**Heme peroxidase-Trapping intermediates by cryo neutron crystallography**

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**Abstract**

By combining the normal practice for X-ray crystallography of collecting diffraction data at 100 K with neutron crystallography the structures of cryo-trapped enzyme intermediates have been determined, revealing the positions of the previously hidden hydrogens that are essential to a better understanding of the involved mechanism.

**Introduction**

Heme-containing enzymes moderate many redox processes *in vivo* including protection from peroxides, drug metabolism and electron transfer, *in vitro* they have many biopharmaceutical applications. The reaction of heme enzymes all proceed through high-valent ferryl (FeIV) intermediates known as Compound I and Compound II. Understanding the chemistry of these intermediates through their structure requires a knowledge of the protonation states of the ferryl heme and the amino acid residues involved in catalysis. Properly characterising these unstable intermediates has only been possible with neutron crystallography at cryogenic temperatures. Here we describe how this work was done and the additional experiments that validate the interpretation of the results.

X-ray crystallography has been used to solve the structures of key examples of these enzymes (Patterson & Poulos, 1995; Pelletier & Kraut, 1992; Poulos et al., 1980) and the X-ray structures of several intermediates had been looked at by various means (Berglund et al., 2002; Bonagura et al., 2003; Fulop et al., 1994), this work provided the framework to understanding the mechanisms of enzyme reactions. However, X-ray methods have important limitations, especially when studying redox enzymes. Firstly, X-ray are scattered by the atomic electrons and so electron-poor hydrogen atoms are difficult or impossible to locate with confidence, even at sub-Ångstrom resolution. Protonation states of bonded atoms can sometimes be deduced from the bond lengths of the connected atoms (as single bonds are longer than double bonds), this requires sufficient accuracy in atomic positions to enable unambiguous distinction. Of course, this still does not necessarily give hydrogen positions. When the environment contains a relatively electron dense atom (as is the case for heme-enzymes) there may be additional distortions in apparent positions (Fülöp, Watmough, & Ferguson, 2000). The electrophilic nature of the heme centre means that the high-valent intermediates discussed here (and in redox enzymes in general (Kwon, Smith, Raven, & Moody, 2017)) are particularly susceptible to the reducing effects of X-rays. Therefore, a trapped intermediate in a crystal may change its nature during the course of data collection, further complicating the interpretation of maps. Unsurprisingly, the nature of the intermediates remained uncertain, with conflicting bond distances for the key iron oxygen distance reported. The use of low doses on multiple crystals, and experiments to exploit the photo-reduction induced chemical changes provided some consensus, but not confidence.

Fortunately, neutron crystallography can place hydrogen (as deuterium) at modest resolutions and the cold or thermal neutrons used do not perturb the chemistry. Neutrons are scattered by the nuclei rather than the electrons and so this is independent of atomic number. Thus deuterium has a similar scattering cross section as compared to the heavier atoms (e.g. carbon, nitrogen, oxygen etc) that form proteins, and can be observed equally well.

However, neutron crystallography has its own problems. The relatively (to X-rays) weak neutron flux available at the limited number of suitable reactors or spallation sources means that obtaining crystal diffraction data requires long experiments on very large crystals on scarce resources. The data recorded are often weak and lacking in completeness. This in turn means that the quality of maps (and their interpretation) may be limited, this may be partially overcome by incorporating X-ray data into joint refinements. Replacing hydrogen with deuterium might also not be trivial. Historians might compare the speed of data collection for neutron crystallography to what was done some 50 years ago with X-rays.

In this article, examples of the heme peroxidases will be discussed, showing how spectroscopic monitoring of intermediate formation and stability has allowed the cryo-trapping of the intermediates, Compound I (Casadei et al., 2014) and Compound II (Kwon et al., 2016). This has allowed the determination of these structures using combined neutron and X-ray crystallography methods. These studies have shown the nature of the ferryl heme and active site in these intermediates, revising the accepted enzyme mechanism.

**Neutron cryo crystallography**

Neutron crystallography is the only technique where diffraction data can be collected at room temperature without radiation damage to the samples. Despite being routine for X-ray work, the collection of neutron data at cryogenic temperatures is usually not required to extend the lifetime of the crystals as neutron beams do not cause any measurable damage to crystals. Nonetheless, the ability to collect data at cryogenic temperatures has several potential advantages, although it also presents some other challenges. As the temperature is reduced the thermal motion of atoms is also reduced, thereby improving the Bragg diffraction of crystals which leads to improvement in nuclear density definition or the potential for reducing the size riquirements of crystals. In cryogenic conditions less stable proteins will not degrade and there is no risk of the crystals drying out. The widespread use of cryogenic environments for protein X-ray crystallography has resulted in robust cooling technology and standard mounting. However, the relatively large size of crystals means that necessarily the cooling will take longer than for smaller crystals, increasing the risk of crystal damage through the stresses on contraction or that vitrification may be slow enough to form ice crystals. The long data collection times also increases the chances of ice formation on the exterior of the sample. In the context of the work described here, cryo-crystallography allows the recording of diffraction data from labile intermediates.

Using neutron cryo-crystallography, we were able to determine the structures of the Compound I of cytochrome *c* peroxidase (C*c*P) and Compound II of ascorbate peroxidase (APX) respectively, resolving the previously ambiguous interpretations of their structures and provoking a re-assessment of the catalytic steps.

**Heme peroxidases**

Heme peroxidases are widespread in biology. They have played key roles in expanding our understanding of structural and mechanistic biochemistry which are used to develop rapid reaction methods. Heme peroxidases share a common catalytic cycle and the general processes involve the catalytic pathway that are well established They catalyse reduction of peroxide to water using high oxidation state intermediates called compound I (CI) and compound II (CII) (Scheme 1). A key challenge has been to clarify the precise nature of the ferryl heme species as the literature reports conflicting evidence (Groves, 2014; Groves & Boaz, 2014; Karlin, 2010; Sligar, 2010).

In our study, we focused on yeast cytochrome *c* peroxidase and soybean ascorbate peroxidase.

Cytochrome *c* peroxidase (C*c*P) is a mitochondrial electron transport chain enzyme with a presumed role in removing the damaging peroxides formed. Originally isolated from yeast in the 40s (Altschul, Abrams, & Hogness, 1940), C*c*P has been studied extensively and there is a well-established protocol to grow large protein crystals (Yonetani, Chance, & Kajiwara, 1966). It was the first heme peroxidase to have its structure solved by X-ray crystallography in 1980 (Poulos et al., 1980). It was one of the first heme enzymes for which oxidised intermediates were observed (George, 1953). Reaction with hydrogen peroxide initially forms the Compound I intermediate, which is metastable in the absence of an electron donor, in this reaction the distal histidine was shown to have a crucial role (Erman et al., 1993). Compound I of C*c*P decays with a half-life of a few hours at room temperature (Fulop et al., 1994)

Soy ascorbate peroxidase (APX) is one of the newest enzymes to have joined the scene of heme peroxidase research. Peroxidases are abundant in plants and scavenge hydrogen peroxide by oxidation of small organic molecules such as ascorbic acid. In particular, a structure of APX-ascorbic acid complex structure provided the first identification of a non-protein physiological substrate binding site for a heme peroxidase (Sharp, Mewies, Moody, & Raven, 2003). Upon reaction with hydrogen peroxide, the Compound I intermediate quickly decays to the less unstable Compound II state, the difference in stability of the reaction intermediates of C*c*P and APX being ascribed to formation of the radical placed on an adjacent tryptophan in the case of C*c*P and on the heme for APX (see Moody and Raven (2018) for a fuller discussion). APX compound II has a half-life of around 500s at room temperature.

The ferryl intermediates are of particular interest as the chemical nature of the ferryl heme group (FeIV-OH or FeIV=O) controls the reactivity of the enzymes (Scheme 2) The transient nature of these intermediates makes a structural study difficult. However, UV-Vis spectroscopy has long been used to characterise and study these enzymes in solution and this can be adapted to follow the reactions in the crystal. This idea was first used to demonstrate that crystals of chymotrypsin did indeed retain enzymatic activity (Rossi & Bernhard, 1970).

Establishing the nature of these transient intermediates had proven to be troublesome. Initially the majority of studies focused on measurement of the Fe-O distance or bond strength using various spectroscopic methods, a short distance being consistent with an Fe=O double bond, and a longer one indicating an Fe-OH single bond. These indirect methods produced ambiguous and conflicting results. In addition, most of the spectroscopic techniques suffer from the complication of laser induced photoreduction which introduced further complications in interpretations of the results.

The ability to react crystals of CcP with hydrogen peroxide to form the Compound I intermediate offered the possibility of measuring the distance directly, if diffraction data could be collected sufficiently quickly. Fulop et al. (1994), using a flow-cell with single-crystal spectroscopy to follow the reaction at 6 °C, collected data rapidly using the Laue method and 300-400 ms exposures, thus mitigating the effects of X-ray photoreduction. Depending on the protocol used they refined a value of 1.7-1.9 Å for the Fe-O distance (Fulop et al., 1994). The use of single-crystal spectroscopy and X-ray induced photoreduction with multiple passes synchrotron data collection was used to create a “movie” of the horse radish peroxidase reaction, interpreting an Fe-O distance of 1.7Å for Compound I and 1.8Å for compound II (Berglund et al., 2002). From a crystal of cryo-trapped C*c*P compound I, monitored before and after data collection by UV-Vis and EPR spectroscopy an Fe-O distance of 1.87 Å was reported (Bonagura et al., 2003). However, subsequent work limiting X-ray exposure using multiple crystals, combined with single crystal spectrophotometry, showed that the Fe-O distance increased from 1.63Å to 2.02Å with photoreduction during data collection, and by using the same technique of minimal exposures and multiple crystals, cryo-trapped and unreduced Compound II of APX was seen to have an Fe-O distance of 1.84Å. These results indicate Compound I is Fe=O , whilst Compound II is Fe-OH (A. Gumiero, Metcalfe, Pearson, Raven, & Moody, 2011; Andrea Gumiero, Murphy, Metcalfe, Moody, & Raven, 2010). However, there remained a suspicion that the Compound II structure was actually ferric hydroxide (FeIII-OH) rather than the FeIV intermediate. Despite the use of SHELX full matrix refinement to give positional ESUs, there remains the caveat that the iron-oxygen distances observed might be distorted by the close proximity of electron-dense iron to the oxygen (Einsle et al., 2002; Fülöp et al., 2000; Seiffert et al., 2007).

**Neutron Structure of Compound I of C*c*P**

Using neutron crystallography to observe hydrogen (as deuterium) is the obvious way to resolve the true nature of the intermediates. Neutron diffraction data was first collected at LADI-III at ILL (Blakeley et al., 2010) on ferric (i.e. resting state) C*c*P crystals that had been soaked in D2O to allow exchange. This used the quasi Laue method, whereby 19 “stills” at different orientations and ~23 hours exposure (each) were recorded using a wavelength range of 3.2 - 4.2 Å and cylindrical image-plate detector to give 2.4 Å data. The joint refinement including X-ray data clearly showed the distinctive banana-shaped density of D2O bound to heme iron. It also confirmed that the distal histidine was in the neutral state. Once the parameters necessary to monitor and cryotrap Compound I in C*c*P crystals had been established, neutron data was collected at the BIODIFF monochromatic neutron beam line at FRM-II (Ostermann & Schrader, 2015). This beam line having established the protocols needed to keep crystals at 100 K for the several hundred hours data collection which it takes to record a set of data from this intermediate. A total of 268 images using the rotation method (0.3° each) gave 2.5 Å data. After joint refinement with X-ray data, the neutron density of an oxygen could be seen clearly, establishing the identity of Compound I of C*c*P as Fe=O (Figure 1). Necessarily complimentary validation was required to establish the stability and identity of the intermediate in the crystal. Accordingly, the single-crystal UV-Vis and EPR spectra were recorded and then re-measured after 20 days storage in liquid nitrogen. Not only did this work establish the identity of the ferryl oxygen intermediate, it also showed that the distal histidine is in the positively charged state, this and the revealed hydrogen-bond structure meant that the mechanism of Compound I formation had to be revised (Casadei et al., 2014; Groves & Boaz, 2014).

**Neutron Structure of Compound II of APX**

Unlike C*c*P, previously grown crystals of APX tended to be small for neutron work (with a volume ~0.001mm3 (Macdonald, Badyal, Ghamsari, Moody, & Raven, 2006), and so the hanging drop crystallisation condition had to be systematically refined, this allowed crystals as large as 1.0 x 0.6 x 0.4 mm to be grown. In order to trap Compound II in APX the ferric enzyme crystals (grown in D2O) were reacted with *m*-chloroperbenzoic acid (CPBA), this first forms Compound I which rapidly decays to Compound II which is stable for long enough (~500s) to trap in liquid nitrogen. This method avoids the possibility of also forming Compound III (the peroxo enzyme) by reaction with excess H2O2. The UV-Vis and EPR spectra confirmed the intermediate formation and that it is stable in liquid nitrogen for at least 20 days. Furthermore, X-ray fluorescence scans of both ferric and Compound II crystals verified the higher oxidation state of the iron with the shift to higher energy. Initial tests on BIODIFF at FRM-II (Ostermann & Schrader, 2015) were followed by neutron data collection at 100 K using LADI-III at ILL (Blakeley et al., 2010), which has been modified to enable a cryogenic gas stream to flow over the crystal. Neutron diffraction data was collected using the quasi-Laue method and 15 exposures of 22.3 hours at different orientations. To minimise the effects of photoreduction, only the first 10° of data from 10 different crystals was collected on the home source and merged to provide the X-ray data needed for joint refinement. The neutron map clearly showed cylindrical density corresponding to OD, with an Fe-O distance of 1.87 Å in close agreement with the earlier multi-crystal work (A. Gumiero et al., 2011). The work confirmed the identity of Compound II in APX is indeed FeIV-OH (Kwon et al., 2016) and that distal histidine is in the positive form, as it is in Compound I in C*c*P (Figure 1).

Neutron crystallography, combined with cryo-trapping and the use of a variety of spectroscopic techniques in the crystal and in solution has resolved long-standing uncertainties about the nature of the ferric intermediates in the two heme peroxidases studied (Scheme 3), and this has revealed further details of the structures that meant a re-consideration of the details of the reaction mechanism was required. This affirms that the extrapolation of hydrogen positions from X-ray work alone may be less reliable than commonly imagined.

Scheme 1

Overall reaction of heme peroxidases

1. FeIII + H2O2 → FeIV-O P∙ + H2O (Compound I)
2. FeIV-O P∙ + S → FeIV-O P + S∙ (Compound II)
3. FeIV-O P + S∙ → FeIII + H2O + S

Reaction with hydrogen peroxide first forms Compound I, formally oxidised by two electron equivalents over the ferric enzyme (where P∙ is a tryptophan radical in the case of CcP and the heme in APX). Compound I is then reduced to Compound II (oxidised by one electron equivalent) and then reduced to the ferric form with the release of water.

Scheme 2

The different stages in the reaction of C*c*P and APX, the possible Compound I and Compound II centres are circled.

Scheme 3

The nature of the intermediates of the heme peroxidases resolved by neutron crystallography.

Figure 1

The structures of the active centres of ferric CcP (left) showing bound water in the distal pocket, Compound I of CcP (middle) showing oxygen bound to form Fe=O, Compound II of APX (right) showing the Fe-OH structure.



Figure 1. Neutron crystal structure of (left) C*c*P Ferric at room temperature, showing bound water in the distal pocket, (centre) C*c*P compound I at 100 K showing oxygen bound to form Fe=O, and (right) APX compound II at 100 K showing the Fe-OH structure.

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