure

Full length (membrane-bound)



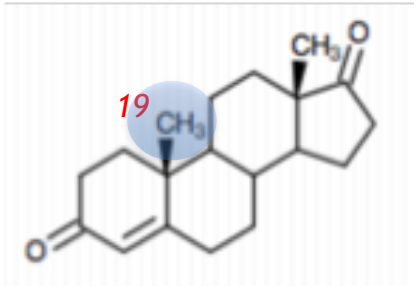
Full length Aro from human placenta:
Ghosh et al., Nature, 2009, 457, 219-224

Recombinant (soluble)



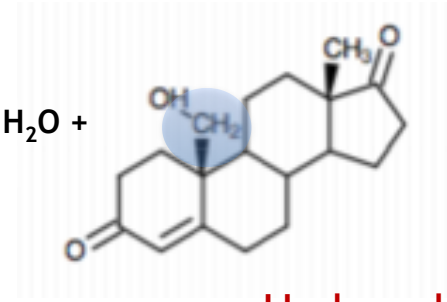
Cyan: pArom, Ghosh et al., Nature, 2009, 457, 219-224
Magenta: rArom, Lo et al., Biochemistry, 2013, 52, 5821−5829
rmsd: 0.36Å

The overall reaction requires 3 cycles:



O_2 + NADPH → NADP+ → H_2O + (1)

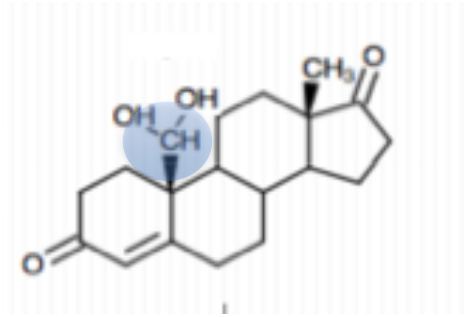
Oxidation at the 19-methyl group



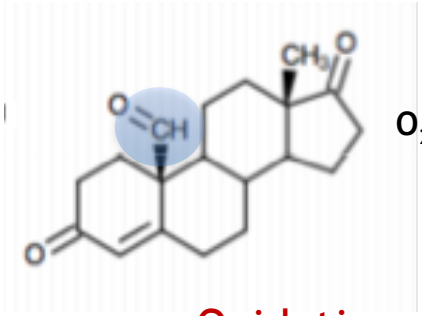
O_2 + NADPH → NADP+ → H_2O + (2)

Hydroxylation which removes the 19-*pro*-R hydrogen to yield 19-*gem*-diol

Androstenedione



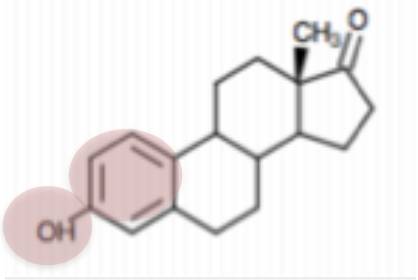
H_2O → spont.



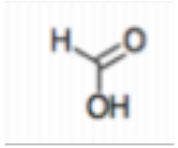
O_2 + NADPH → NADP+ → H_2O + (3)

Dehydration to the 19-aldehyde

Oxidative cleavage of C10-C19 bond to form formic acid and estrone or estradiol

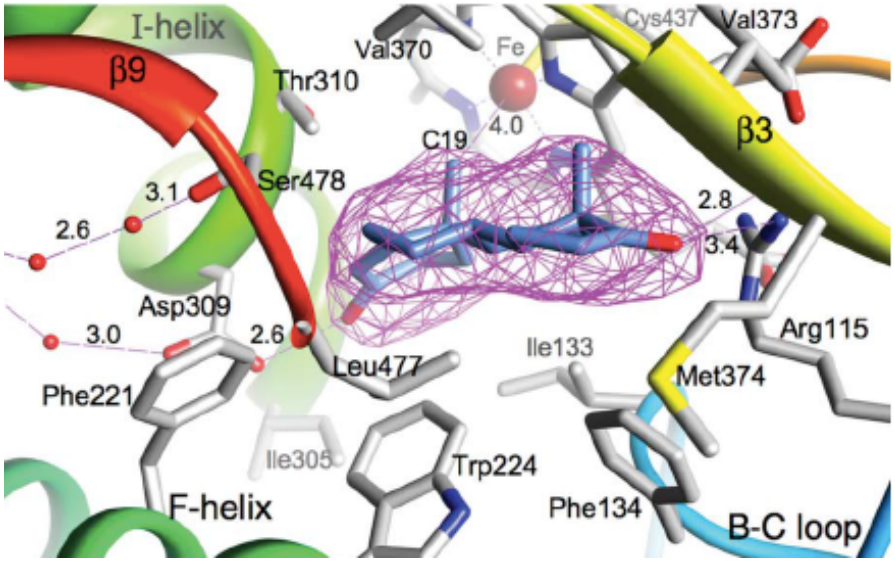


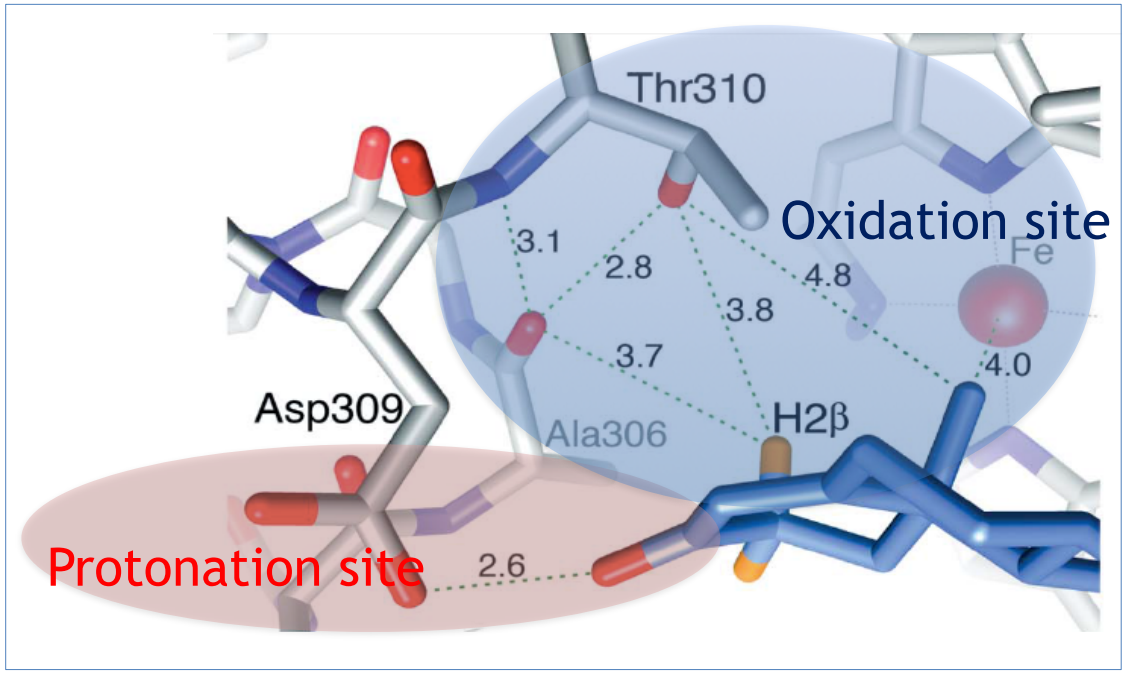
+

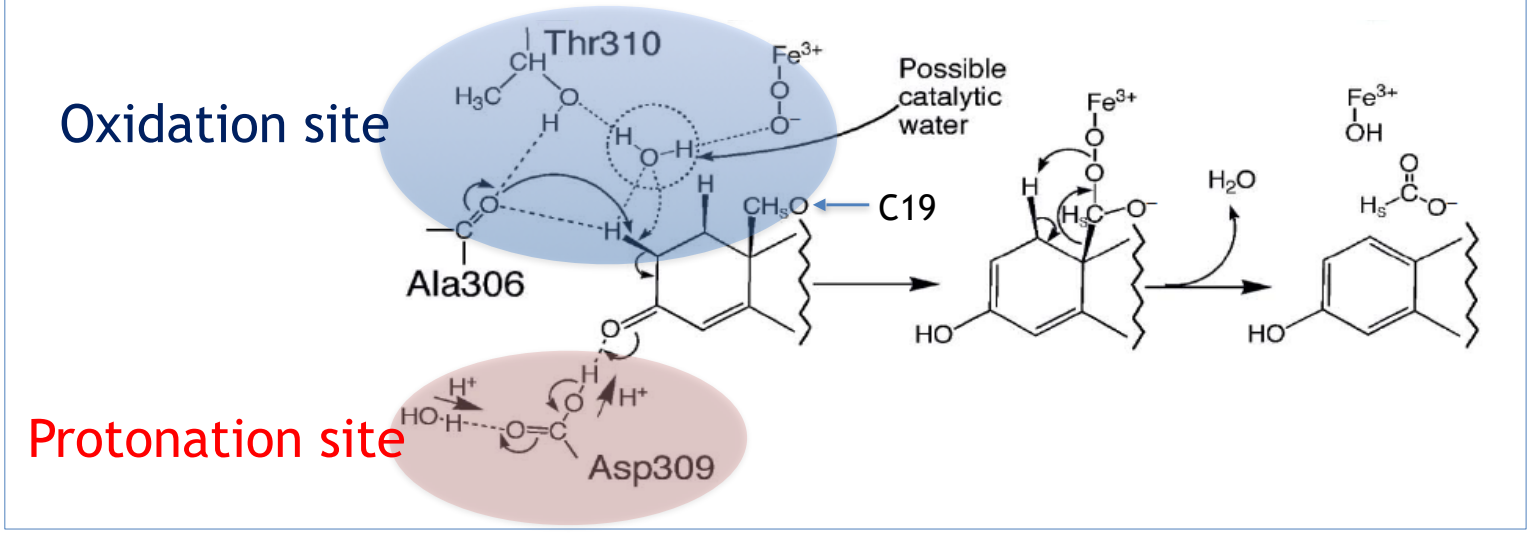


Estrone Formic acid

The binding pocket





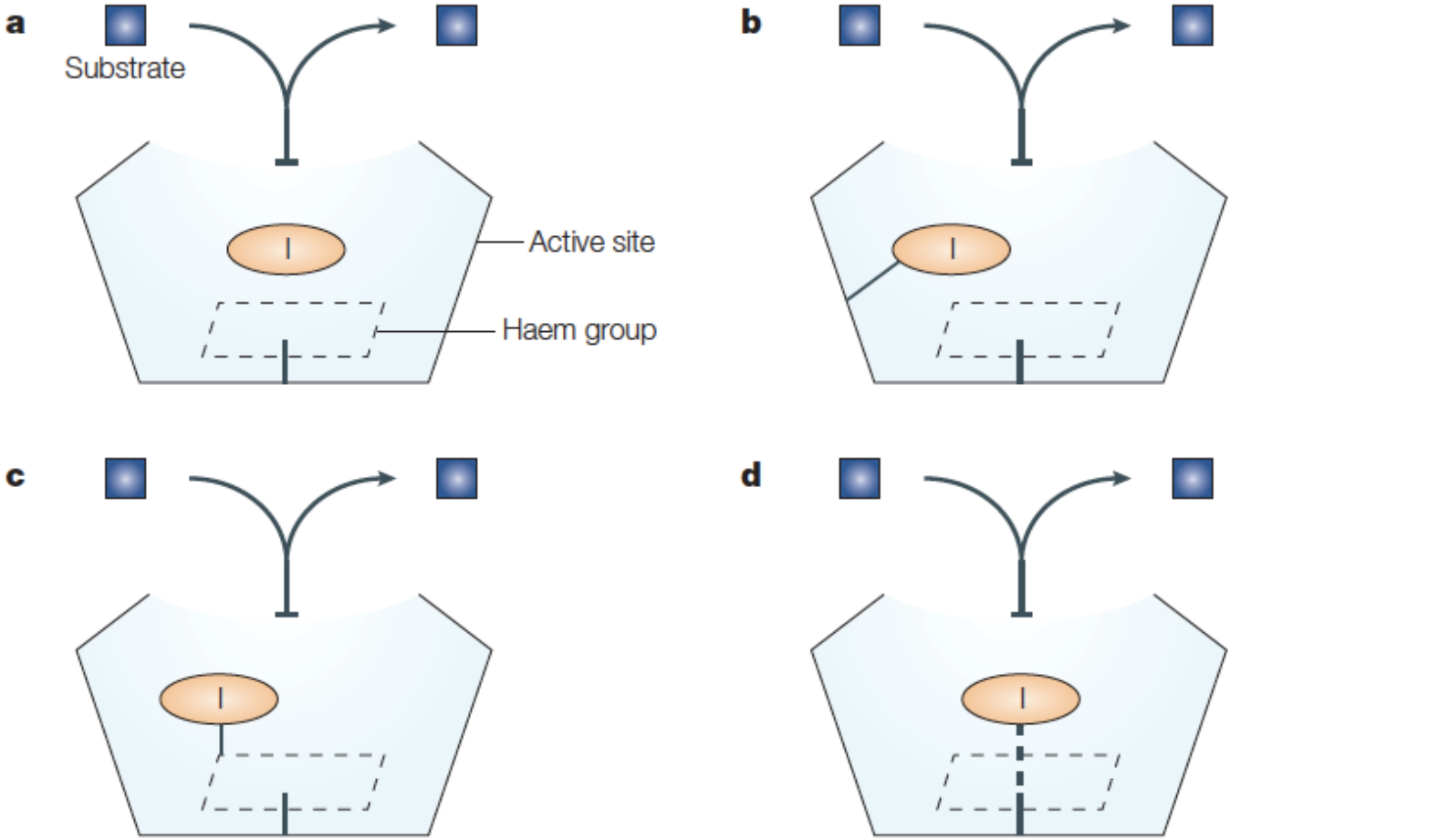


Cytochrome P450: Inhibition

Modulation of activity : inhibition

A) Reversible inhibition

B) Irreversible inhibition - 1

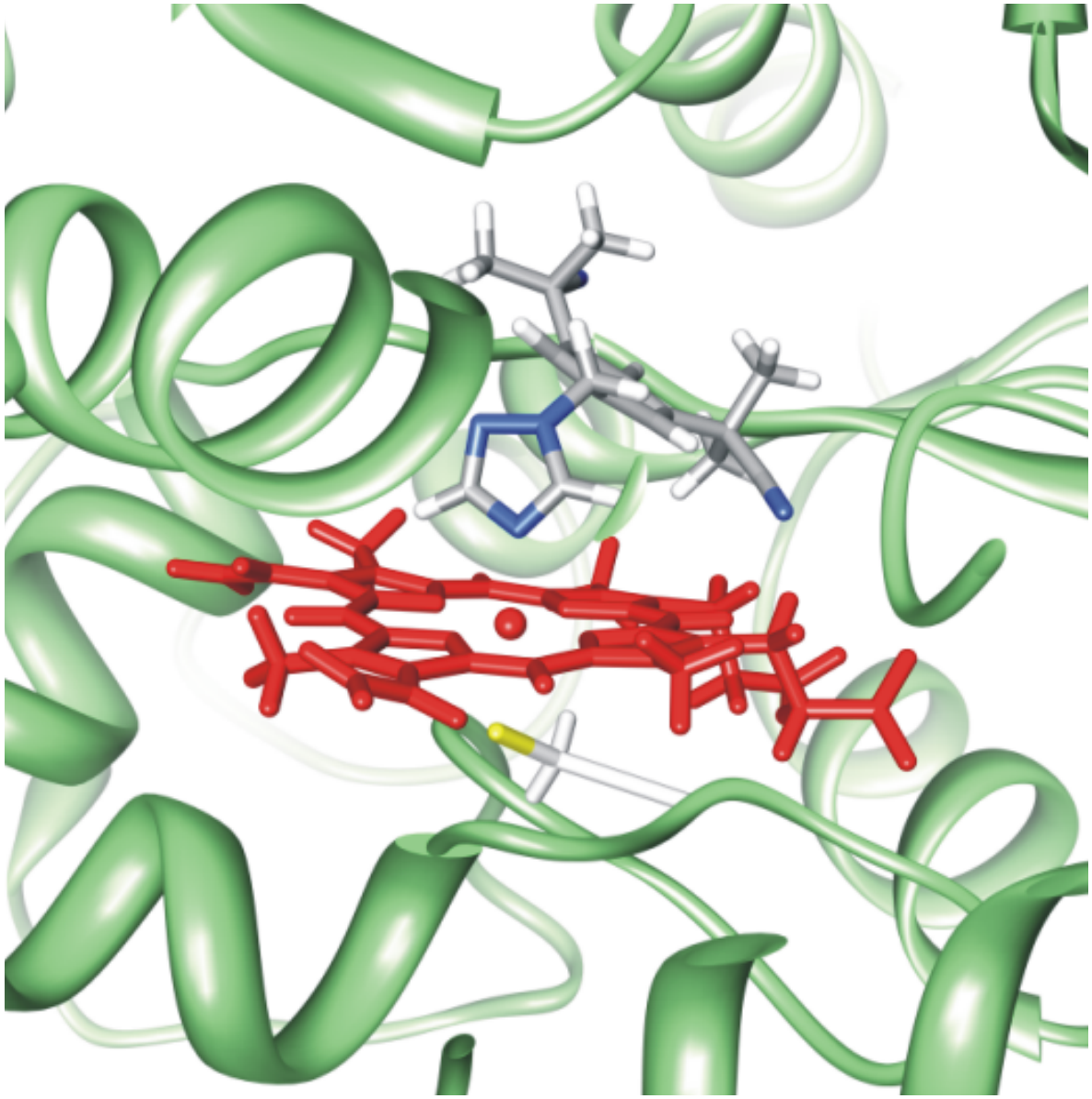


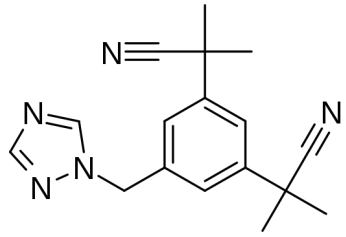
C) Irreversible inhibition - 2

C) Quasi-irreversible inhibition

Wienkers and Heath, Nature Reviews, 2005, 4, 825-833

Quasi-irreversible inhibition of aromatase

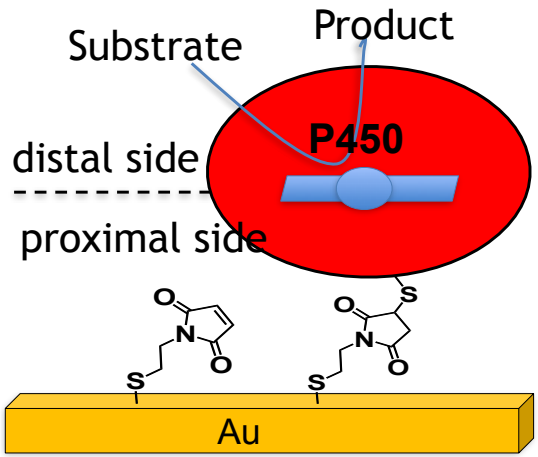




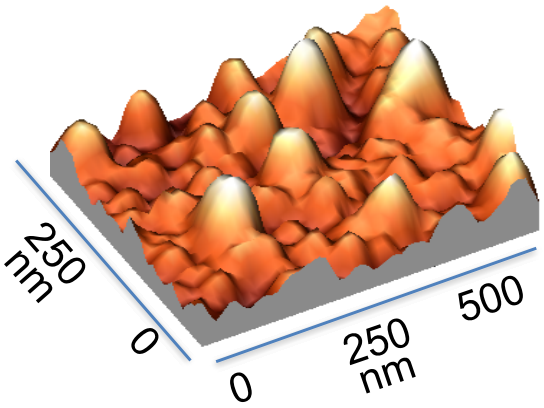
Anastrozole-bound Aro

Morelli *et al*, Chem. Commun.,2011, **47**, 10737–10739

Research in our lab: bioelectrochemistry of P450

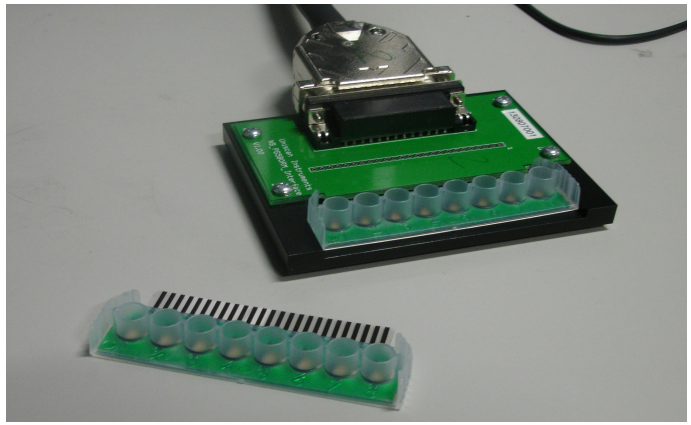


Fantuzzi et al. J. Am. Chem. Soc. (2004), 126 (16), 5040 – 5041

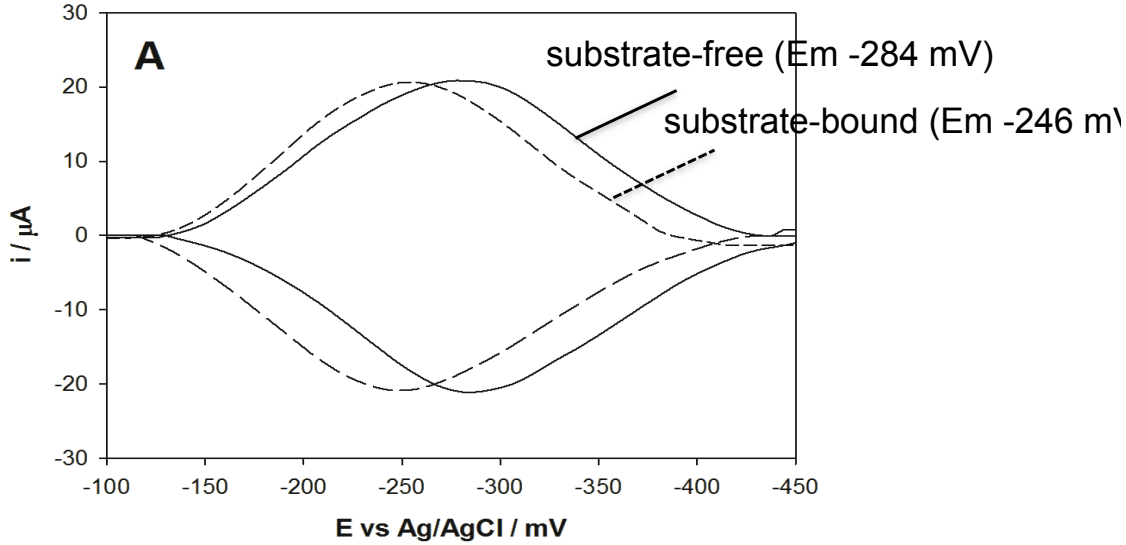


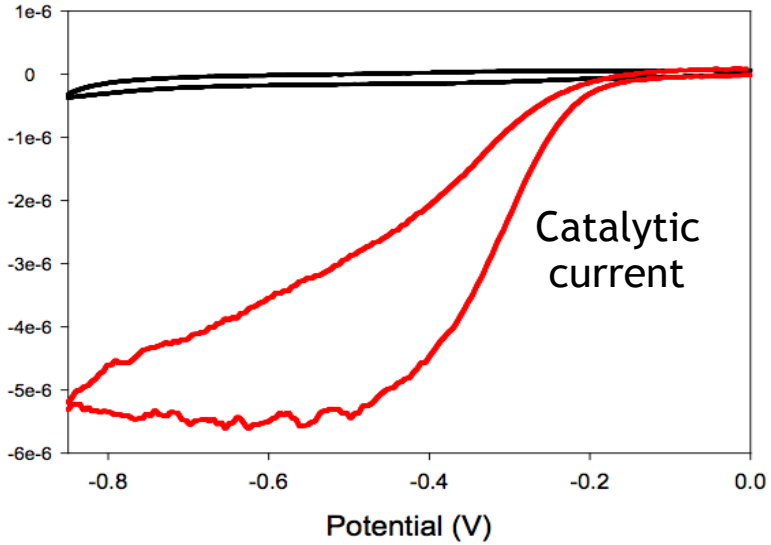
Ferrero et al. Anal. Chem. (2008), 80, 8438-8446

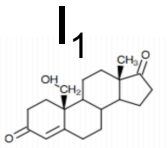
Electrode array

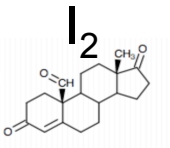


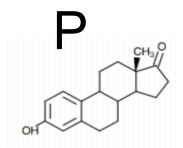
Fantuzzi et al. Anal. Chem. (2011), 83, 3831-3839

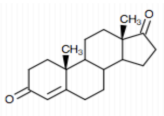




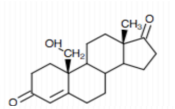


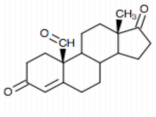


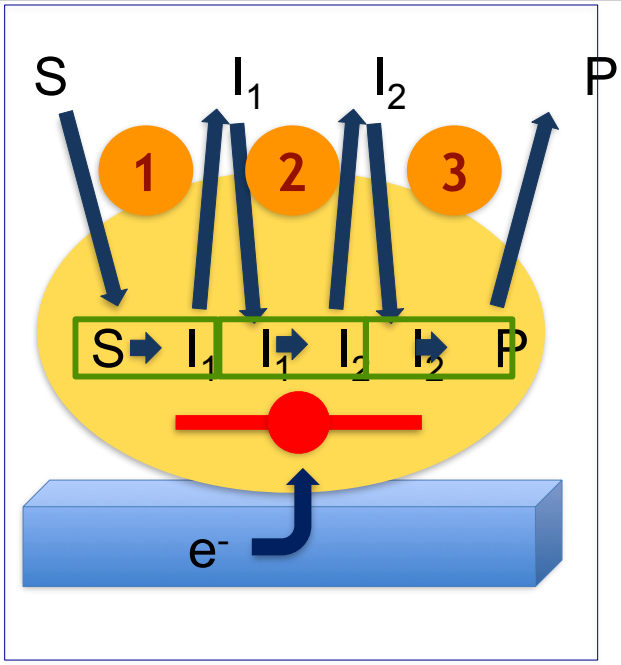



S

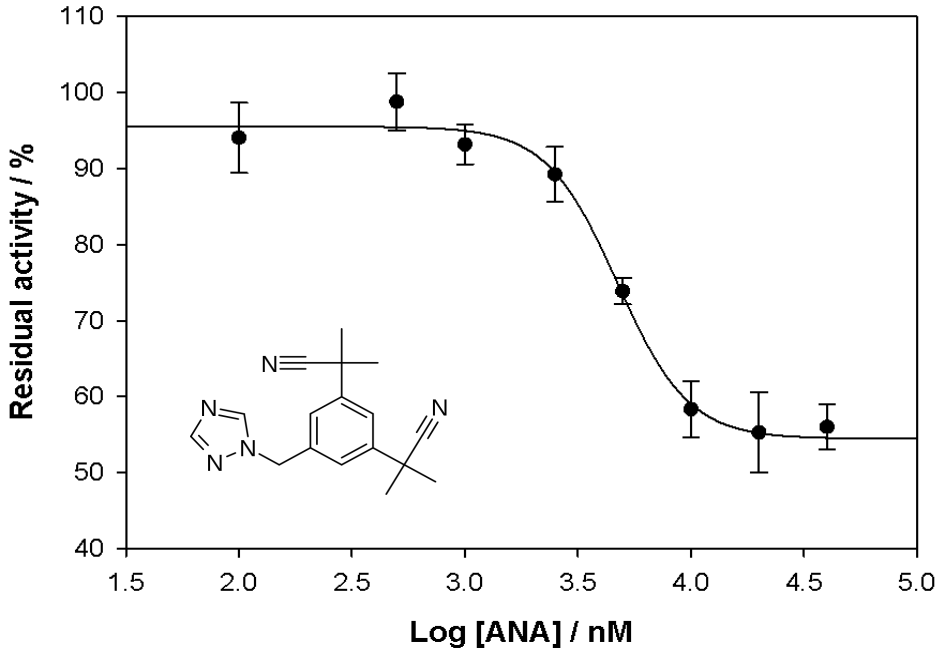
Starting substrate	Concentration (µM)	19-hydroxy (µM)	19-oxo (µM)	Estrone (µM)
Androstenedione	10	0.15 ± 0.05	Not detectable	Not detectable
Androstenedione	1	Below the quantification limits	0.11 ± 0.03	Not detectable
19-hydroxy	150	-	0.31 ± 0.01	0.81 ± 0.08
19-oxo	150	-	-	1.69 ± 0.17


I_1


I_2



Distributive behaviour of catalysis



Inhibition by anastrozole

Di Nardo *et al.* Electrochem. Comm. 2015, **52**, 25-28

Cytochrome P450: drug-drug interactions

Drug-drug interactions

- Early predictions are important:
  - (1) TERFENADINE: H_1-histamine antagonist
  - (2) KETOCONAZOLE: Antifungal agent

(1) good substrate of CYP3A4, metabolised at normal rate;

(2) potent inhibitor of CYP3A4;

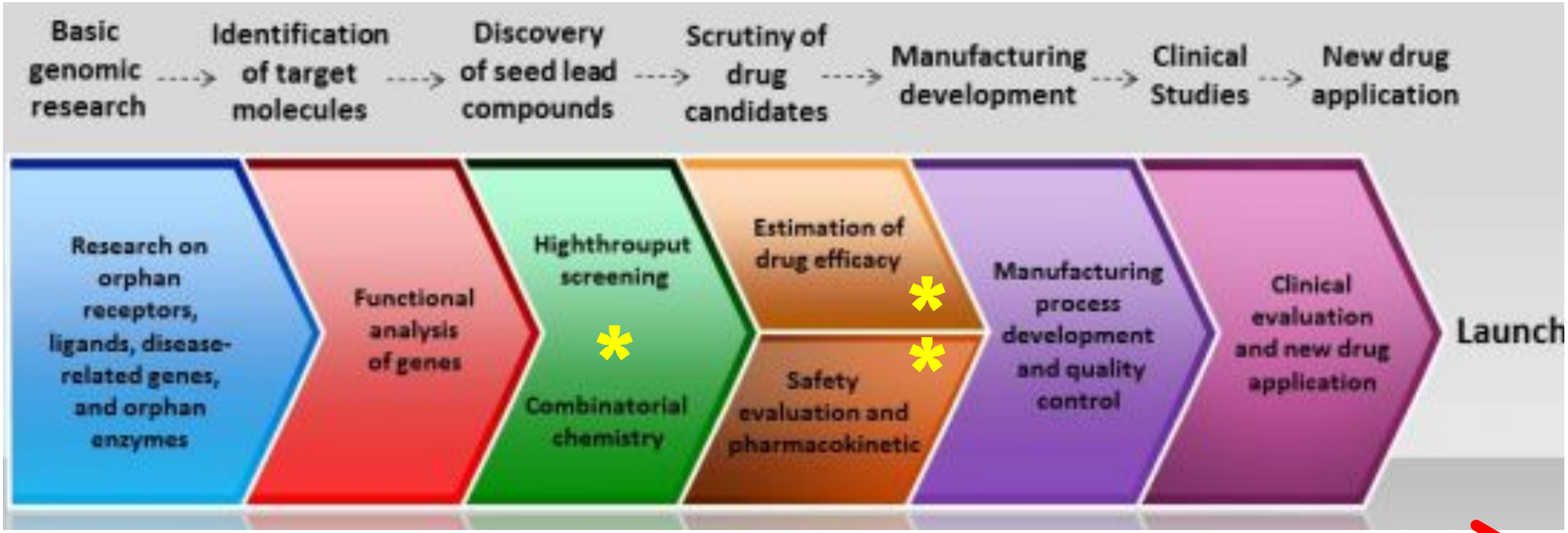
(1) + (2), terfenadine remains at high levels in plasma, leading to LETHAL VENTRICULAR ARRHYTHMIAS.

P450 related withdrawals

Drug (Company)	Use	P450 involved	Effect of interaction
Terfenadine (Hoechst Marion Roussel)	Antihistamine	CYP3A4	Fatal arrhythmias
Cisapride (Janssen)	Heartburn	CYP3A4	Fatal arrhythmias
Cerivastatin (Bayer)	Lipid lowering drug	CYP2C8	Rhabdomyolysis (muscle breakdown)
Astemizole (Janssen)	Antihistamine	CYP3A4	Fatal arrhythmias
Mibefradil (Roche)	Hypertension and angina	CYP3A4	Fatal arrhythmias
Perhexiline	Angina	CYP2D6	Nerve toxicity
Nefazodone (Bristol-Myers Squibb)	Antidepressant	CYP3A4	Liver toxicity
Grepafloxacin (Glaxo)	Antibiotic	CYP3A4	Fatal arrhythmias

Role of human cytochromes P450 in drug development

P450s responsible for 95% of drug metabolism
25-35% of Drug failures are due to P450s
50% of ADR's due to P450s
Role of P450 testing in Drug Discovery



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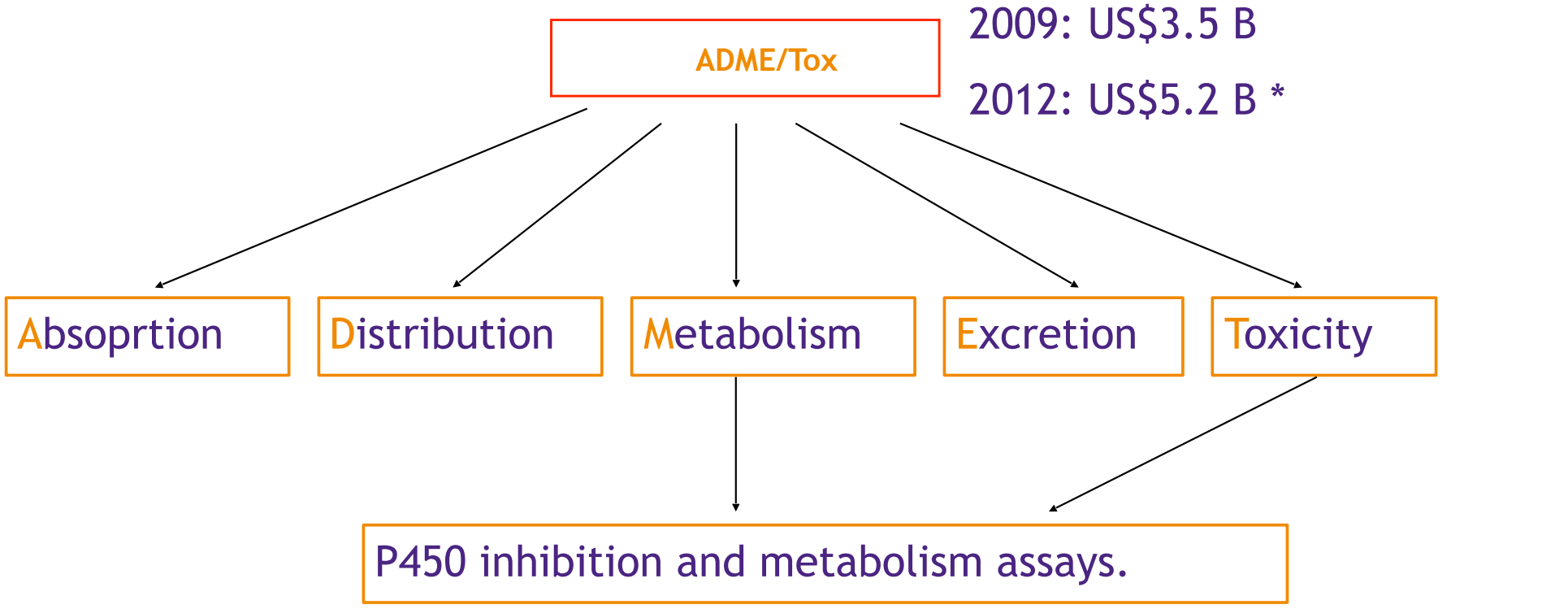
The ADME/Tox and *in vitro* P450 Markets



* Predictive ADME/Tox Strategies.

Dr N. Patel, Business Insights, 2006.

In vitro methods for metabolic studies on NCE

- Human liver microsomes:
  - Source rare
  - Contains mixtures of DMEs (P450s, FMOs, UGTs) - requires the use of mixtures of isoform specific substrates/inhibitors
- Human hepatocytes and liver slices
  - Closely mimics *in vivo* situation
  - Must be fresh; long term storage is a problem
- Recombinant-engineered DMEs:
  - *E.coli:*
    - N-ter modifications. 1991 Henry Barnes, bovine CYP17A, changed first 7 codons - silent mutations - increased AT richness (codons 4 and 5) and minimised potential mRNA IIary structure (codons 6 and 7).
    - Best vector pCWori with tandem lac promoter plus unusual spacing between the ribosome binding site and the initial codon (3 bases instead of 8-12).
    - Some different K_m have been observed

How drug metabolising P450s are studied

Enzyme	Chrom. loc.	Polymor.	Inducible	Marker activity	Inhibitor
CYP1A2	15q22	YES	YES	Ethoxyresorufin-O-deethylation Phenacetin-O-deethylation	Fluvoxamine Furafylline
CYP2A6	19q13	YES	YES	Coumarin-7-hydroxylation	
CYP2C9	10q24	YES	YES	Diclofenac-4'- hydroxylation S-Warfarin-7- hydroxylation	Sulfaphenazole
CYP2C19	10q24	YES	YES	S-Mephenytoin-4'- hydroxylation R-Omeprazole-5- hydroxylation	
CYP2D6	22q13	YES	NO	Debrisoquine-4- hydroxylation Bufuralol-1'- hydroxylation Dextromethorphan-N-demethylation	Quinidine
CYP2E1	10q24	(YES)	YES	Chlorzoxazone-6- hydroxylation	4-Methylpyrazole Diethyldithiocarbamate
CYP3A4	7q22	YES	YES	Testosterone-6ß- hydroxylation Nifedipine oxidation	Ketoconazole

Some information on different P450s….

- CYP1A2:
  - Hydrophylic compounds (acetaminophen, arom. amines, caffeine, theophylline);
  - Induced by cigarette smoke, Brussels sprouts, charbroiled beef;
- CYP2A6:
  - Coumarinhydroxylase; metabolises nitcotine and carcinogenic compounds;
  - 2 defective alleles are present in 2-30% population;
- CYP2C9:
  - Metabolism of both acid/base drugs; Highly induced by rifampicin;
  - 2 allele variants (R144C, impaired inter. with CPR, I359L, impaired S inter.) clinically very very important;
- CYP2C19:
  - Induced by rifampicin and barbiturates
  - 4 allele variants, two very important for individual variability

- CYP2D6:
    - Metabolises more than 30% of drugs (bases with planar hydrophobic rings)
    - Induced during pregnancy
    - 7% of Caucasian population lack a functional 2D6 enzyme;
    - 2-30% of different populations is ultraeffective in drug metabolism, due to multiple gene copies of 2D6;
- CYP2E1:
    - Involved in the metabolism of acetone, precursor in gluconeogenesis (phys. role);
    - Induced by ethanol and isoniazid - role in alcoholism
- CYP3A4:
    - Most relevant in drug metabolism: more than 50% of drugs are metabolised
    - Large substrates