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Redox-linked domain movements in the catalytic cycle of cytochrome P450 reductase

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Supervisors: Emma Raven (chemistry), Gordon Roberts (biochemistry) and Anne Martel (ILL)

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Big molecule changes shape after gaining more electrons

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All You Need is Neutrons (etc…)

- Introduction
  - Background
  - Earlier studies
  - Aims of this work
- Work in Leicester
- Work at the ILL
- Conclusions
- Future experiments

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The cytochrome P450 mono-oxygenase system

- Consists of an electron transport chain terminated by a cytochrome P450
- The most studied oxygenase system in nature – since the 1950s
- A large number of roles. Primarily responsible for metabolism of a very large proportion of exogenous compounds as well steroid metabolism/biosynthesis and vitamin degradation

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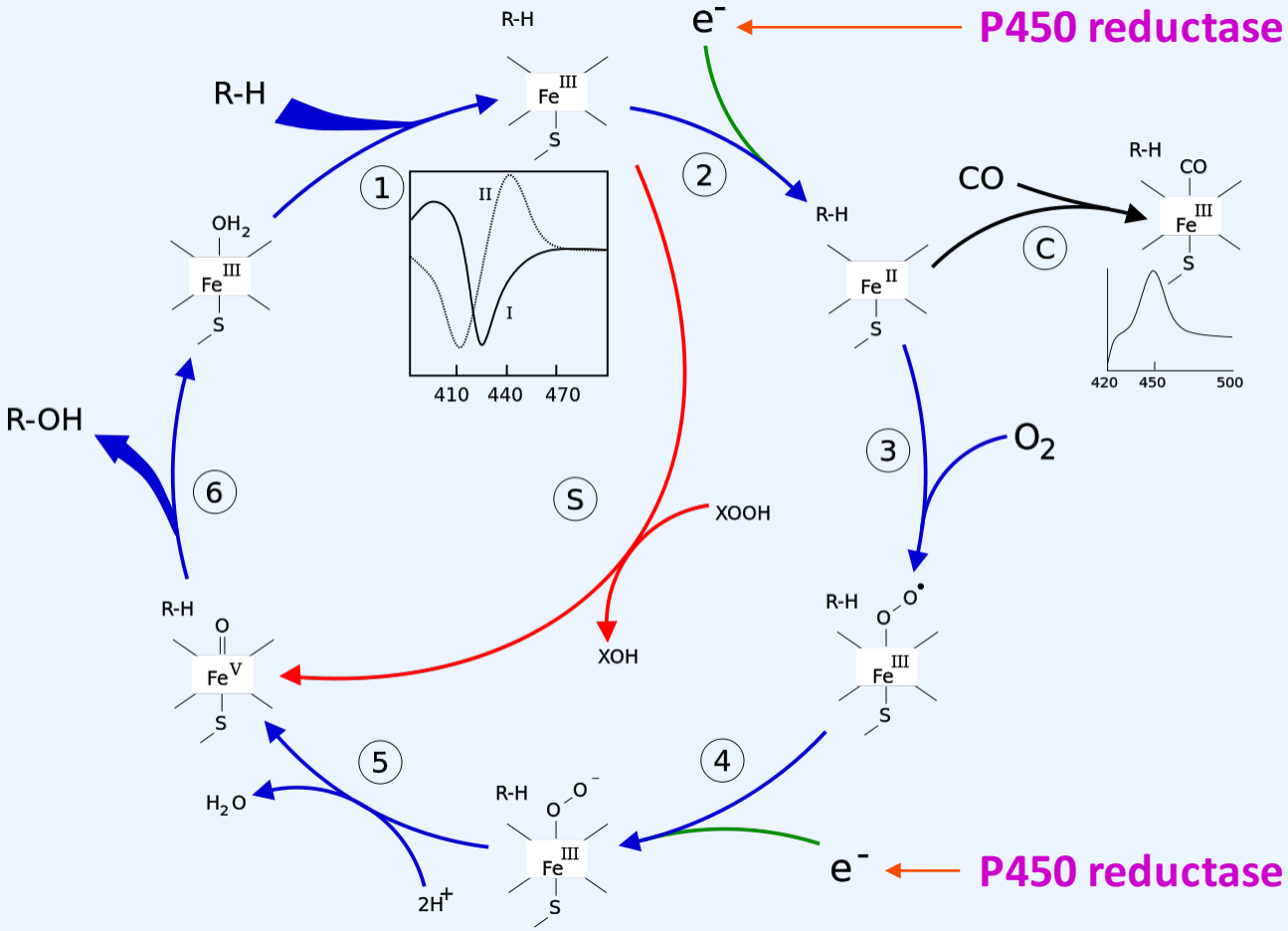
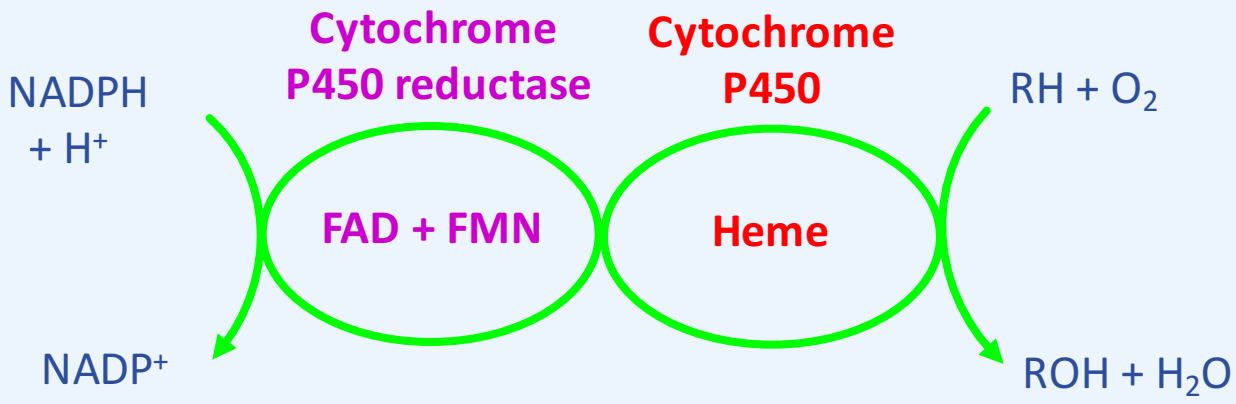
Cytochromes P450 (family)

- Occur across all domains of life - haemproteins responsible for carrying out oxidation reactions
- Located mostly in the mitochondria or on the surface of the endoplasmic reticulum in mammals
- Requires a supply of single electron equivalents from cytochrome P450 reductase, the electron transfer partner
- **$RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O$**

Cytochromes P450 catalytic cycle



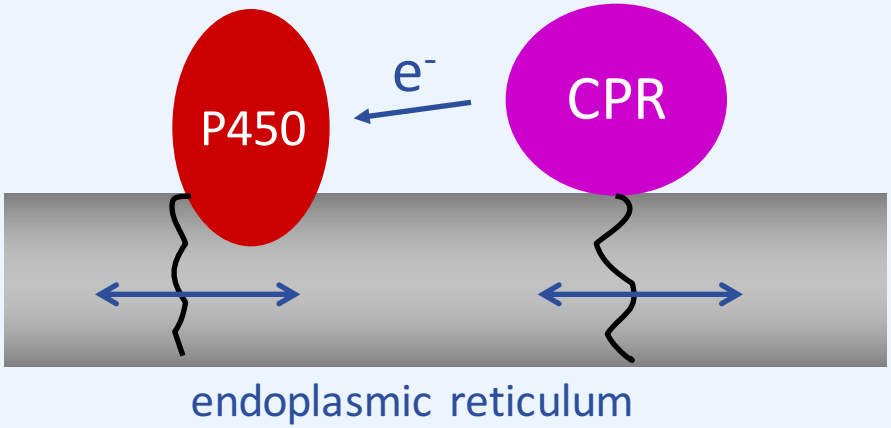
System schematic



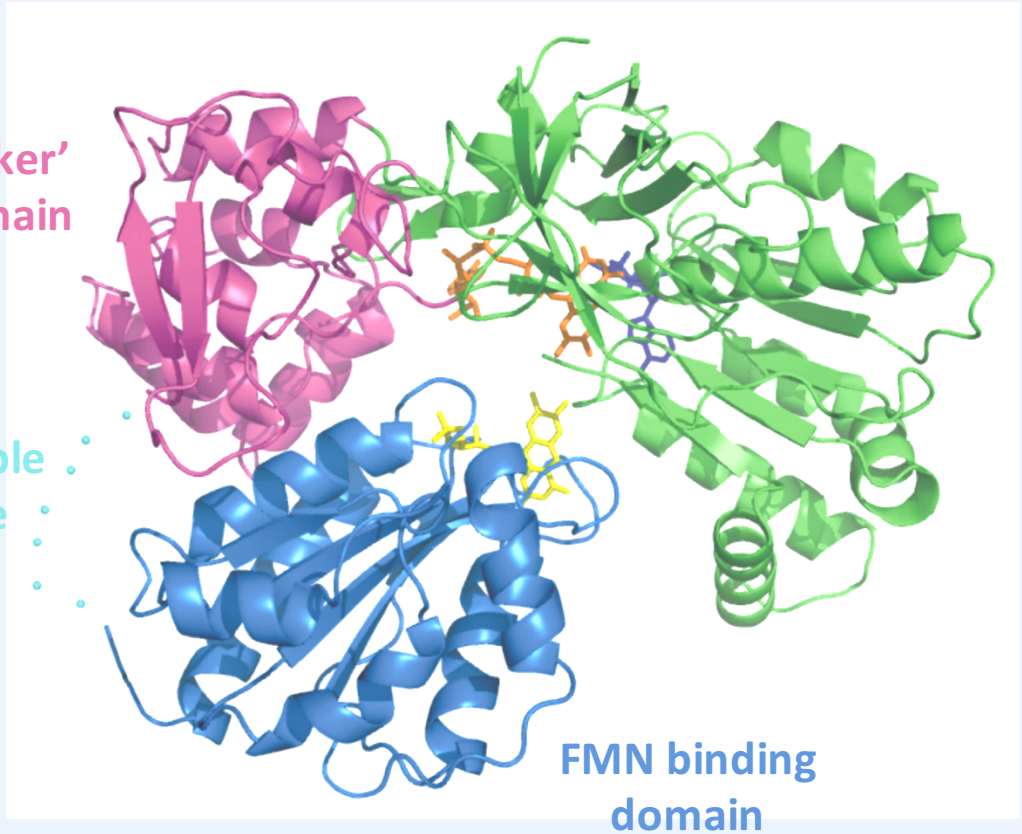
In the endoplasmic reticulum membrane

P450:CPR ~ 20:1

Diffusion and collision



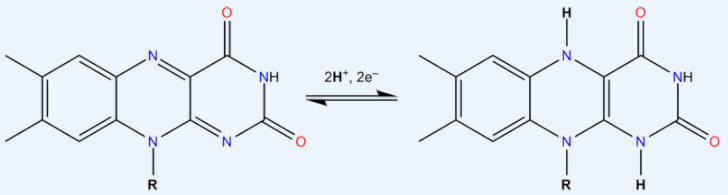
NADPH-cytochrome P450 reductase (CPR)



Three-dimensional structure of NADPH–cytochrome P450 reductase: Prototype for FMN- and FAD-containing enzymes (Wang *et. al.* 1997)

The electron flow in the P450 mono-oxygenase system is:

NADPH → FAD → FMN → P450



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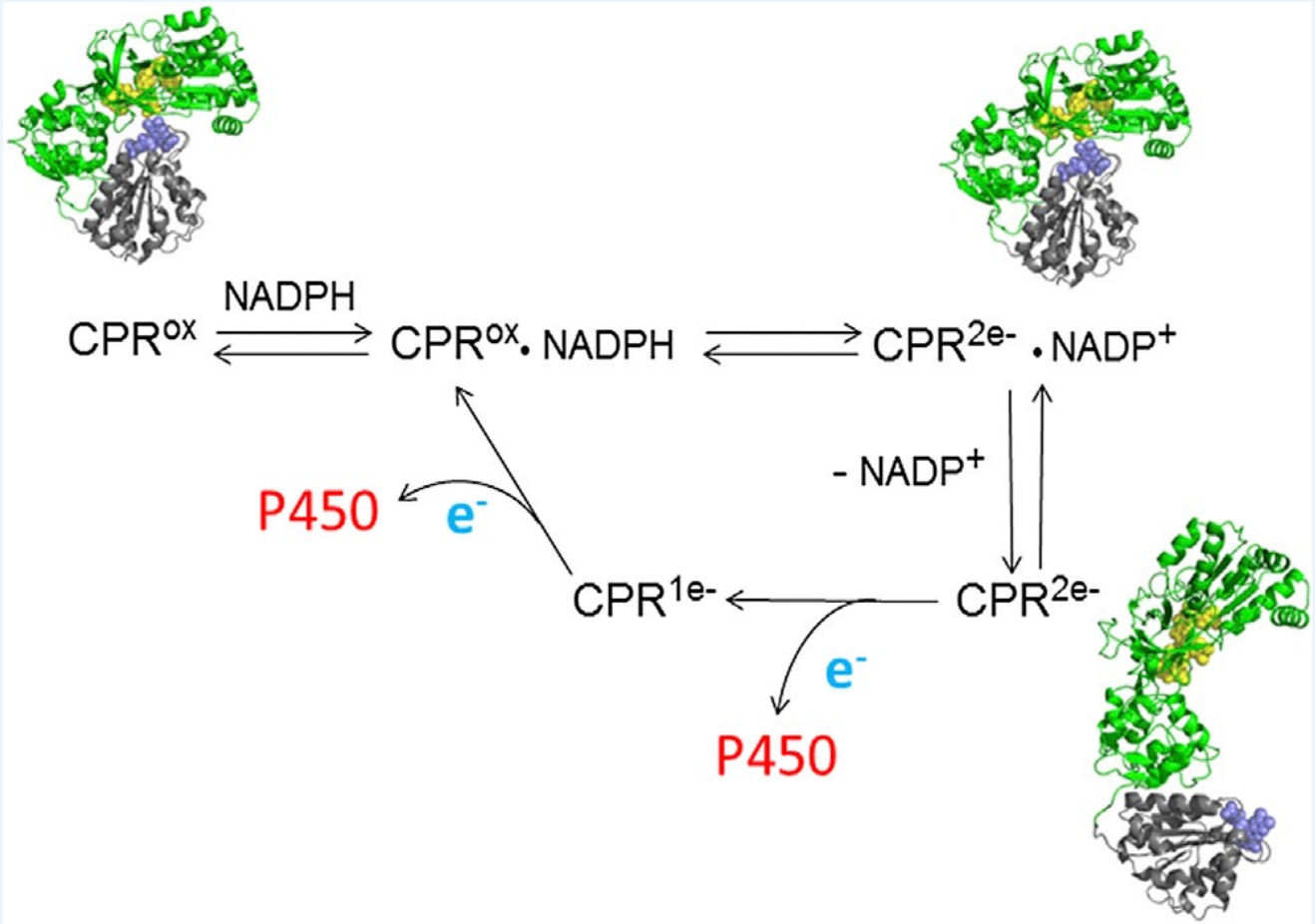
Domain motions

- Well established idea (Frauenfelder *et. al.* 1991)
- Both small and large scale motions have been shown to facilitate biological function
- The importance in electron transfer is a recent discovery
- Plenty of evidence of domain movement in diflavin reductase family
  - Large and dynamic complexes make this easily conceivable

CPR catalytic cycle



Redox-Linked Domain Movements in the Catalytic Cycle of Cytochrome P450 Reductase (Huang *et. al.* 2013)

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Preceding work

Author's Choice

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 52, pp. 36628–36637, December 25, 2009
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Domain Motion in Cytochrome P450 Reductase

CONFORMATIONAL EQUILIBRIA REVEALED BY NMR AND SMALL-ANGLE X-RAY SCATTERING

Received for publication, August 10, 2009, and in revised form, October 21, 2009 Published, JBC Papers in Press, October 26, 2009, DOI 10.1074/jbc.M109.054304

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From the [‡]Henry Wellcome Laboratories for Structural Biology, Department of Biochemistry, University of Leicester, Leicester LE1 9HN and the [§]Molecular Biophysics Group, Science and Technology Facilities Council Daresbury Laboratory, Warrington, Cheshire WA4 4AD, United Kingdom

NADPH-cytochrome P450 reductase (CPR), a diflavin reductase, plays a key role in the mammalian P450 mono-oxygenase system. In its crystal structure, the two flavins are close together, positioned for interflavin electron transfer but not for electron transfer to cytochrome P450. A number of lines of evidence suggest that domain motion is important in the action of the enzyme. We report NMR and small-angle x-ray scattering experiments addressing directly the question of domain organization in human CPR. Comparison of the ^{1}H-^{15}N heteronuclear single quantum correlation spectrum of CPR with that of the isolated FMN domain permitted identification of residues in the FMN domain whose environment differs in the two situations. These include several residues that are solvent-exposed in the CPR crystal structure, indicating the existence of a second conformation in which the FMN domain is involved in a different interdomain interface. Small-angle x-ray scattering experiments showed that oxidized and NADPH-reduced CPRs have different overall shapes. The scattering curve of the reduced enzyme can be adequately explained by the crystal structure, whereas analysis of the data for the oxidized enzyme indicates that it exists as a mixture of approximately equal amounts of two conformations, one consistent with the crystal structure and one a more extended structure consistent with that inferred from the NMR data. The correlation between the effects of adenosine 2′,5′-bisphosphate and NADPH on the scattering curve and their effects on the rate of interflavin electron transfer suggests that this conformational equilibrium is physiologically relevant.

The cytochrome P450 mono-oxygenase system in the mammalian endoplasmic reticulum is responsible for oxidative metabolism of both endogenous compounds, including fatty acids, steroids, and prostaglandins, and exogenous compounds ranging from therapeutic drugs and environmental toxins to carcinogens (1). Within this enzyme system, NADPH-cytochrome P450 reductase (CPR[4]; EC 1.6.2.4) plays a central role by catalyzing the transfer of electrons from NADPH, via its two flavin cofactors FMN and FAD, to the cytochromes P450 (2). CPR is also an electron donor to heme oxygenase (3), the fatty acid elongation system (4), and cytochrome b_5 (5). More recently, CPR has been found to carry out *in vivo* reductive activation of anticancer prodrugs in a hypoxia-specific manner, resulting in a markedly cytotoxic effect on tumors (6, 7), and has thus also become a target in anticancer therapy (8, 9). CPR is a member of a small family of diflavin reductases, including the isoforms of nitric-oxide synthase (10), methionine synthase reductase (11), protein NR1 (12), cytochrome P450 BM3 (13), and sulfite reductase (14), each of which catalyzes electron transfer through the pathway NAD(P)H → FAD → FMN → acceptor.

Sequence analysis indicates that CPR comprises three identifiable domains: a hydrophobic N-terminal domain that anchors the enzyme to the membrane, an FMN-binding domain homologous to bacterial flavodoxins, and an (FAD + NADPH)-binding domain homologous to ferredoxin-NADP$^+$ reductases, leading to the proposal that the enzyme evolved as the product of a fusion of two ancestral flavoproteins (15). The latter two putative domains were expressed separately and shown to fold correctly and to bind their respective cofactors (16). The crystal structure of a soluble form of the enzyme lacking the membrane-anchoring N-terminal 70 residues (see Fig. 1) (17) confirmed the existence of these domains and their structural and sequence homology to flavodoxin and ferredoxin-NADP$^+$ reductase, respectively. The structure reveals two additional important features: (i) the existence, as an insert in the FAD-binding domain, of a "linker domain" that may serve to determine the mutual orientation of the FMN- and FAD-binding domains and (ii) the fact that the FMN-binding domain is connected to the rest of the protein through a loop or hinge of 13 linker domain which may serve to orient the other two domains. The FMN-binding domain is connected to the rest of the protein by a 14-residue "hinge", thus providing the flexibility that is thought to be important for the function of the protein. An N-terminal 57 amino acid peptide is responsible for anchoring CPR to the endoplasmic reticulum membrane; recombinant CPR, which lacks this N-terminal peptide, is both soluble and functional, thus facilitating detailed structure–activity studies.

The CPR-mediated electron transfer from NADPH to cytochrome P450 proceeds in a stepwise fashion: NADPH→FAD→FMN→P450. Interflavin electron transfer requires spatial proximity of the two prosthetic groups, and the X-ray crystal structure of CPR (PDB file: 1AMO[18]) confirms this is the case (closest approach of the FAD and FMN methyl groups: 3.85 Å (C–C)).[8] However, in this compact or "closed" conformation, the FMN cofactor appears to be inaccessible to the large cytochrome P450 molecule, and so the need for domain movement as an essential part of the catalytic cycle has been widely assumed.[16] Recently, NMR spectroscopy, small-angle X-ray scattering (SAXS), and crystallographic evidence for this movement has been obtained,[19] thus suggesting that in solution, CPR exists in an equilibrium between a compact conformation appropriate for interflavin electron transfer and an extended conformation appropriate for electron transfer to P450 (Figure 1).

Herein we show that two major conformations of wild-type CPR are present in the gas phase, and that their relative abundance can be influenced by the ionic strength of the solution from which they are electrosprayed, by removal of key intramolecular ionic interactions, and, crucially, by the redox state of the flavin groups. This study demonstrates the ability of ESI-IMS-MS to detect a protein conformational

* This work was supported by grants from the Biotechnology and Biological Sciences Research Council and the Wellcome Trust.

✖ *Author's Choice*—Final version full access.

* This article was selected as a Paper of the Week.

[S] The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure.

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[4] The abbreviations used are: CPR, NADPH-cytochrome P450 reductase; CPR$_{ox}$, oxidized CPR; CPR$_{hq}$, 4-electron reduced CPR; CPR$_{sq}$, 2-electron reduced CPR (CPR semiquinone); SAXS, small-angle x-ray scattering; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; HSQC, heteronuclear single quantum correlation; EOM, ensemble optimization method.

Angewandte International Edition Chemie

DOI: 10.1002/anie.201101077

Protein Conformation

Detection of a Protein Conformational Equilibrium by Electrospray Ionisation-Ion Mobility-Mass Spectrometry**

*Matthew Jenner, Jacqueline Ellis, Wei-Cheng Huang, Emma Lloyd Raven, Gordon C. K. Roberts, and Neil J. Oldham**

Ion mobility spectrometry (IMS) is emerging as a promising technique for providing low-resolution protein-structure information, particularly in combination with electrospray ionization (ESI) and mass spectrometry (MS).[1] There is currently much debate on the structure of protein ions in the gas phase, as summarized by Breuker and McLafferty.[2] Whilst it appears probable that some structural collapse occurs within picoseconds of dehydration, the onset of gross structural rearrangement may require tens of milliseconds.[3] This time provides a potential window for the observation of "near-native" structures that may retain some elements of the solution structure, with the ability to provide biologically relevant information. A number of recent applications have used IMS to study the conformation and stoichiometry of proteins and their complexes. Structural changes to amyloid and prion proteins, as well as steps in amyloid fibril assembly, have been detected by using this approach,[4] and the calcium-dependent conformational change in calmodulin has been probed by IM-MS,[5] as has the relationship between tertiary structure and chemotactic activity in antibacterial peptides.[6] Insights into the structures of large multiprotein complexes, such as the RNA-binding TRAP protein, GroEL, and the 20S proteasome have also been provided by ion mobility measurements.[7]

We postulated that IM-MS may be used to study the dynamic equilibrium between well-characterized conformations of a monomeric multidomain protein. To test this hypothesis, we have examined NADPH-cytochrome P450 reductase (CPR) using ESI-IM-MS. CPR is a 76 kDa membrane bound flavoprotein that catalyses the transfer of electrons from NADPH to a number of oxygenase enzymes.[8] CPR consists of three folded domains,[8] an FAD- and NADPH-binding domain, an FMN-binding domain, and a

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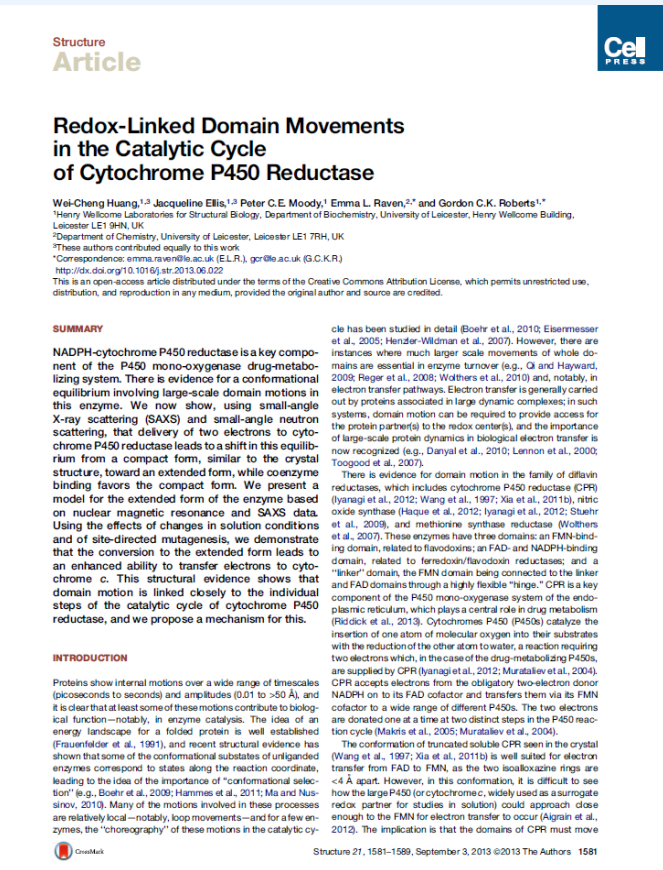
[**] We are grateful to the Wellcome Trust and the University of Nottingham for financial support.

Supporting information for this article (detailed experimental methods and instrumental conditions) is available on the WWW under http://dx.doi.org/10.1002/anie.201101077.

Figure 1. Compact and extended forms of CPR showing the FMN binding domain (blue), the FAD binding and linker domains (magenta), FMN (orange), and FAD (yellow). NADPH is omitted for clarity. FAD = flavin adenine dinucleotide, FMN = flavin mononucleotide, NADPH = nicotinamide adenine dinucleotide phosphate.



Structure

Article

Cell Press

Redox-Linked Domain Movements in the Catalytic Cycle of Cytochrome P450 Reductase

Wei-Cheng Huang,[1,3] Jacqueline Ellis,[1,3] Peter C.E. Moody,[1] Emma L. Raven,[2,*] and Gordon C.K. Roberts[1,*]
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http://dx.doi.org/10.1016/j.str.2013.06.022

SUMMARY

NADPH-cytochrome P450 reductase is a key component of the P450 mono-oxygenase drug-metabolizing system. There is evidence for a conformational equilibrium involving large-scale domain motions in this enzyme. We now show, using small-angle X-ray scattering (SAXS) and small-angle neutron scattering, that delivery of two electrons to cytochrome P450 reductase leads to a shift in this equilibrium from a compact form, similar to the crystal structure, toward an extended form, while coenzyme binding favors the compact form. We present a model for the extended form of the enzyme based on nuclear magnetic resonance and SAXS data. Using the effects of changes in solution conditions and of site-directed mutagenesis, we demonstrate that the conversion to the extended form leads to an enhanced ability to transfer electrons to cytochrome *c*. This structural evidence shows that domain motion is linked closely to the individual steps of the catalytic cycle of cytochrome P450 reductase, and we propose a mechanism for this.

INTRODUCTION

Proteins show internal motions over a wide range of timescales (picoseconds to seconds) and amplitudes (0.01 to >50 Å), and it is clear that at least some of these motions contribute to biological function—notably, in enzyme catalysis. The idea of an energy landscape for a folded protein is well established (Frauenfelder et al., 1991), and recent structural evidence has shown that some of the conformational substates of unliganded enzymes correspond to states along the reaction coordinate, leading to the idea of the importance of "conformational selection" (e.g., Boehr et al., 2009; Hammes et al., 2011; Ma and Nussinov, 2010). Many of the motions involved in these processes are relatively local—notably, loop movements—and for a few enzymes, the "choreography" of these motions in the catalytic cycle has been studied in detail (Boehr et al., 2010; Eisenmesser et al., 2005; Henzler-Wildman et al., 2007). However, there are instances where much larger scale movements of whole domains are essential in enzyme turnover (e.g., Qi and Hayward, 2009; Reger et al., 2008; Wolthers et al., 2010) and, notably, in electron transfer pathways. Electron transfer is generally carried out by proteins associated in large dynamic complexes; in such systems, domain motion can be required to provide access for the protein partner(s) to the redox center(s), and the importance of large-scale protein dynamics in biological electron transfer is now recognized (e.g., Danyal et al., 2010; Lennon et al., 2000; Toogood et al., 2007).

There is evidence for domain motion in the family of diflavin reductases, which includes cytochrome P450 reductase (CPR) (Iyanagi et al., 2012; Wang et al., 1997; Xia et al., 2011b), nitric oxide synthase (Haque et al., 2012; Iyanagi et al., 2012; Stuehr et al., 2009), and methionine synthase reductase (Wolthers et al., 2007). These enzymes have three domains: an FMN-binding domain, related to flavodoxins; an FAD- and NADPH-binding domain, related to ferredoxin/flavodoxin reductases; and a "linker" domain, the FMN domain being connected to the linker and FAD domains through a highly flexible "hinge." CPR is a key component of the P450 mono-oxygenase system of the endoplasmic reticulum, which plays a central role in drug metabolism (Riddick et al., 2013). Cytochromes P450 (P450s) catalyze the insertion of one atom of molecular oxygen into their substrates with the reduction of the other atom to water, a reaction requiring two electrons which, in the case of the drug-metabolizing P450s, are supplied by CPR (Iyanagi et al., 2012; Muralidev et al., 2004). CPR accepts electrons from the obligatory two-electron donor NADPH on to its FAD cofactor and transfers them via its FMN cofactor to a wide range of different P450s. The two electrons are donated one at a time at two distinct steps in the P450 reaction cycle (Makris et al., 2005; Muralidev et al., 2004).

The conformation of truncated soluble CPR seen in the crystal (Wang et al., 1997; Xia et al., 2011b) is well suited for electron transfer from FAD to FMN, as the two isoalloxazine rings are <4 Å apart. However, in this conformation, it is difficult to see how the large P450 (or cytochrome *c*, widely used as a surrogate redox partner for studies in solution) could approach close enough to the FMN for electron transfer to occur (Agrain et al., 2012). The implication is that the domains of CPR must move

Ellis *et. al.* (JBC, 2009), Jenner *et. al.* (Angewande Chemie, 2011), Huang *et. al.* (Structure, 2013)

Preceding work

- SAXS and initial SANS measurements

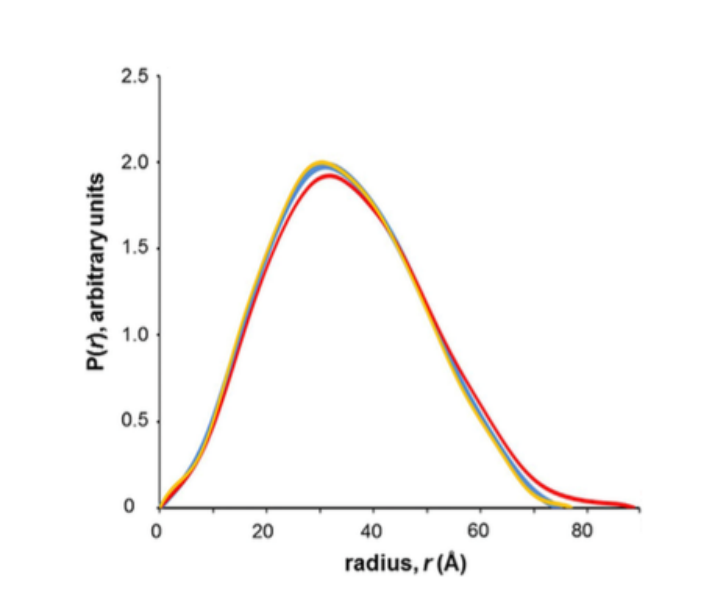

Table 1. Hydrodynamic Parameters for Oxidized and Reduced CPR Obtained from Solution Scattering Experiments

Sample	R_g (SAXS) (Å)	D_{max} (SAXS) (Å)	R_g (SANS) (Å)
Oxidized	26.4	73	24.8
Dithionite $2e^-$-reduced	28.8	80	29.0
NADPH $2e^-$-reduced	27.6	76	27.8
NADH $2e^-$-reduced	29.3	83	–
Dithionite $2e^-$-reduced + $NADP^+$	27.7	78	–

Errors were in the range ±0.1–0.4 Å for SAXS R_g values, ±0.4–0.6 Å for SANS R_g values, and ±1–2 Å for D_{max} values.
See also Figures S1 and S3.

Blue: Oxidised
Red: 2e- Reduced (dithionite)
Yellow: 2e- Reduced (NADPH)

Redox-Linked Domain Movements in the Catalytic Cycle of Cytochrome P450 Reductase (Huang *et. al.* 2013)

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Aims

- Relate structurally-defined domain motion to individual steps in the catalytic cycle
- Investigate the influence of mutagenesis on the position of conformational equilibrium
- Investigate an electron transfer complexes in solution and in the membrane

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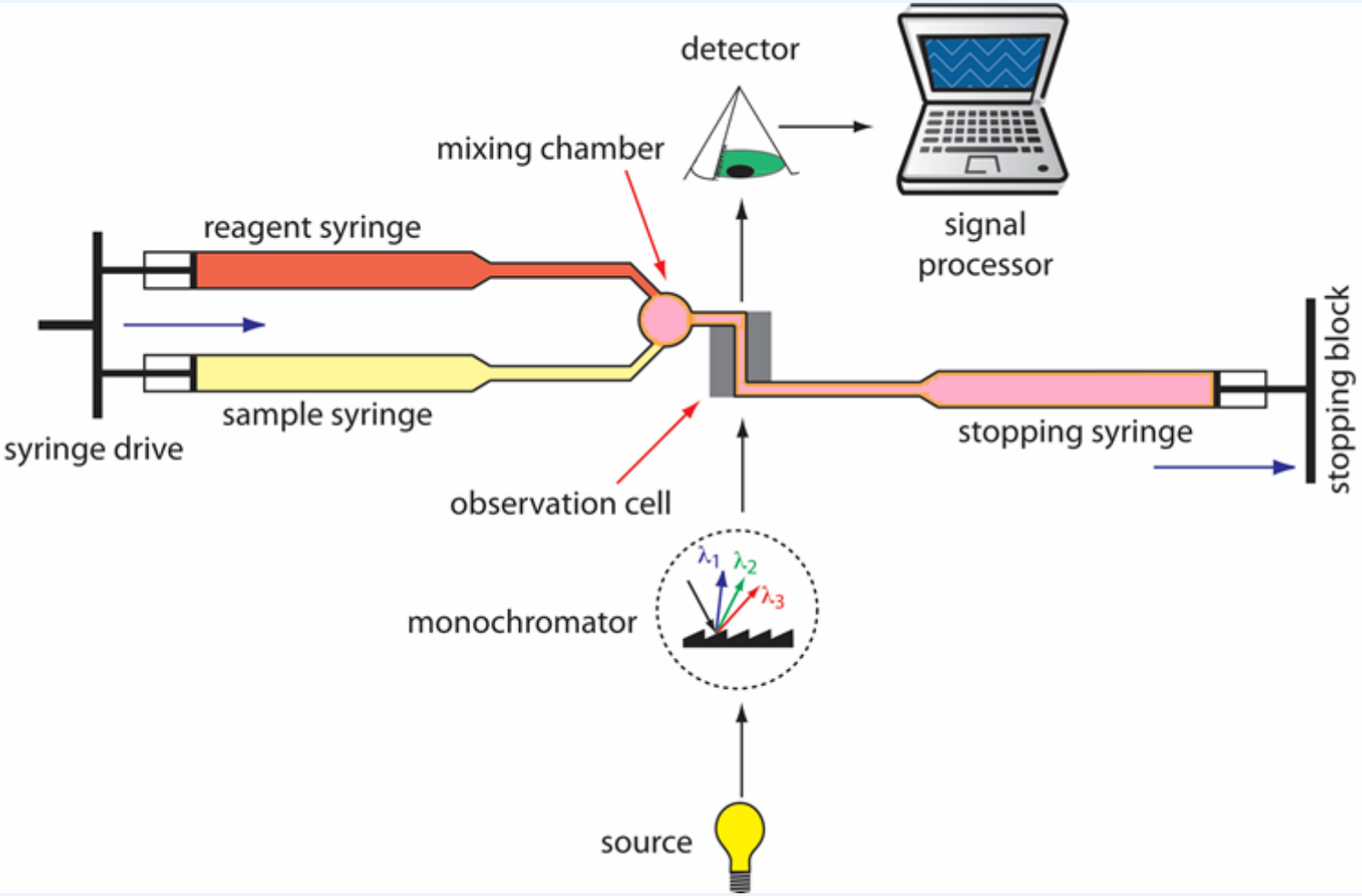
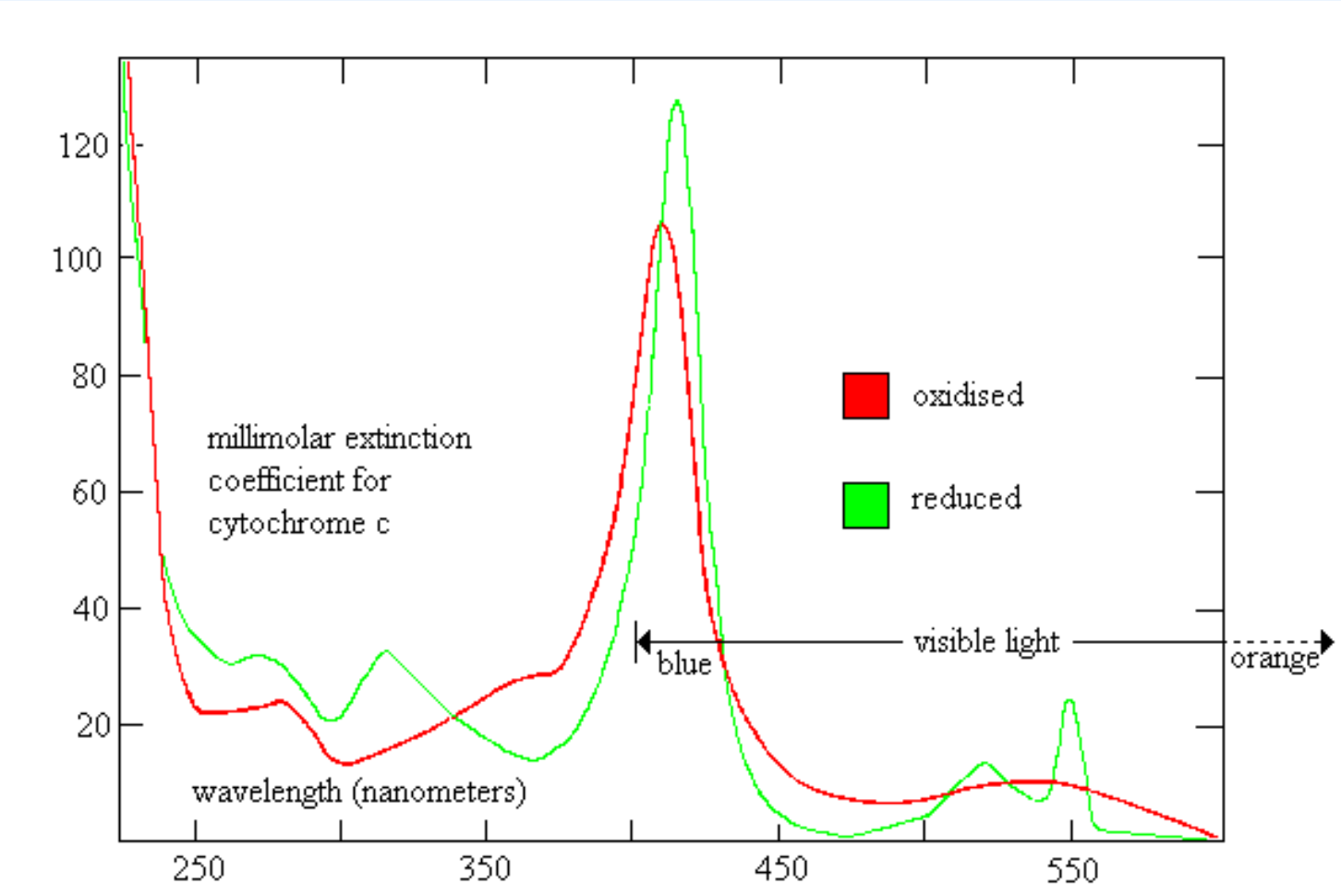
Summary of work completed to date

- Expression of soluble CPR in *E. coli*
- Purification (of wildtype and mutants)
- Spectroscopic and kinetic characterisation
- Redox titrations (anaerobic)
- Stopped-flow studies (anaerobic)
- SANS
- Deuteration of soluble CPR (almost)

Stopped-flow studies (kinetics)



The cytochrome c assay



http://www.bmb.leeds.ac.uk/teaching/icu3/lecture/20/

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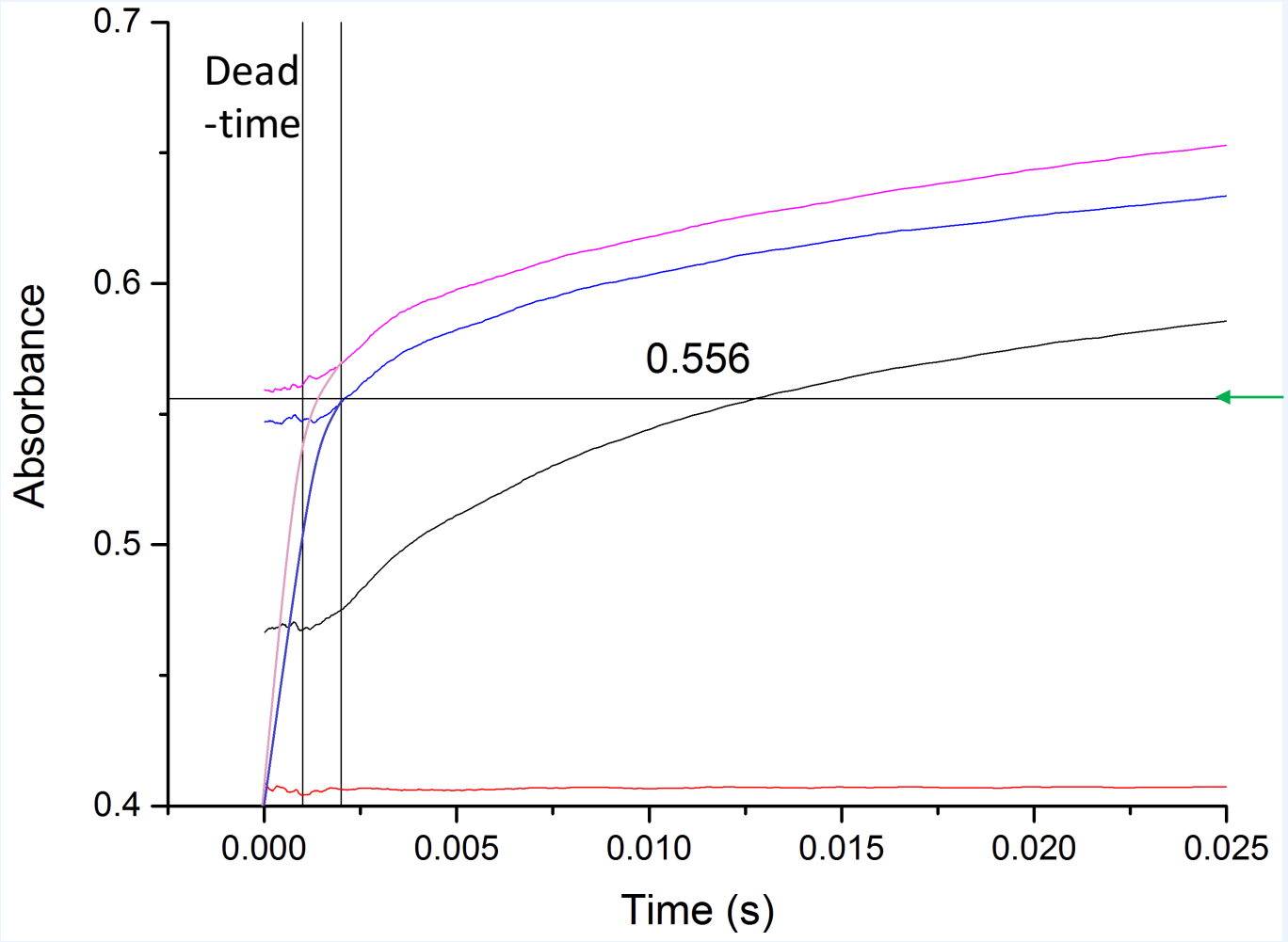
Stopped-Flow Studies



PKR

EPVP

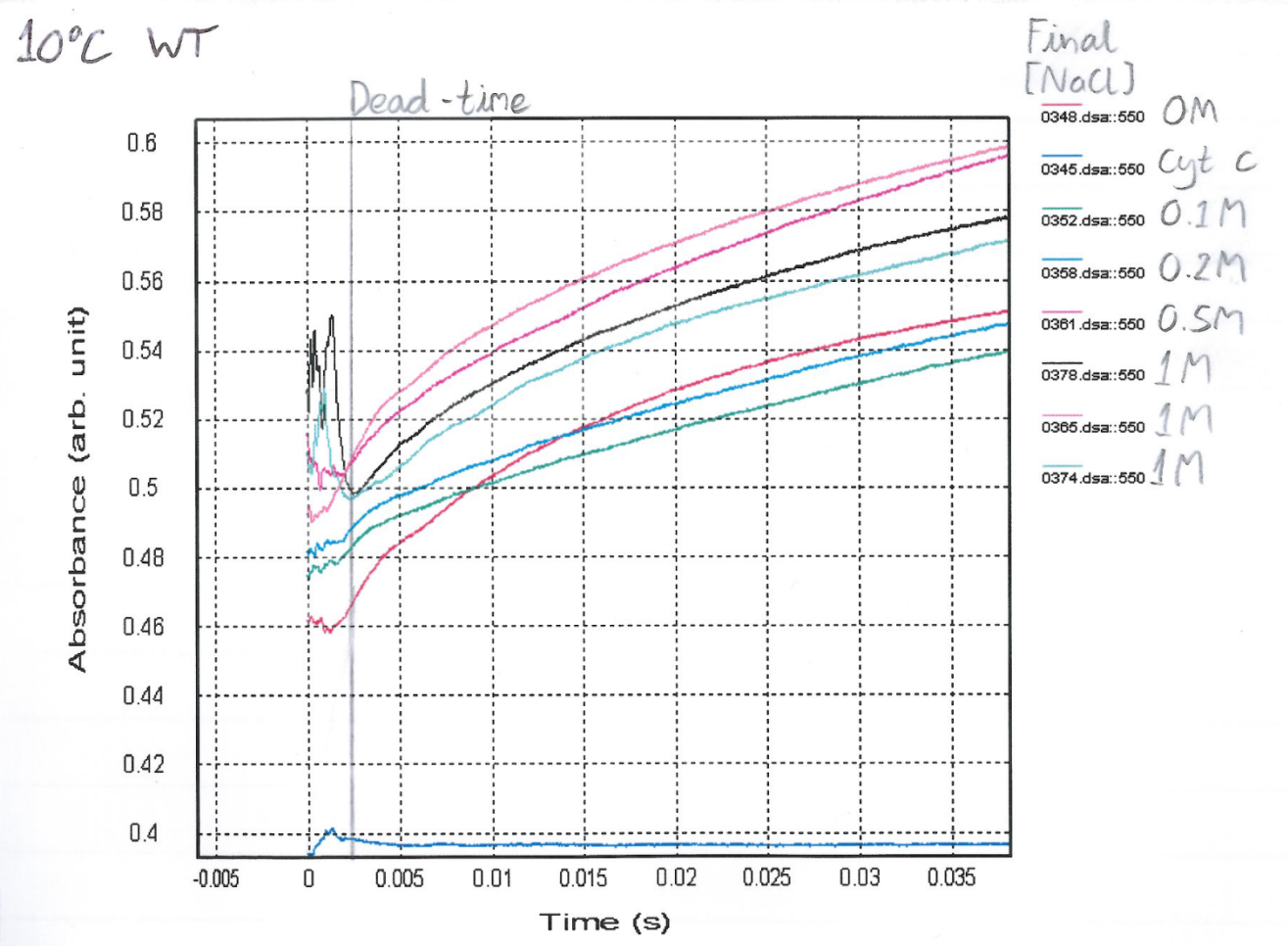
Wildtype

First turnover

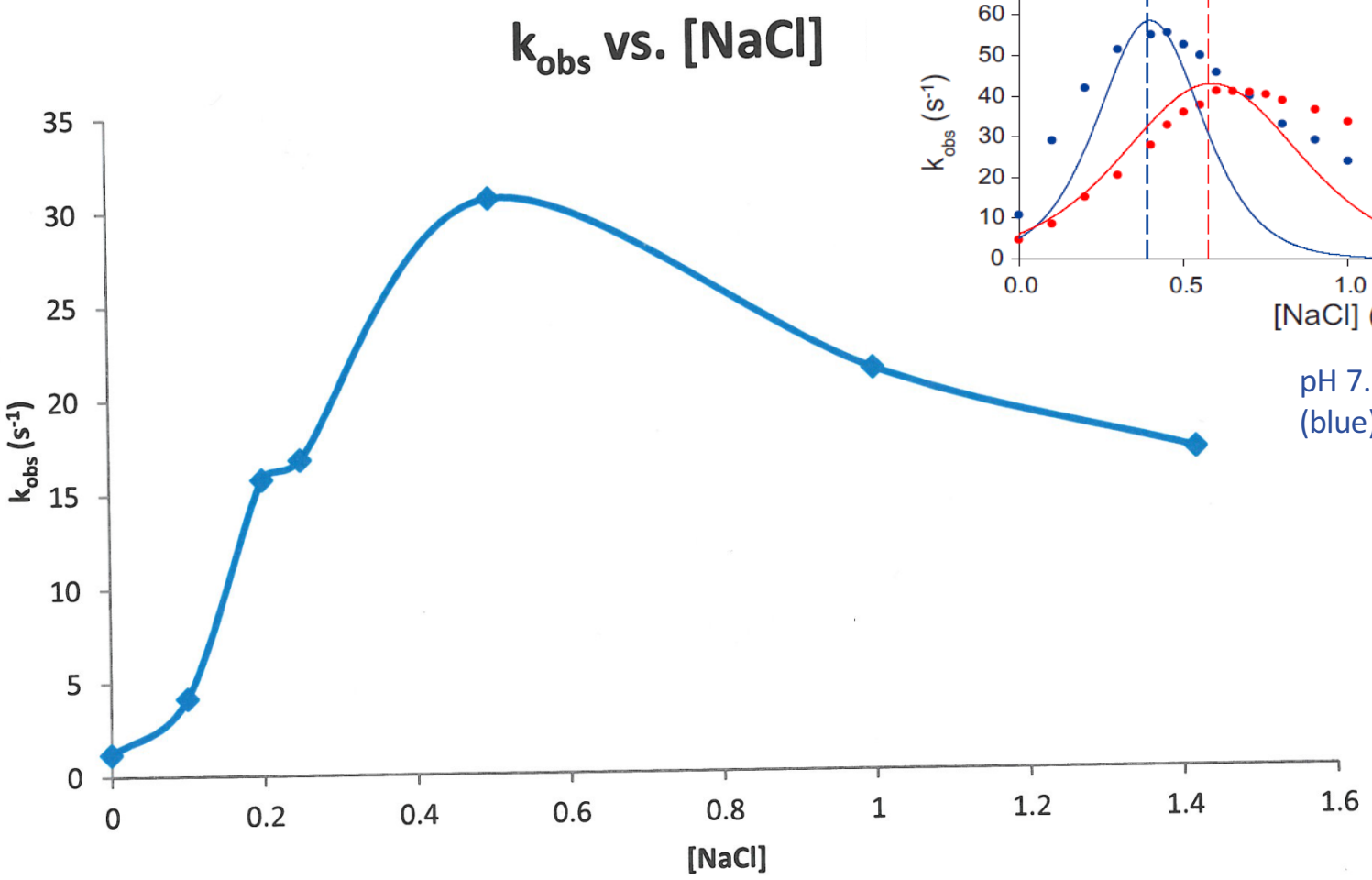
Cytochrome c baseline

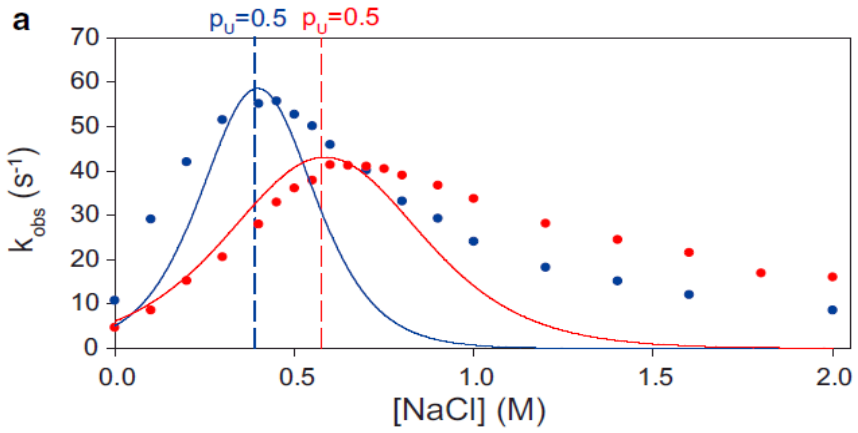
10ºC, pH 7.0

Variation of ionic strength



Variation of ionic strength

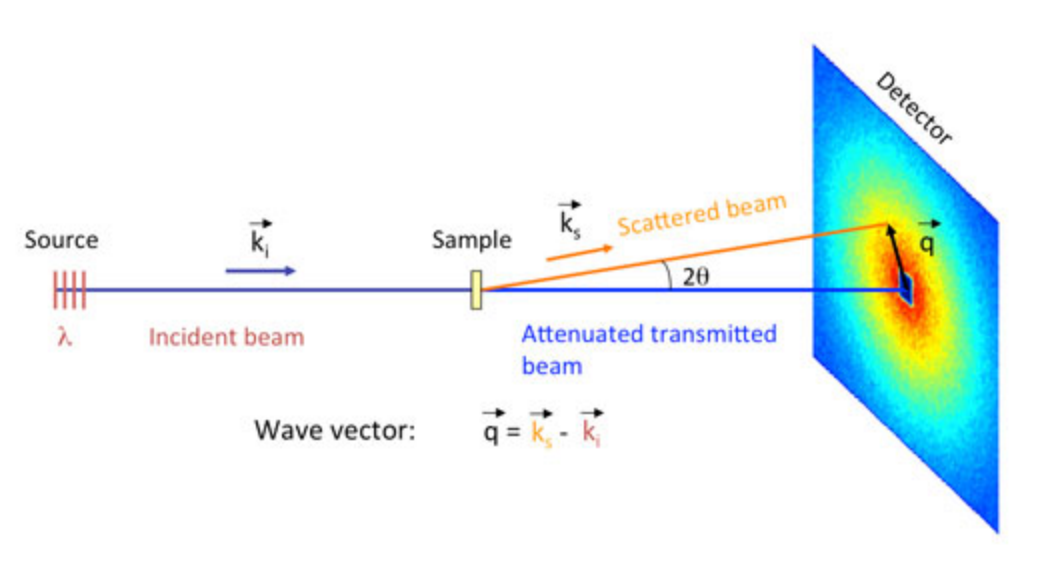


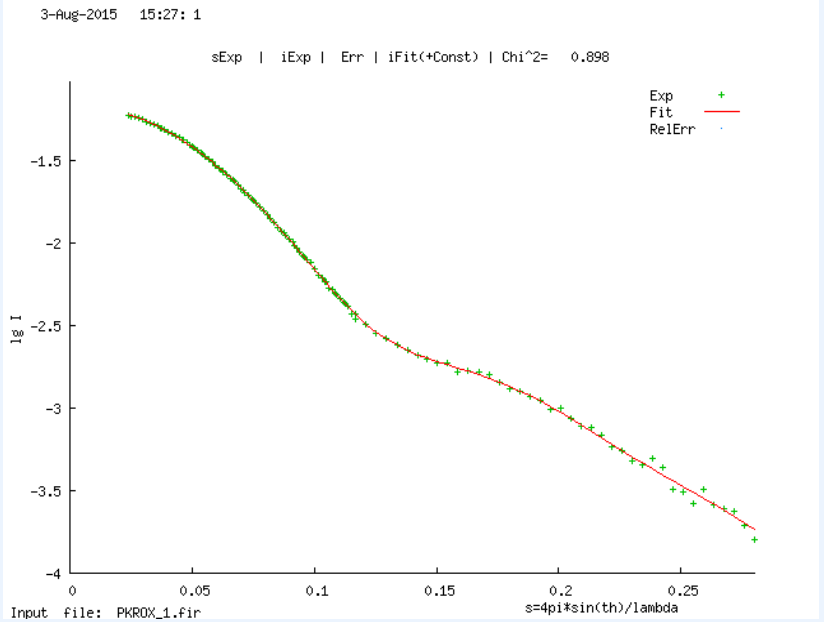


pH 7.4 (red) and pH 6.7 (blue), Frances *et. al.* 2015

Solution SANS

- Solution Small Angle Neutron Scattering
- Non-atomic resolution structure determination

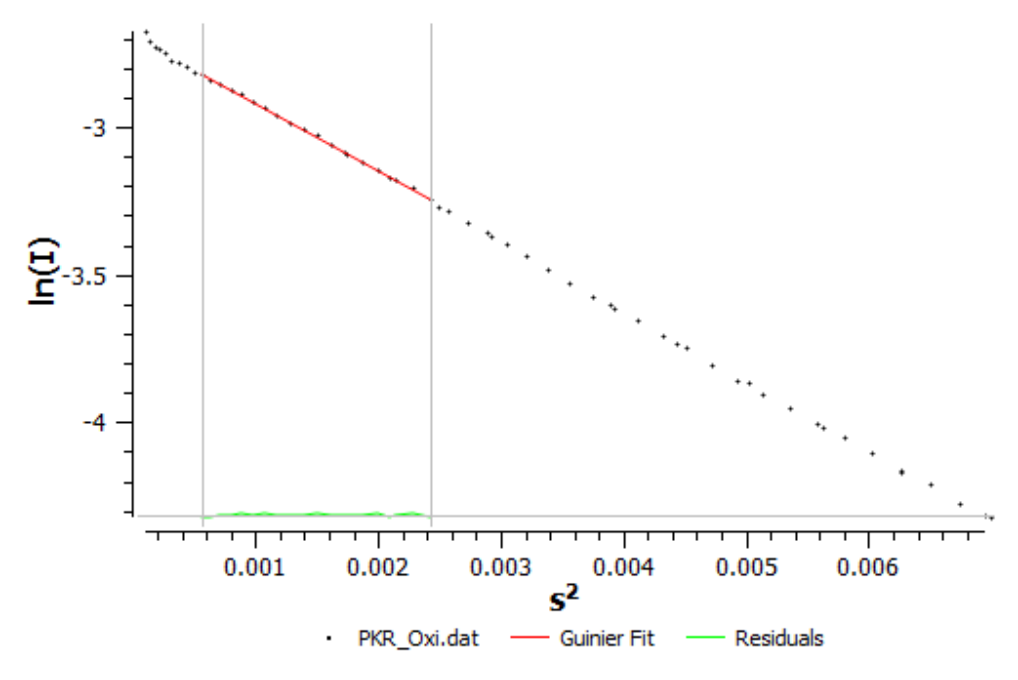


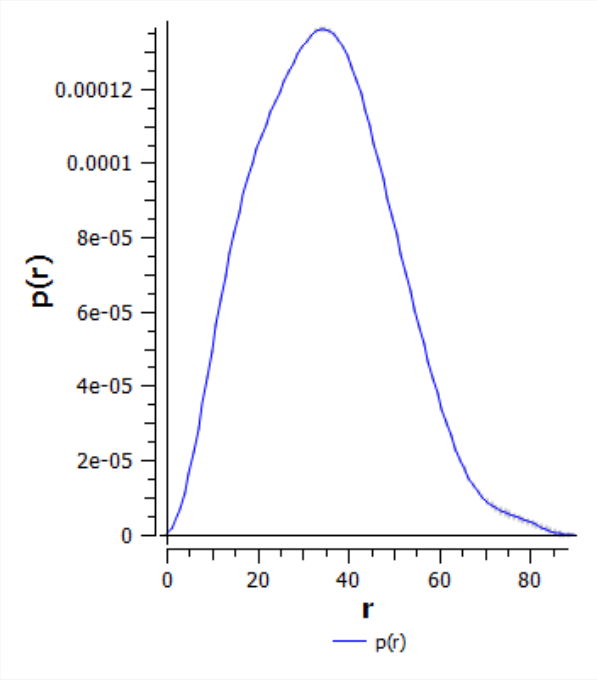


Scattering vector $(q) = 4\pi \sin\theta \,/\lambda$

SANS data analysis

- Guinier plot (slope = radius of gyration)
- P(r) plot (pair distance distribution)





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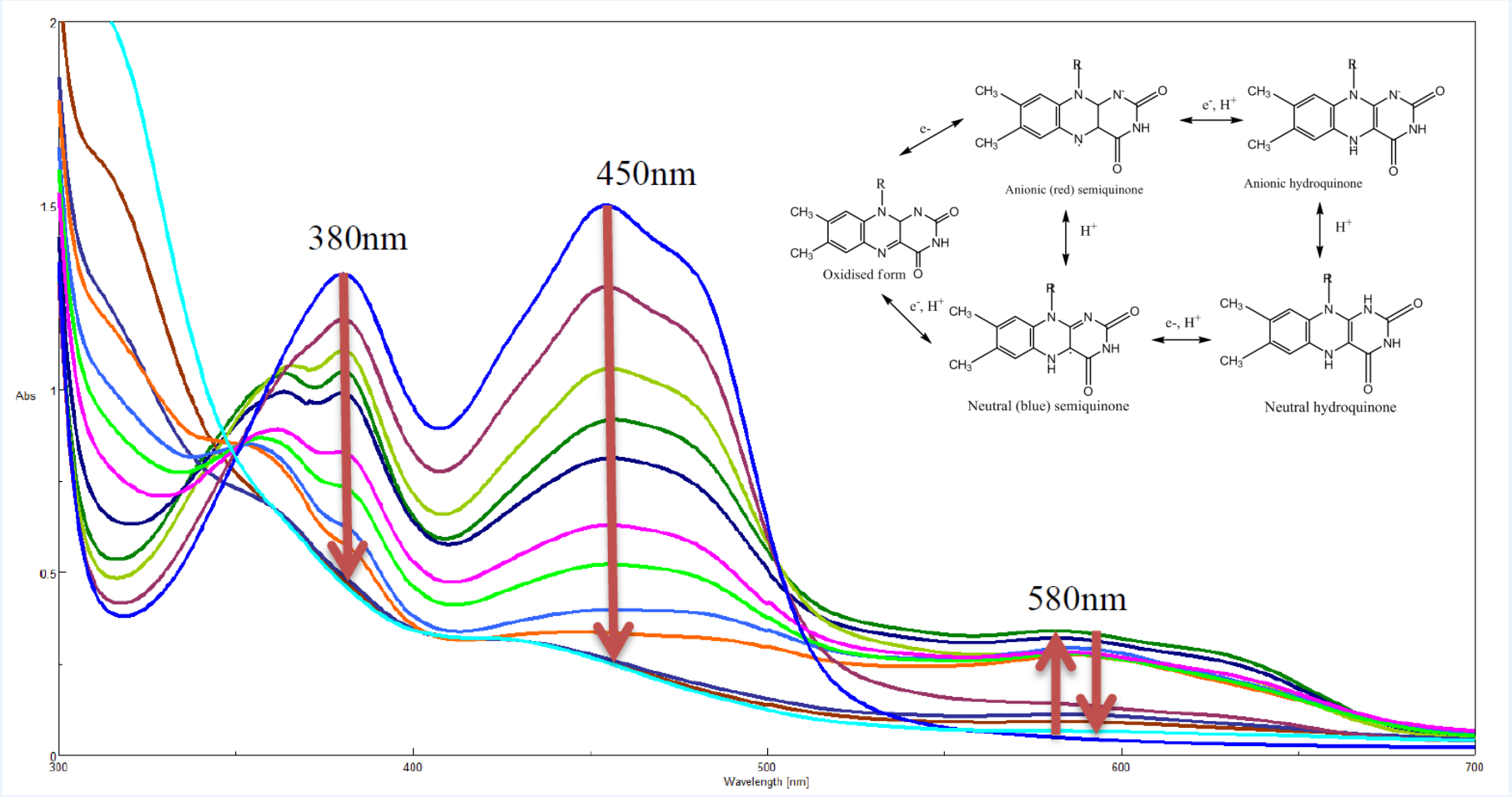
Redox titrations (anaerobic)



Quantitative analysis

- Determination of Rg and Dmax
- Appears to follow the pattern observed in earlier studies

PKR								
Sample	Rg	Rg Error	I0	I0 Error		Dmax	Quality (%)	Manual Rg Range
Oxidised	26.117	0.365	0.068	0.000		89.450	93.500	10-131
Dithionite 1e-	27.200	0.112	0.068	0.000		90.620	96.400	10-131
Dithionite 2e-	29.000	0.171	0.083	0.000		99.500	93.000	10-131
Dithionite 4e-	29.200	0.197	0.076	0.000		95.730	93.400	13-131
Dithionite 2e- + NADP+	27.800	0.128	0.076	0.000		95.160	94.500	10-110
NADPH 1e-	25.800	0.131	0.061	0.000		87.170	80.700	1-139
NADPH 2e-	27.200	0.118	0.071	0.000		93.590	94.000	10-134

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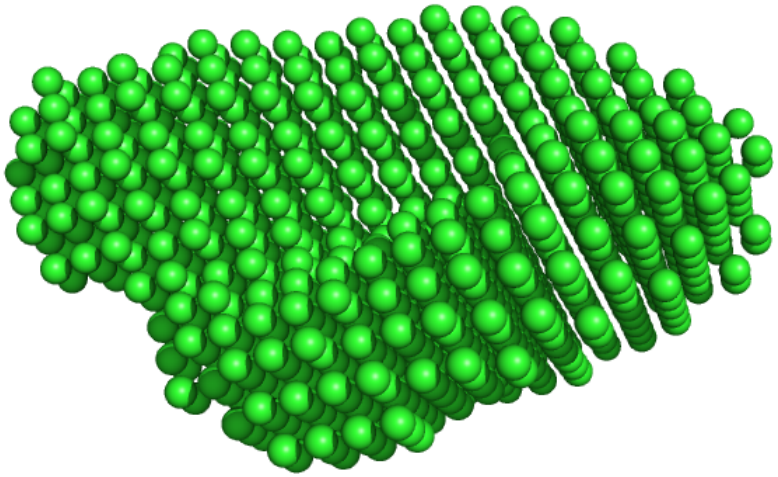
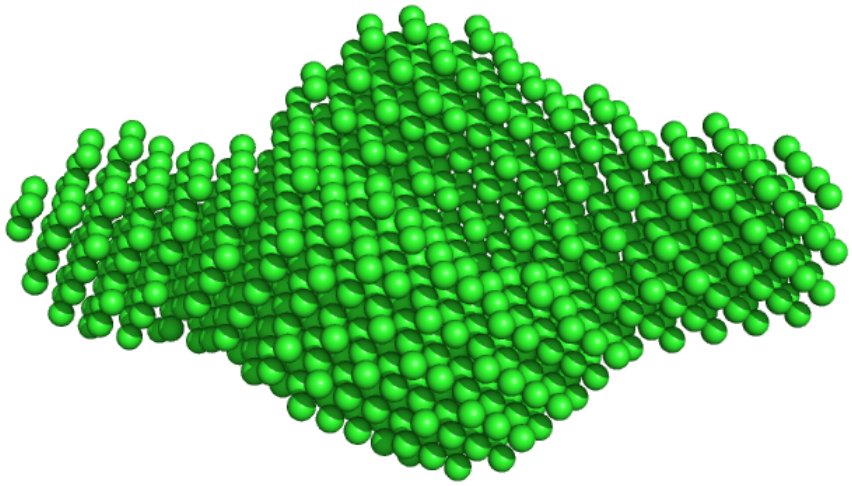
Ab initio modelling

- DAMMIF (Rapid shape determination)

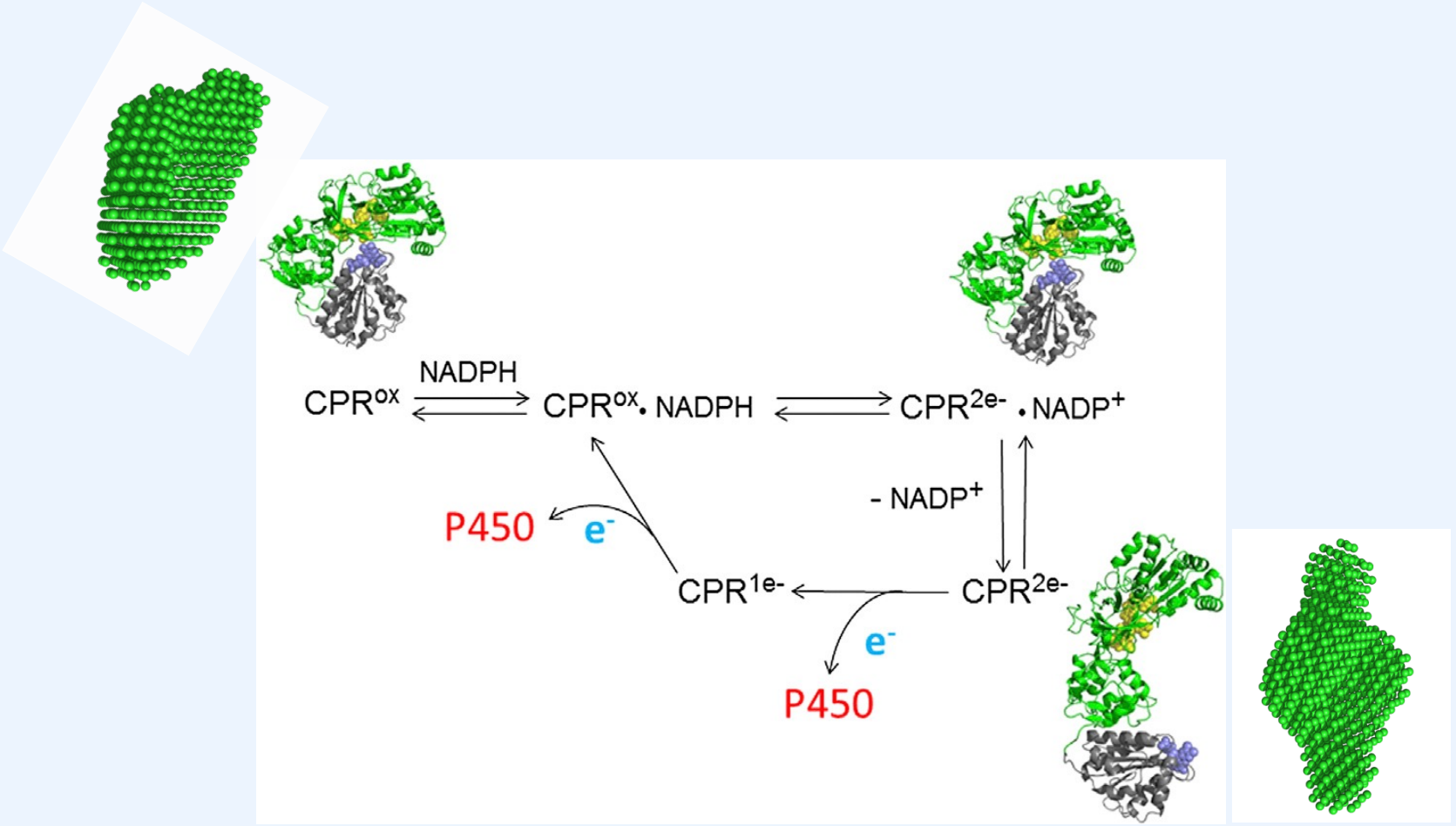
Oxidised



Dithionite $2e^-$

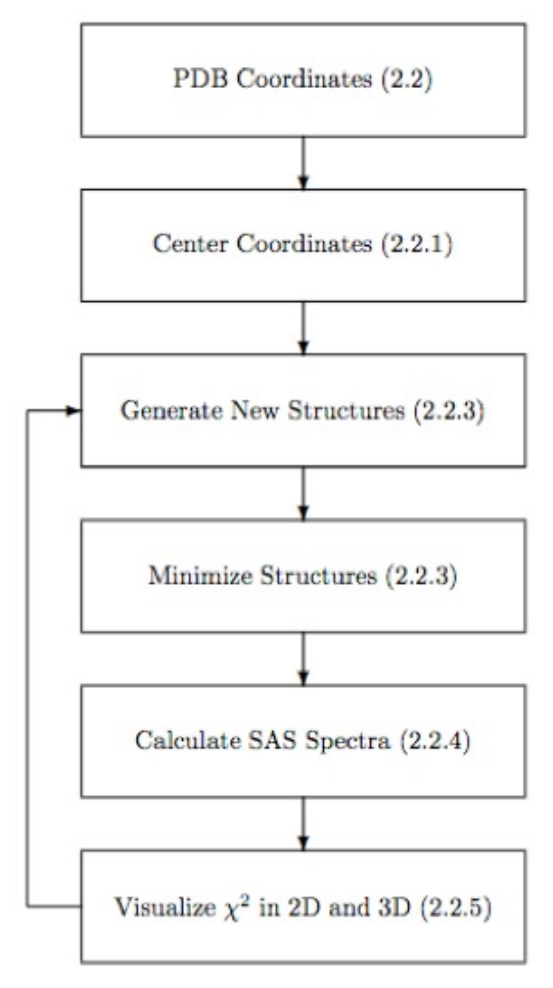


Comparison with predicted conformations



Conformational modelling

- EOM
- SASSIE
- MultiFoXS



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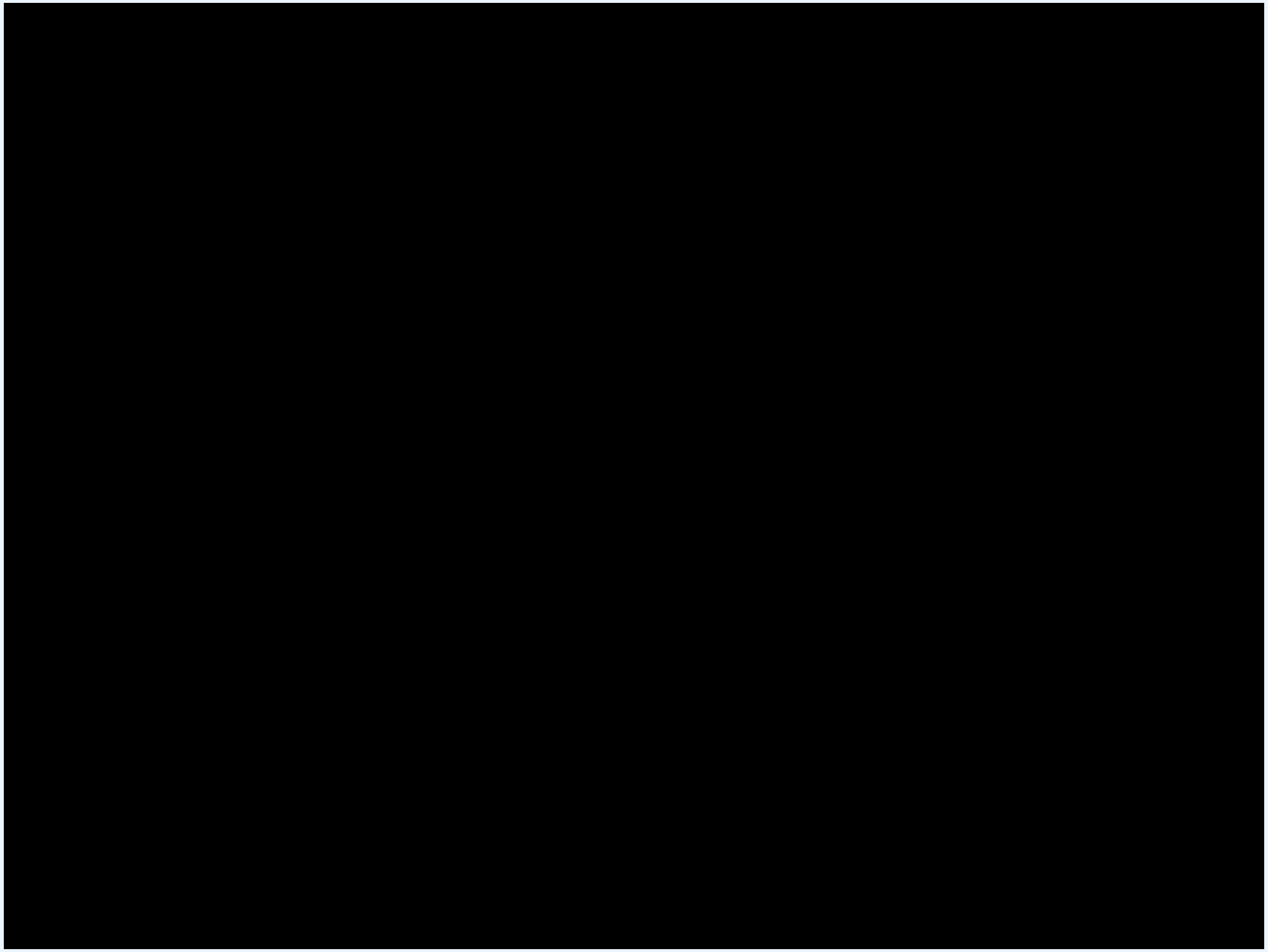
Monomer monte carlo structure generation



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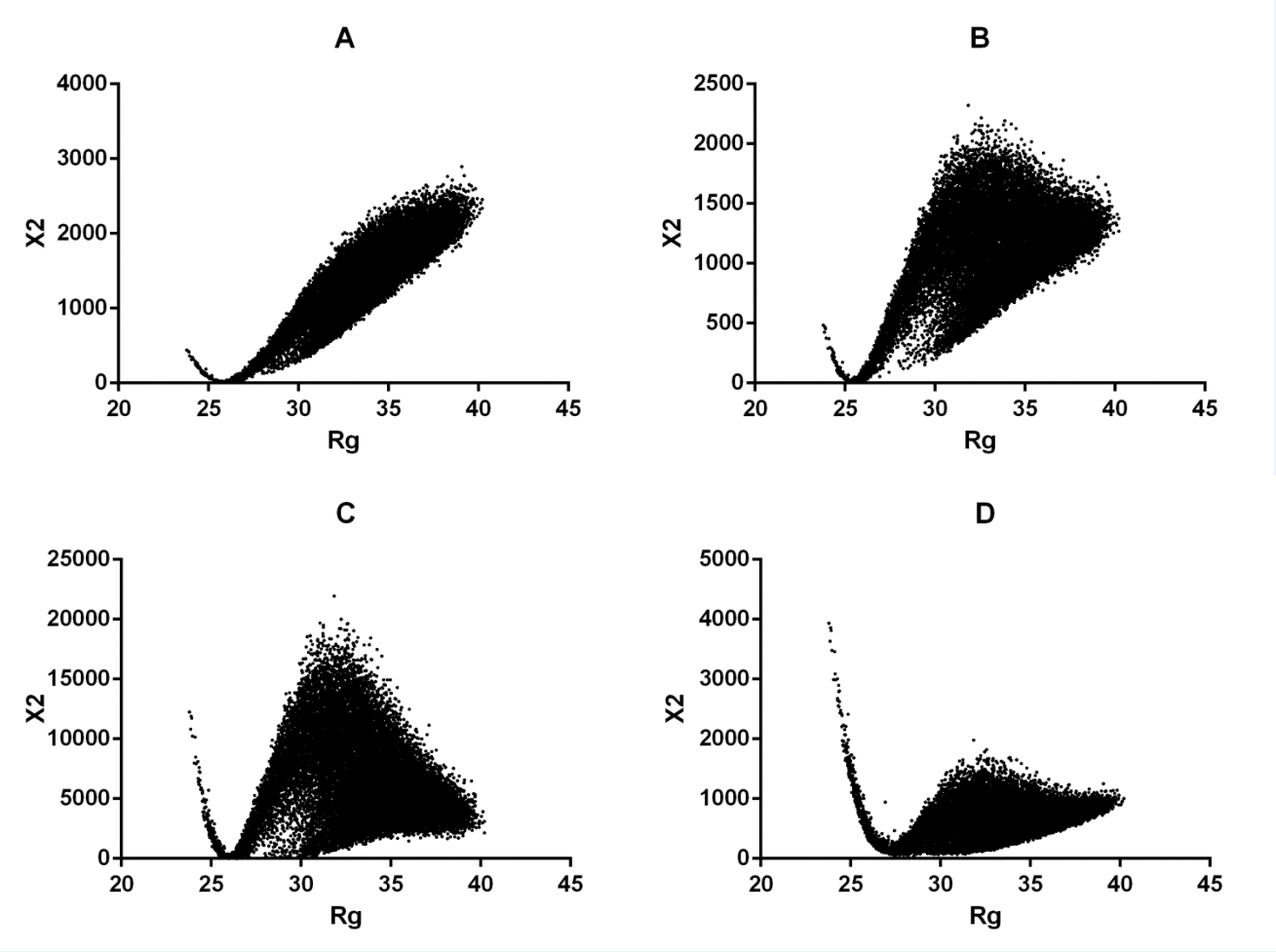
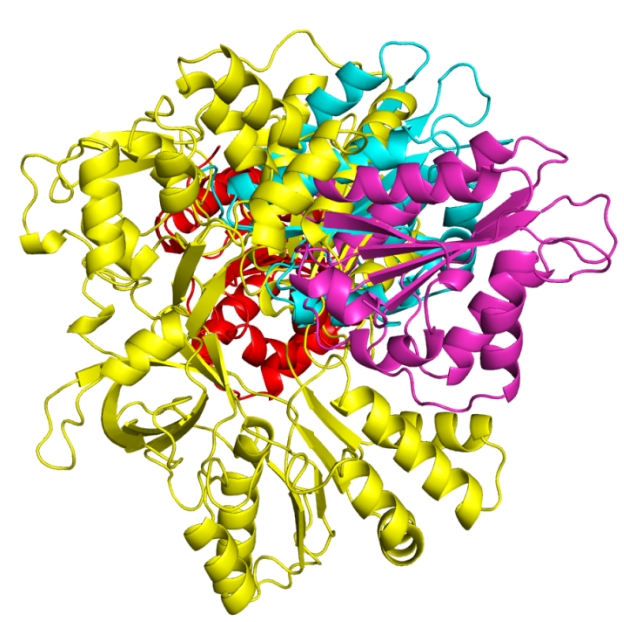
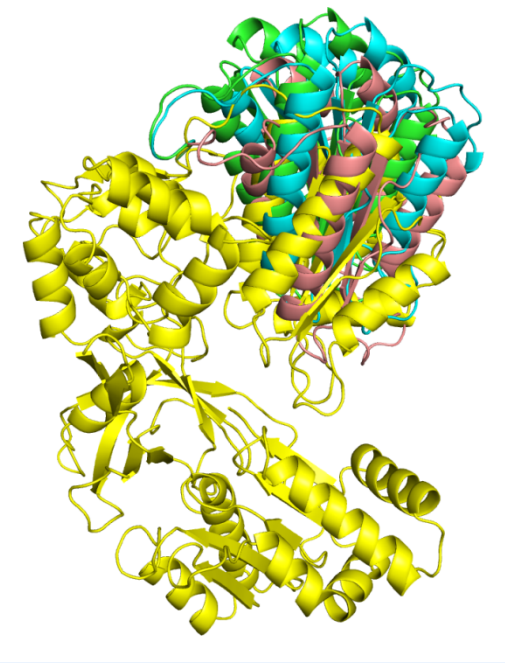
SASSIE



(A) Oxidised
(B) 1e$^-$ NADPH
(C) 2e$^-$ NADPH
(D) 2e$^-$ Dithionite

SASSIE

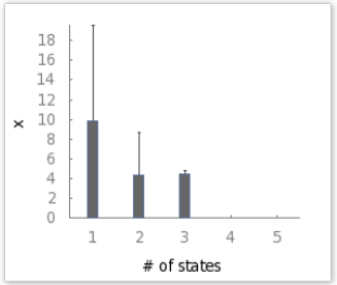


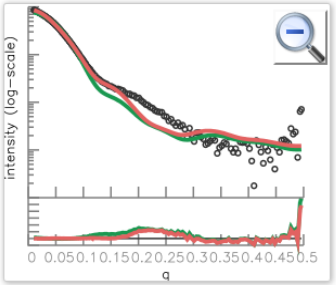


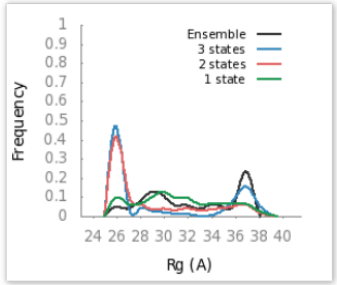
Best Fit Structures Determined Using the Chi-Squared Filter Functionality in SASSIE
The left hand structures are those that best fit the oxidised sample curve and the in the right hand best fit the $2e^-$ dithionite sample. The yellow structure in both represents the crystal structure (PDB code: 3QE2) as a point of reference

MultiFoXS

Input protein	Flexible residues	SAXS profile	e-mail address	# of conformations	Download results	
input.pdb	3qe2flexibleregions.txt	PKR_Dithio_2emod22222222.dat	slf24@le.ac.uk	103	conformations	MultiFoXS output files

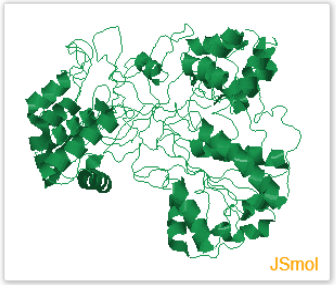






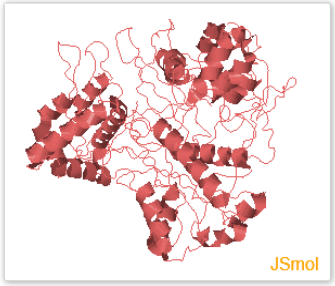
Best scoring 1-state model $\chi = 9.78$ $c_1 = 0.99$ $c_2 = -0.50$ ☑ show/hide weighted profile

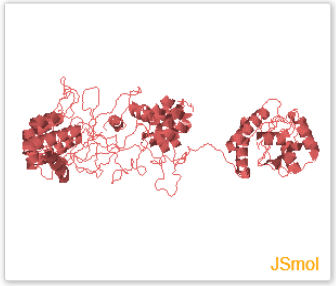
PDB1 $R_g = 26.3644$ $w_1 = 1$



Best scoring 2-state model $\chi = 4.39$ $c_1 = 0.99$ $c_2 = -0.50$ ☑ show/hide weighted profile

PDB1 $R_g = 26.8166$ $w_1 = 0.567$ PDB2 $R_g = 37.7858$ $w_2 = 0.433$



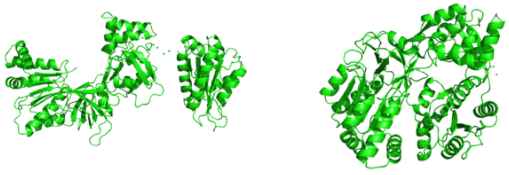


MultiFoXS

Full MultiFoXS Dataset using pool of 10,000 structures (generated using RANCH from the ATSAS suite)
Max q-value = 0.3
WILDTYPE
Oxidised

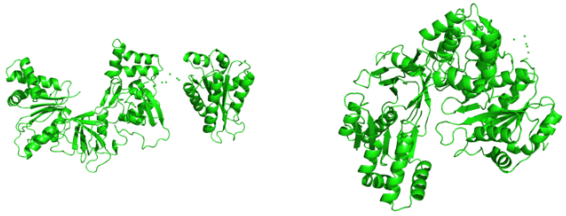
0.747 left: 0.253 right

2e- Dithionite



0.849 left: 0.154 right

2e- NADPH



0.579 left: 0.421 right

- Offline protocol modified for SANS data
- Better fits and 2-state system always fit best
- 2-model “pool” also yields good fits

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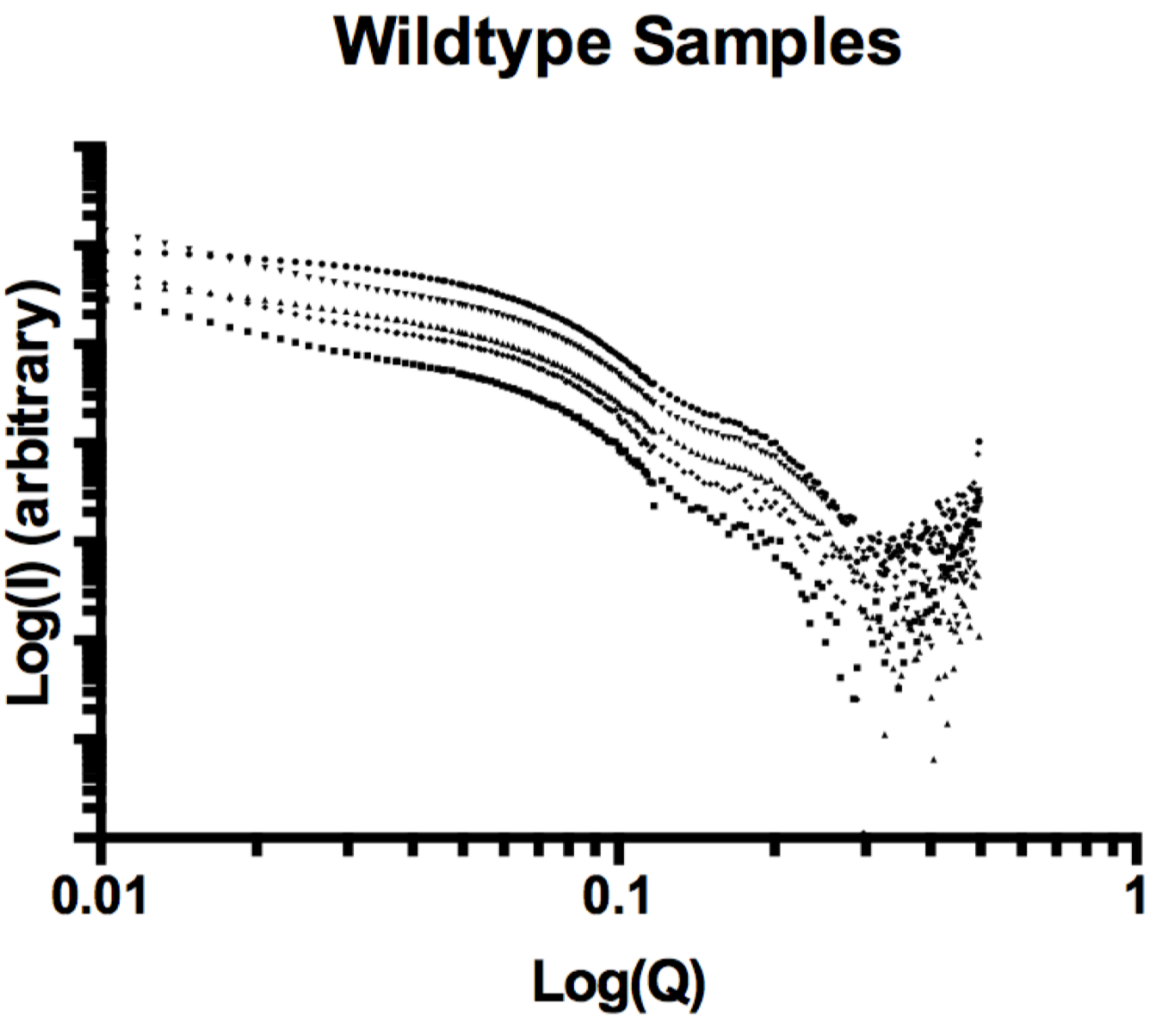
Problems



- WT
- Dithio 2e
- Dithio 4e
- NADPH 2e
- Dithio 2e + NADP+

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Conclusions

- A better understanding of the conformational equilibrium
  - Defined shapes
  - The position of the equilibrium
  - Unambiguously defined redox state (no x-rays)
- A revised catalytic cycle
  - The role of domain motion in each step
- Use of mutants to understand the relation of kinetic parameters to the conformational equilibrium

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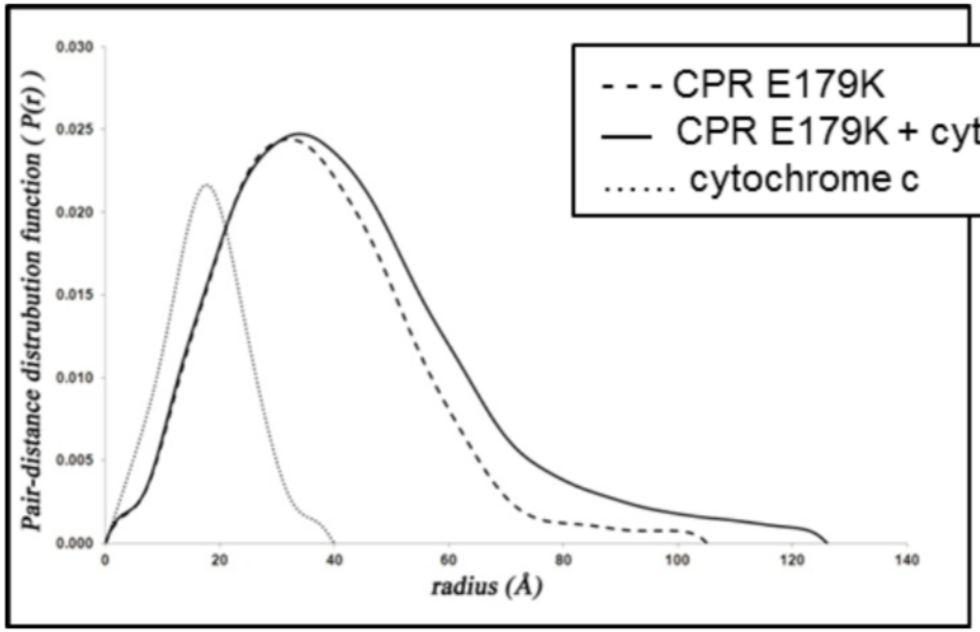
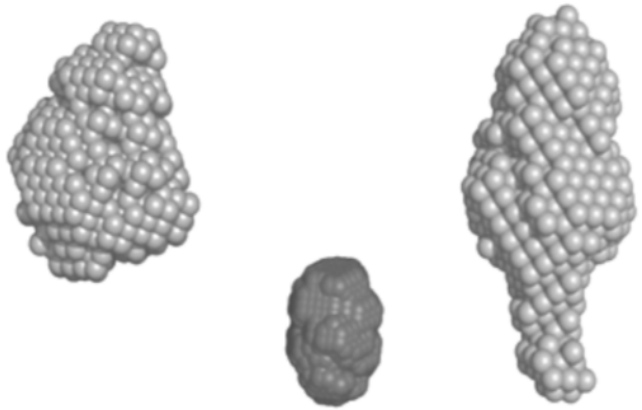
The grand scheme

- Completion of the definition of the conformational equilibrium – repeats of some earlier samples and possible investigation of the full length protein.
- Solution studies of CPR + cytochrome c complex in solution (deuterated CPR)
- Integration of CPR into the membrane (nanodisc or traditional bilayer) to study with cytochromes P450 (physiological partner); SANS or reflectometry may be possible

Cytochrome c complex experiment





DAMMIN models from SAXS data. *Left*, CPR E179K alone; *centre*, cytochrome c; *right*, the CPR E179K – cytochrome c complex

- Deuteration of mutant sCPR (almost) completed
- SANS will yield an unambiguous result

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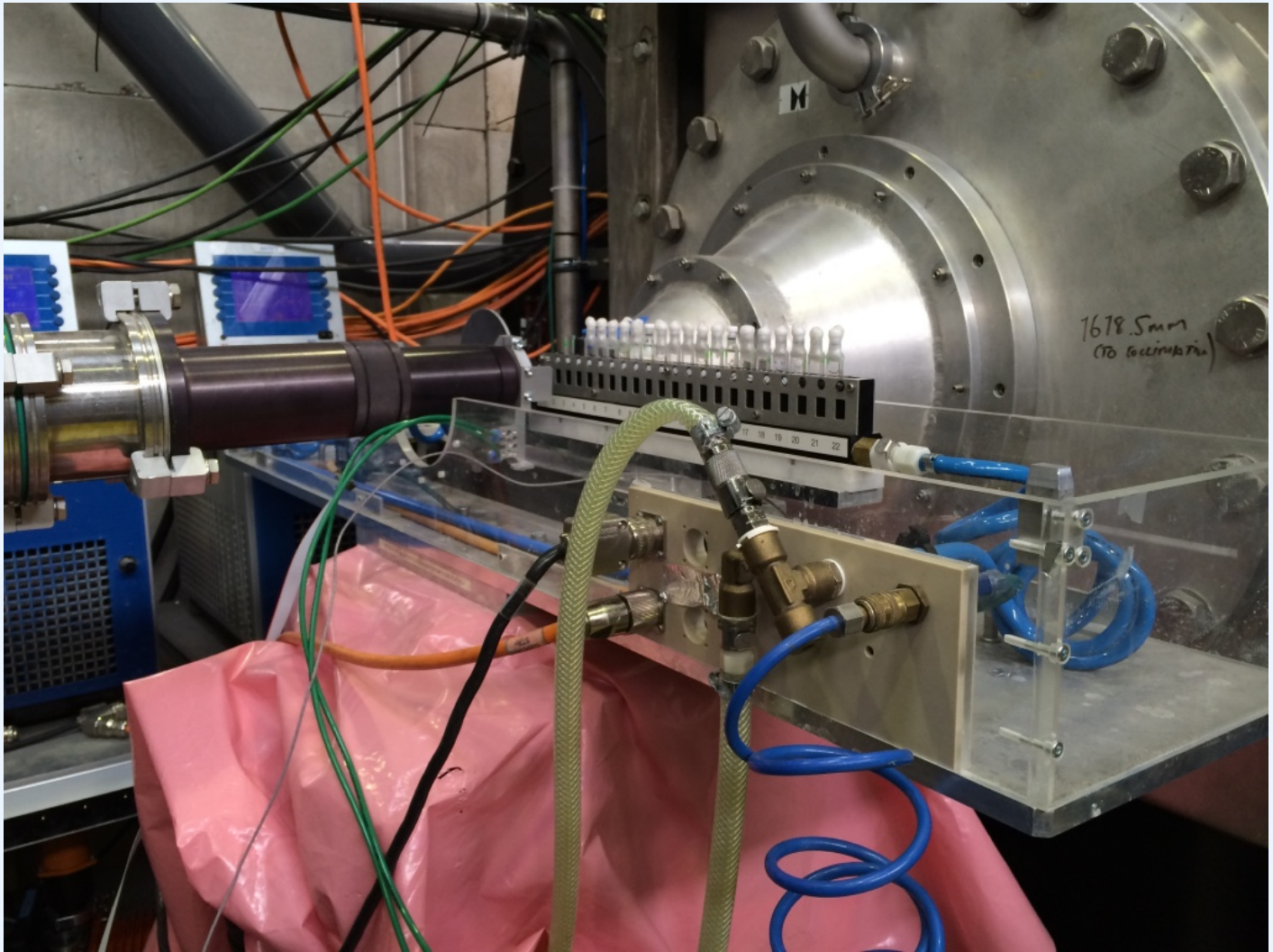
Thank you for listening



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Wildtype						
Sample	Rg	Rg Error	I0	I0 Error	Dmax	Quality (%)
Oxidised	27.800	0.150	0.076	0.000	75.000	66.500
Dithionite 1e-	28.700	0.284	0.700	0.001	76.920	66.400
Dithionite 2e-	30.000	0.582	0.051	0.001	84.000	73.100
Dithionite 4e-	30.000	0.451	0.068	0.001	76.500	66.500
Dithionite 2e- + NADP+	27.900	0.476	0.023	0.000	82.550	95.900
NADPH 1e-	28.700	0.284	0.070	0.001	75.500	66.400
NADPH 2e-	28.943	0.272	0.081	0.001	77.120	66.300
EPVP						
Sample	Rg	Rg Error	I0	I0 Error	Dmax	Quality (%)
Oxidised	27.445	0.518	0.062	0.000	90.320	92.900
Dithionite 1e-	28.522	1.049	0.054	0.000	97.830	91.500
Dithionite 2e-	28.450	0.618	0.084	0.000	91.570	92.100
Dithionite 4e-	28.662	1.334	0.069	0.000	98.670	66.900
Dithionite 2e- + NADP+	30.409	1.221	0.089	0.001	76.470	66.500
NADPH 1e-	27.002	0.542	0.058	0.000	95.010	89.700
NADPH 2e-	27.551	1.484	0.073	0.000	90.030	71.100
PKR						
Sample	Rg	Rg Error	I0	I0 Error	Dmax	Quality (%)
Oxidised	26.117	0.365	0.068	0.000	89.450	93.500
Dithionite 1e-	27.200	0.112	0.068	0.000	90.620	96.400
Dithionite 2e-	29.000	0.171	0.083	0.000	99.500	93.000
Dithionite 4e-	29.200	0.197	0.076	0.000	95.730	93.400
Dithionite 2e- + NADP+	27.800	0.128	0.076	0.000	95.160	94.500
NADPH 1e-	25.800	0.110	0.061	0.000	83.500	80.700
NADPH 2e-	27.200	0.118	0.071	0.000	93.590	94.000

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Confirmation of fit (CRYSON)