Finding Hydrogens: 
Elucidating the Redox Chemistry of Cholesterol Oxidase Through Neutron Diffraction Studies

Emily Golden BSc (Hons)

This thesis is presented for the degree of Doctor of Philosophy of Biochemistry of The University of Western Australia

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ABSTRACT

Cholesterol oxidase (COx) is a bacterial flavoenzyme catalysing the oxidation and isomerisation of cholesterol to choleste-4-en-3-one. This enzyme is utilised by bacteria in the first step of the degradation of cholesterol as a carbon source or in the depletion of cholesterol from eukaryotic membranes. In the oxidative reaction (reductive half-reaction) a proton is abstracted from the substrate hydroxyl group and a hydride transferred from the substrate to the tightly bound cofactor resulting in reduction of the cofactor and oxidation of the substrate. These reducing electrons are then passed to molecular oxygen to form hydrogen peroxide. While X-ray diffraction studies have provided significant insights into the role of the protein structure in the reaction mechanism, many questions still remain unanswered. A knowledge of the positions of hydrogen atoms in the reduced and oxidised forms of the enzyme would provide insights into the redox chemistry of COx, which involves the transfer of hydrogen atoms either in the form of hydrogen, hydrides or protons. X-ray scattering by atoms is proportional to the atomic number, therefore hydrogen atoms can only be seen in X-ray crystal structures at atomic resolution. Neutron protein crystallography (NPC) is a technique which can be used for visualising hydrogen atoms in crystal structures, as hydrogen atoms have a similar scattering magnitude to the heavier atoms in proteins (C, O, N, S) and thus can be visualised at moderate resolutions (1.5 Å - 2.5 Å). NPC studies of COx were pursued to determine the positions of these hydrogen atoms in the reduced and oxidised forms to investigate the redox chemistry of COx.

NPC requires very large crystal volumes compared to those used for X-ray diffraction at synchrotron radiation beamlines. The predominant reason for requiring large crystals is due to the low flux of beamlines. One way to increase the signal to noise ratio (SNR) of the experiment and therefore decrease the required crystal volume, is to use deuterium instead of hydrogen in the sample. Hydrogen has a negative neutron
scattering length, which causes density cancellation in regions where hydrogen is bonded to a positive neutron scatterer (C, N, O and S). Hydrogen also has a large incoherent scattering component which increases the noise in the experiment and therefore lowers the SNR. Deuterium, an isotope of hydrogen, has a positive scattering length, low incoherent scattering component and twice the scattering magnitude of hydrogen, eliminating density cancellation problems and increasing the SNR. Deuterium can be incorporated into protein structures using H/D exchange - where protein is soaked in D2O-containing buffers causing exchange of labile hydrogen atoms for deuterium- or by perdeuterating the protein - where only deuterium is available for protein expression resulting in a protein with all hydrogen atoms exchanged for deuterium.

To pursue the neutron diffraction structures of COx a process of macroseeding was developed to produce large crystals suitable for neutron diffraction. A 2.2 Å resolution structure of H/D-exchanged COx was obtained from one of these macroseeded crystals and revealed insights into substrate binding for efficient hydride transfer in COx. In particular, a deuteron was observed stabilised between the protein Gly120-N and FAD-N5 reactive centre. The negative dipole over the Gly120/Asn119 peptide bond which is inferred by the position of the deuteron, is induced by a positively charged lysine side chain. Furthermore, both of these interactions are conserved in other oxidoreductase enzymes indicating that this configuration is important for enzyme function. Additionally, an X-ray diffraction structure of the reduced enzyme was obtained revealing the location of the hydride transferred to FAD in the oxidation reaction. The hydride position was in a tetrahedral geometry on the N5 atom of FAD and density functional theory calculations revealed that the interaction between Gly120 and FAD-N5 serves to tetrahedralise the N5 centre by stabilising the lone pair of electrons of N5. Taken together, these results revealed an elegant
pre-formed active site which acts to position the substrate hydride donating orbital and the FAD receiving orbital simultaneously for efficient hydride transfer.

Neutron diffraction structures of the perdeuterated enzymes were also pursued. Perdeuterated protein was successfully expressed, purified and crystallised and a modified macroseeding technique produced large protein crystals. Functional and structural characterisation of the perdeuterated enzyme revealed no significant effects due to perdeuteration and confirmed that perdeuterated protein crystals were suitable for neutron diffraction studies. A 2.1 Å neutron diffraction structure was obtained which showed nuclear density for many of the deuterium atoms, however, crystal twinning decreased the quality of the maps limiting the structural insights that could be obtained from this structure. However, this indicated the viability of neutron diffraction studies of perdeuterated COx and further work will be conducted to improve the size and quality of perdeuterated crystals.
DECLARATION

I declare that this thesis comprises my own original work except where otherwise indicated in the statement of contributions.

_______________
Emily Golden
ACKNOWLEDGEMENTS

When I began this PhD I didn’t realise how deeply involved I would get with the project, how difficult it would be or how fulfilling it would be to finish. Now, at the end, I can see how much I have learnt in all aspects of my life and realise that I could not possibly achieve this without the support I have had from my mentors, family and friends.

First and foremost I would like to thank my supervisor, Professor Alice Vrielink. Alice has been a fantastic supervisor and mentor. She gave me close guidance especially when learning something new and then encouraged me to take control and direct my own work. Alice was always available for discussions and advice and had incredible patience as I improved.

I would also like to thank Dr. Anthony Duff for producing the perdeuterated protein and giving me the opportunity to visit and work in his lab at ANSTO. Anthony has also provided much advice and feedback about the results. Anthony’s help has been invaluable.

Dr. Flora Meilleur, who has spent a lot of neutron beamtime testing and collecting diffraction data on my crystals. She has spent a lot of time and effort helping me with this project and provided valuable advice for neutron diffraction which is especially helpful as neutron protein crystallography is a relatively small field.

Dr. Matthew Blakeley, who provided me the opportunity to visit one of the few neutron beamlines for protein crystallography in the world and produced the first neutron diffraction data of my crystals with all the inevitable challenges of this novel project.

All the members of the lab. I have spent many hours discussing the problems and solutions with all of you, and often it was these discussions that lead to a way
forward when it felt like I was at a dead-end. This has been an incredibly supportive, happy and fun lab to work in - discoveries need cake breaks!

My family who brought me up. Mum ignited my curiosity about the natural world, a love of the environment and a strong sense of social justice - you were not here for long but have had the strongest impact on my life. Dad, who provided a stable and loving home, especially through the rough times, and always encouraged me to aim high. Kelly and all my siblings who created a big, noisy family to come home to from school. Thank you for your love and support. You all helped me to get here in the first place.

And most of all my partner, Tim, who provided love and support and bore the brunt of the emotional rollercoaster. Who cooked and cleaned for me and put up with my mess. You have always been by my side. Without your support this would not have been possible.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$^1$H-COx</td>
<td>protiated COx</td>
</tr>
<tr>
<td>$^2$H-COx</td>
<td>perdeuterated COx</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ANSTO</td>
<td>Australian Nuclear Science and Technology Organisation</td>
</tr>
<tr>
<td>APV</td>
<td>amprenavir</td>
</tr>
<tr>
<td>AS</td>
<td>Australian Synchrotron</td>
</tr>
<tr>
<td>AZM</td>
<td>acetazolamide</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BZB</td>
<td>benzothiophen</td>
</tr>
<tr>
<td>CA</td>
<td>carbonic anhydrase</td>
</tr>
<tr>
<td>COx</td>
<td>cholesterol oxidase</td>
</tr>
<tr>
<td>COx-H6</td>
<td>hexa-histidine tagged COx</td>
</tr>
<tr>
<td>DAAO</td>
<td>D-amino acid oxidase</td>
</tr>
<tr>
<td>DFT</td>
<td>density functional theory</td>
</tr>
<tr>
<td>DHA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DHDFT</td>
<td>double-hybrid density functional theory</td>
</tr>
<tr>
<td>DPI</td>
<td>diffraction precision index</td>
</tr>
<tr>
<td>DSF</td>
<td>differential scanning fluorimetry</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FRM</td>
<td>Forschungsreaktor München (Munich research reactor)</td>
</tr>
<tr>
<td>GMC</td>
<td>glucose-methanol-choline</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HCA-II</td>
<td>human carbonic anhydrase II</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HFIR</td>
<td>high flux isotope reactor</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HR</td>
<td>high resolution</td>
</tr>
<tr>
<td>ILL</td>
<td>Institut Laue-Langevin</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>JAERI</td>
<td>Japanese Atomic Energy Research Institute</td>
</tr>
<tr>
<td>J-PARC</td>
<td>Japan Proton Accelerator Research Complex</td>
</tr>
<tr>
<td>LADI</td>
<td>Laue diffractometer</td>
</tr>
<tr>
<td>LANSCE</td>
<td>Los Alamos Neutron Science Centre</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LBHB</td>
<td>low-barrier hydrogen bond</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix assisted laser desorption ionization time-of-flight</td>
</tr>
<tr>
<td>MaNDi</td>
<td>macromolecular neutron diffractometer</td>
</tr>
<tr>
<td>NDF</td>
<td>National Deuteration Facility</td>
</tr>
<tr>
<td>NIP</td>
<td>neutron image plate</td>
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<tr>
<td>NMC</td>
<td>neutron macromolecular crystallography</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPC</td>
<td>neutron protein crystallography</td>
</tr>
<tr>
<td>ORNL</td>
<td>Oak Ridge National Laboratory</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PCS</td>
<td>protein crystallography station</td>
</tr>
<tr>
<td>PYP</td>
<td>photoactive yellow protein</td>
</tr>
<tr>
<td>RMSD</td>
<td>root-mean-square deviation</td>
</tr>
<tr>
<td>SHB</td>
<td>short hydrogen bond</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>SIHB</td>
<td>short ionic hydrogen bond</td>
</tr>
<tr>
<td>SNR</td>
<td>signal-to-noise ratio</td>
</tr>
<tr>
<td>SNS</td>
<td>spallation neutron source</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight</td>
</tr>
<tr>
<td>XI</td>
<td>xylose isomerase</td>
</tr>
</tbody>
</table>
AIMS

To obtain insight into the redox chemistry of cholesterol oxidase through comparison of the positions of hydrogen atoms in the reduced and oxidised enzyme states using the technique of neutron protein crystallography.

Neutron structures of protiated COx ($^{1}$H-COx) and perdeuterated COx ($^{2}$H-COx) in the oxidised and reduced states were pursued. In order to achieve the overall aim of the project the following sub-aims were pursued:

1. Obtain sufficient quantity and quality of protein for crystallisation
   a. Express, purify and crystallise $^{1}$H-COx and $^{2}$H-COx.
   b. Functional characterisation of $^{2}$H-COx for comparison with $^{1}$H-COx.

2. Produce large crystals suitable for neutron diffraction
   a. Macroseed $^{1}$H-COx and $^{2}$H-COx crystals.
   b. Structural characterisation of $^{2}$H-COx using X-ray diffraction for comparison with $^{1}$H-COx.

3. Develop techniques for the reduction of COx crystals
   a. Structural characterisation of reduced COx crystals using X-ray diffraction.

4. Obtain and compare the neutron diffraction structures of oxidised and reduced COx
   a. Neutron diffraction of $^{1}$H-COx and $^{2}$H-COx in the oxidised stated.
   b. Neutron diffraction of $^{1}$H-COx and $^{2}$H-COx in the reduced state.
The following people have contributed to the work contained in this thesis:

E.G.  Emily Golden
A.V.  Prof. Alice Vrielink
A.P.D.  Dr. Anthony Duff
P.V.A.  Prof. Paul Attwood
A.K.  Dr. Amir Karton
F.M.  Dr. Flora Meilleur
M.P.B.  Dr. Matthew Blakeley

**Chapter 1.** Presents a brief overview of the structural and functional understanding of COx with emphasis on COx from *Streptomyces SA-Coo*. Further information is also presented in the introductions of later chapters.

**Chapter 2.** Presents a review of the literature of single crystal neutron macromolecular crystallography with emphasis on the previous five years of research in the field. An overview of the development of neutron protein crystallography is given, followed by a discussion of some recent structure examples. This review was published in the *Australian Journal of Chemistry*. E.G. wrote the paper with revisions provided by A.V.

**Chapter 3.** Presents the reduced X-ray structure of cholesterol oxidase. This chapter is published in *Acta Crystallographica D*. This work presents the cloning, expression, purification, and crystallisation of protiated COx (\(^1\)H-COx), as well as describing the technique for reducing COx crystals. E.G. performed all experimental work and wrote the paper. A.K.
performed the computational work and analysis (DFT) and provided the
text describing the computational work. A.V. provided critical revisions.

Chapter 4. Presents the expression, purification, and crystallisation of perdeuterated
COx (\(^2\text{H-}\text{COx}\)). Also, presented is the technique for macroseeding of
both \(^1\text{H-}\text{COx}\) and \(^2\text{H-}\text{COx}\) crystals. Characterisation of \(^2\text{H-}\text{COx}\) and
X-ray structures of \(^2\text{H-}\text{COx}\) crystals are presented to show the viability of
perdeuteration for structural studies of COx. This chapter has been
published in *Analytical Biochemistry*. Perdeuterated enzyme was
produced by A.P.D. and E.G. E.G. did all other experimental work.
Determination of kinetic parameters for the enzyme assays were provided
by P.V.A. E.G. wrote the paper with critical revisions provided by A.V.,
A.P.D. and P.V.A.

Chapter 5. Presents the neutron structure of \(^1\text{H-}\text{COx}\). This chapter has been
submitted to the *Journal of the American Chemical Society*. E.G
produced the protein crystals for neutron diffraction. F.M performed the
neutron diffraction data collection and data processing. E.G performed
the refinement and analysis of the data with guidance and suggestions
from A.V., F.M., A.P.D., and M.P.B. E.G. wrote the paper with critical
revisions provided by A.V., F.M., A.P.D., and M.P.B.

→ Note: A \(^1\text{H-}\text{COx}\) neutron dataset was also collected by M.P.B. at
the Institute Laue-Langevin where E.G. also learnt to process
quasi-Laue neutron diffraction data. However, due to technical
issues only the data that was subsequently obtained at the ORNL
was included in this thesis.
Chapter 6. Presents the neutron diffraction structure of oxidised $^2$H-COx. E.G. produced the crystals for neutron diffraction. F.M. performed the neutron diffraction and data processing. E.G. analysed the neutron maps and wrote the chapter with revisions provided by A.V.

Chapter 7. Presents a general discussion of the thesis.
Chapter 1

INSIGHTS FROM THE X-RAY STRUCTURES OF CHOLESTEROL OXIDASE
1.1. Introduction

Cholesterol oxidase (COx) is a bacterial flavin adenine dinucleotide (FAD) dependent enzyme which catalyses the oxidation and isomerisation of cholesterol to cholest-4-en-3-one with the concomitant production of hydrogen peroxide ($H_2O_2$) (E.C 1.1.3.6). COx was first identified in *Norcadia erythropolis* (Turfitt, 1944) but has since been found to be expressed by a variety of bacteria including many soil bacteria. COx catalyses the first step in the degradation of cholesterol which allows the bacteria to use cholesterol as a carbon and/or energy source. The enzyme is most often expressed as a secreted enzyme (*Schizophyllum commune* (Fukuyama & Miyake, 1979), *Pseudomonas sp.* (Rhee *et al.*, 1991), *Streptomyces hygroscopius* and *Brevibacterium sterolicum* (Gadda *et al.*, 1997) and *Rhodococcus equi*) but several intracellular, membrane-associated forms of COx have also been identified (*Corynebacterium cholesterolicum* (Shirokane *et al.*, 1977) *Nocardia rhodocrous* (Buckland *et al.*, 1976) and *Brevibacterium sterolicum* (Uwajima *et al.*, 1974)). COx is widely used in laboratories for the determination of serum cholesterol levels (Richmond, 1976) which is particularly relevant for the diagnoses and monitoring of arteriosclerotic diseases. COx has also been used to probe the cholesterol content of high density lipoprotein (Sugiuchi *et al.*, 1995), erythrocytes (Teng & Smith, 1995), as well as gallstones (Wei *et al.*, 1989) and bile (Bocos *et al.*, 1992). COx immobilised on biocompatible matrices (Ahmad *et al.*, 2012; Ruecha *et al.*, 2014) offers a fast and reliable method for cholesterol determination for a range of applications such as a disposable biosensor for determination of whole blood cholesterol (*Fang et al.*, 2011). Recently, a highly sensitive and specific cholesterol biosensor was developed using COx immobilised on $\alpha$-Fe$_2$O$_3$ nanoparticles (Umar *et al.*, 2014). Additionally, COx from *Streptomyces SA-COO* is effective against boll weevil larvae which are particularly damaging to cotton crops; ingestion of COx by the larvae results in lyses of the gut epithelia and
therefore death of the larvae (Purcell et al., 1993). One of the advantages of using COx in clinical and biotechnological applications is the high temperature optimum (~40-60°C) and large pH stability range (~pH 4-10) as well as the ability to overexpress the enzyme. This also makes COx a particularly attractive enzyme as a model for flavin-dependent oxidoreductases and flavin-dependent enzymes in general.

COx catalyses three chemical reactions; the reductive half-reaction, isomerisation and the oxidative half-reaction (Fig. 1.1). The reductive half-reaction is catalysed by the FAD cofactor and results in the oxidation of the 3β hydroxyl group of the substrate and reduction of the FAD cofactor. This is followed by the isomerisation of the substrate double bond at the Δ5-Δ6 position. The oxidative half-reaction involves transfer of the redox equivalents from the reduced cofactor to molecular oxygen (O₂), producing H₂O₂. The FAD cofactor which catalyses the redox reactions of COx is usually tightly but non-covalently bound (Type I) but may also have a covalent attachment to the protein through a histidine residue to the 8-methyl group FAD (Type II). While the Type I and Type II enzymes catalyse the same reaction, they share little sequence similarity with only 11% identity between the COx from Streptomyces SA-COO and Brevibacterium sterolicum*. A Type I COx from Streptomyces hygroscopius and Type II COx from Brevibacterium sterolicum were characterised and showed marked differences in the FAD absorption spectra and redox midpoint potentials (Gadda et al., 1997). The covalent linkage in the Type II COx increases the redox potential of the flavin compared to a mutant form without this linkage (Motteran et al., 2001). These differences are due to structural variations between the two enzymes in both the protein microenvironment around the cofactor which modulates the redox potential, and the active site residues which are important for catalysis.

* Based on ClustalW2 alignment of UniprotKB accession numbers P12676 and Q7SID9
Only the Type I COx from *Streptomyces SA-COO* (Lario *et al*., 2003) and *Brevibacterium sterolicum* (Li *et al*., 1993) and the Type II COxs from *B. sterolicum* (later reclassified as *Rhodococcus equi*) (Coulombe *et al*., 2001) and *Chromobacterium sp. DS-1* (Sagermann *et al*., 2010) structures have been determined by X-ray crystallography. Along with the low sequence similarity, the structures of the Type I and Type II COxs show a different overall topology with the two forms of enzyme having a different fold and active site architecture, and utilizing different protein residues for the redox reactions. The Type I COx is a member of the

**Figure 1.1. The reactions catalysed by COx**

Cholesterol is oxidised by COx in the reductive half-reaction resulting in reduced FAD. This reaction is followed by isomerisation and the enzyme is regenerated by molecular oxygen to produce hydrogen peroxide.
glucose-methanol-choline (GMC) oxidoreductase family and shows similarities in sequence and fold to other members of this family, although they catalyse different chemical reactions (Fig. 1.2). In particular, the key catalytic residue for oxidation of the substrate (His447, discussed later) is conserved in hydroxynitrile lyase (Dreveny et al., 2001), glucose oxidase (Kiess et al., 1998), pyranose-2-oxidase (Bannwarth et al., 2004), cellobiose dehydrogenase (Hallberg et al., 2003) and formate oxidase (Doubayashi et al., 2011). COx from Streptomyces SA-COO has been the most extensively characterised COx with several high resolution X-ray structures in combination with kinetic analysis of active site mutants (Chen et al., 2008; Lyubimov et al., 2009; Lyubimov et al., 2007; Yin et al., 2001; Yue et al., 1999). COx from Streptomyces SA-COO is the subject of this thesis and the remainder of this chapter will focus on the structural insights into the mechanism of this enzyme.

Figure 1.2 Secondary structure and fold of COx and GMC-oxidoreductases

(a) Superposition of COx (magenta), aryl-alcohol dehydrogenase (green) and hydroxynitrile oxidase (yellow). (b) The tertiary structure of a Type II COx. PDB IDs are 1N1P (Lario & Vrielink, 2003), 3FIM (Fernandez et al., 2009), 1KDG (Hallberg et al., 2002) and 1I19 (Coulombe et al., 2001), respectively.
1.2. Reaction Mechanism

COx is the only bi-functional member of the GMC oxidoreductase family which catalyses a second reaction after oxidation. Extensive characterisation of the COx from *Streptomyces SA-COO* has provided detailed insight into the role of the protein structure in the reactions catalysed by COx and provides a good model for understanding flavoprotein redox chemistry. Figure 1.3 shows some of the catalytically important residues in the active site region of COx.

![The active site of COx](image)

**Figure 1.3. The active site of COx**

The FAD cofactor and catalytically important residues are shown in sticks with green carbon atoms. Helix 10 and helix 1 are also indicated as they play a role in stabilisation of negative charges on the FAD.

1.2.1. Substrate Oxidation (Reductive Half-Reaction)

Substrate oxidation occurs via a hydride transfer mechanism. This mechanism proceeds via a concerted rupture of the substrate hydroxyl bond and the C3-H bond. An active site base abstracts the hydroxyl proton, causing delocalisation of electrons onto the C3 carbon, which enhances the transfer of the hydride to the FAD (Fig. 1.4). This
would not otherwise occur due to the high pK$_a$ of the hydroxyl group (>15). Initial X-ray structures suggested that His447 was the general base for proton abstraction during the oxidation reaction based on its proximity to the substrate hydroxyl group (Lario et al., 2003; Vrielink et al., 1991; Yue et al., 1999) and due to its conservation in the active site of other GMC oxidoreductases. Mutagenesis studies were consistent with this hypothesis showing abolition of oxidation activity if His447 was mutated either to acidic residues (Asp, Glu), or to another base (Lys) (Kass & Sampson, 1998).

Figure 1.4. The reductive half-reaction of COx

The hydride transfer mechanism displaying the transfer of the hydride from the substrate to N5 of the isoalloxazine moiety of FAD. The three possible locations of the negative charge on the FAD are shown.
However, atomic resolution structures at various pH points resulted in a reanalysis of the role of His447 as a base. Structures were obtained of the enzyme at pH values from 4.0 – 9.0 and revealed that the Ne2 atom of His447 remains protonated to at least pH 7.0, but the proton is absent in the structure at pH 9.0 (Fig. 1.5) indicating that this residue could not act as a base at the physiologically relevant pH (Lario et al., 2003; Lyubimov et al., 2006). Instead, it was proposed that His447 acts as a hydrogen bond donor for the substrate hydroxyl group. This is consistent with the mutagenesis studies that showed that the enzyme was completely inactive when His447 is mutated to glutamic acid and aspartic acid (residues which cannot act as hydrogen bond donors) as well as to lysine whose side chain is unlikely to be in the correct position to act as a hydrogen bond donor. Conversely, those mutations that introduced residues that could act as hydrogen bond donors did retain some oxidation activity, albeit the activity was lower. The lower activity in the latter case was due to misalignment of the hydrogen bond donor as seen in the X-ray structures of these mutants (Yue et al., 1999). These kinetic and crystal structure studies of the mutants suggest positioning of the substrate is very important for the oxidation reaction (Vrielink & Ghisla, 2009).

**Figure 1.5. Protonation state of His447 at various pH values**

Omit maps of the His447 side chain with hydrogen atoms removed at pH 4.5 (a), pH 7.0 (b) and pH 9.0 (c). $2F_o - F_c$ density is shown in purple mesh and is contoured at 4.5 $\sigma$ and $F_o - F_c$ is shown as green mesh and contoured at 2.0 $\sigma$. Figure taken from (Lyubimov et al., 2006).
In light of these studies, it was unclear then, what was acting as the base for substrate hydroxyl deprotonation. Only one other basic residue in the active site, Glu361, is in close enough proximity to perform this reaction and indeed mutagenesis studies indicate a reduction in oxidation activity when this residue is mutated (20-fold and 11-fold reduction for the Glu361Gln and Glu361Asp, respectively) (Kass & Sampson, 1998; Sampson & Kass, 1997). It is interesting to note that His447 is the most important residue for the oxidation activity despite the fact it does not act as the general base for oxidation. This suggests that positioning of the substrate is very important for the oxidation reaction to occur. Oxidation occurs as a concerted rupture of the hydroxyl and C-H bonds, therefore a mis-positioning of the substrate due to mutation of His447 likely affects both substrate deprotonation and hydride transfer.

### 1.2.2. Isomerisation

A glutamate residue (Glu361) is the only basic residue in the vicinity of the flavin in the active site binding pocket and indeed mutation of this residue to a glutamine results in a 20-fold reduction in oxidation activity (Sampson & Kass, 1997). However, Glu361 has also been identified as the base for the isomerisation reaction. Deuterium exchange studies demonstrated that the isomerisation reaction is stereospecific for the β-face of the steroid and is catalysed by a single base which deprotonates the steroid at the C4 position and reprotonates at the C6 position (Kass & Sampson, 1995). Glu361 is perfectly positioned to perform this reaction and also adopts two conformations in the crystal structure (Fig 1.3) which conceivably correlate with the deprotonation and reprotonation steps of isomerisation (Li et al., 1993). Mutation of this residue to the neutral glutamine results in ~10,000 fold decrease in the catalytic activity but only an ~20 fold decrease in oxidation activity (Sampson & Kass, 1997) while mutation to aspartic acid results in 320 fold decrease in isomerisation and an ~11 fold decrease in oxidation activity. However, as Glu361 is certainly the base for
isomerisation, if it also deprotonates the substrate in the preceding oxidation reaction, then it must transfer the proton somewhere else before it can catalyse the isomerisation reaction.

1.3. Cofactor-Protein Interactions

Cofactor stabilisation is an important function of flavin dependent enzymes. Protein-FAD interactions are formed to modulate the redox potential of the flavin, stabilise negative charge on the flavin and to tightly bind the flavin to the protein. The very large range of redox potentials of flavoproteins is due to the differing protein microenvironment around the FAD and therefore, these interactions are important to understand. The interactions include hydrogen bonding interactions, charged interactions and also hydrophobic/steric interactions.

Asn485 was identified as an important residue for modulation of the redox potential in COx. Mutation of this residue to leucine results in a 76 mV decrease in reduction potential and a 1300-fold decrease in oxidation activity (Yin et al., 2001). The high resolution structures indicated that Asn485 exists in two discrete conformations, with one conformation closer to the isoalloxazine and forming an NH•••π bond with the cofactor (Fig. 1.3). Asn485 also gates a narrow hydrophobic tunnel which is proposed to allow oxygen access to the active site in order to regenerate the FAD in the oxidative half-reaction. When Asn485 is further away from the isoalloxazine, the tunnel is closed and oxygen cannot access the active site, while the alternate conformation causes the tunnel to be open and O₂ may then enter to oxidise the reduced flavin (Lario et al., 2003). Formation of the NH•••π bond therefore could stabilise the reduced cofactor and simultaneously allow O₂ to access the active site only when the flavin is reduced.
A negative charge is introduced to the FAD upon reduction that could be stabilised on O4, O2, or N1 (Fig. 1.4). In COx, the O4 atom forms a hydrogen bond with the hydrogen atom of Met122 while the O2 atom is positioned over the end of helix 10 (Fig. 1.3). Helices have a positive dipole that can stabilise negative charge, such as helix 1 which stabilises the negative charge on the diphosphate moiety of FAD (Fig. 1.3). N3-H of FAD also forms a hydrogen bond with Met122-O which likely helps to stabilise the isoalloxazine ring system. Interestingly, the high resolution structure of COx shows a hydrogen atom at pH 7.3 that appears as two peaks in the pH 9.0 structure. A similar splitting of the peak is observed also between the FAD-N3H atom and Met122-O atom (Fig. 1.6). The pH 9.0 condition may mimic the changes that would occur in the reduced anionic form.

Figure 1.6. Multiple populations of hydrogen atoms involved in FAD cofactor - protein interactions

Electron density $2F_o - F_c$ (magenta) and $F_o - F_c$ (green) between the FAD-N3 atom and Met122-O atoms and between the FAD-O4 atom and Met122-N atom. Hydrogen atoms have been omitted. Difference density is clearly seen at the expected position of the hydrogen atoms at pH 7.3 (a) and pH 9.0 (b). The $2F_o - F_c$ density is contoured at 4.0 $\sigma$ and the $F_o - F_c$ density is contoured at 3.0 $\sigma$. Figure taken from (Lyubimov et al., 2006).
1.4. Conclusion

The reaction mechanism of COx involves the transfer of a hydride and protons between the cofactor, protein, and substrates. The specific chemical reactions that are catalysed by flavoproteins are highly dependent on the protein microenvironment, in particular, the hydrogen bonding between the protein and FAD in addition to pi-bonding and hydrophobic interactions that act to modulate the redox potentials of flavins. X-ray diffraction is an excellent tool for determining the positions of the heavy atoms in protein structures but is not useful for the determination of hydrogen atom positions in proteins that are essential to the fine chemical detail of the protein mechanism. For this reason neutron diffraction studies of COx were pursued. Of greatest interest was to visualise the positions of hydrogen atoms in the reduced and oxidised forms of the protein to gain insight into the redox cycle of COx and other flavoproteins. This thesis presents the work and insights gained while working toward this ultimate goal. An overview of the aims and chapter layout is given on page xviii and xix.
Looking for Hydrogen Atoms: Neutron Crystallography Provides Novel Insights Into Protein Structure and Function
2.1. **Authors and Contributions**

This chapter is published in the *Australian Journal of Chemistry* (Golden & Vrielink, 2014). The authors of the manuscript are Emily Golden and Alice Vrielink. E.G wrote the initial draft and incorporated critical revisions by A.V.

2.2. **Abstract**

Neutron crystallography provides unique opportunities to directly observe hydrogen atoms in biological macromolecules. Within enzymes, hydrogen atoms play a pivotal role in catalysis. Recent advances in terms of instrumentation and sample preparation have helped to overcome the difficulties of performing neutron diffraction experiments on protein crystals. The application of neutron protein crystallography to a growing number of proteins has yielded novel structural insights. The ability to accurately position water molecules, hydronium ions, and hydrogen atoms within protein structures has helped in the study of low-barrier hydrogen bonds and hydrogen bonding networks. The determination of protonation states of protein side-chains, substrates, and inhibitors in the context of the macromolecule provide important insights into ligand binding affinities and will assist in the design of potent therapeutic agents. In this review, we give an overview of the method and highlight advances in knowledge attained through the application of single crystal neutron diffraction on protein samples.

2.3. **Introduction**

Since the first X-ray diffraction images from zincblende and copper sulfate crystals were obtained by von Laue in 1912, the field of crystallography has advanced exponentially (Friedrich *et al.*, 1912). Proof that crystals could act as diffraction gratings was first demonstrated by von Laue who diffracted X-rays through copper...
sulfate crystals and these observations were used by William Henry Bragg and his son William Lawrence Bragg to develop Bragg’s Law and determine the first X-ray crystal structures (Bragg, 1913). X-ray diffraction from crystals would later be used to solve the first protein structures, myoglobin (Kendrew et al., 1958) and haemoglobin (Muirhead & Perutz, 1963). Initially, the resolution obtained by protein crystallography was low (<4.0 Å) and could only resolve the overall fold of the protein (Johnson & Phillips, 1965). Improved X-ray sources such as synchrotron facilities, advanced crystallisation techniques, improvements in detector technology, and developments in data processing software are a few of the prominent factors that have resulted in more than 89000 protein crystal structures being available in the Protein Data Bank (Berman et al., 2000). These developments have allowed the precise determination of structural folds of proteins and in most cases the positions of amino acid side-chains, giving unprecedented insights into the relation between protein structure and function. The development of neutron macromolecular crystallography (NMC) has followed a similar path, albeit far less pronounced, beginning with small inorganic molecules and progressing to the structures of small unit cell proteins (Borah et al., 1985; Schoenborn, 1969; Wlodawer et al., 1984). Now improvements in data collection methods, instrumentation, detectors, crystallisation techniques, and sample preparation (e.g. perdeuteration) allow structures of protein crystals with larger asymmetric units (>10^5 Å^3) to be characterised (Bryan et al., 2013; Meilleur et al., 2006; Oksanen et al., 2014). One hundred years after the first crystal structure was reported and 50 years after the first protein crystal structure was solved, we are beginning to gain unique insights from neutron protein crystallography that complement the information available from X-ray crystallographic analyses of macromolecules.

The magnitude of the atomic scattering by X-rays is directly proportional to the number of electrons the atom contains. Hence, atoms such as carbon, nitrogen, oxygen,
and sulfur exhibit stronger scattering than hydrogen atoms, which only have a single electron. Owing to their weak scattering, the ability to visualise hydrogen atoms in electron density maps typically requires atomic resolution (>1.2 Å) data and critically, well-ordered hydrogen atoms with low atomic displacement factors (<~13 Å²) (Howard et al., 2004).

In contrast to X-ray scattering, neutrons interact with the nucleus of an atom. The magnitude of the scattering is dependent on the complex structure of the nucleus and on the isotope of the atom. A unique aspect of neutron diffraction is that it provides the opportunity to determine the atomic positions of atoms with low atomic number, such as hydrogen atoms. The relative scattering of X-rays and neutrons by atoms commonly found in biological macromolecules is shown in Table 2.1. Although hydrogen atoms are more visible by neutron diffraction than X-ray diffraction, the large incoherent scattering cross-section (~80 barns) results in a significant background noise and thus a lower overall signal-to-noise ratio (SNR). This background noise can be reduced by replacing the hydrogen atoms within the structure by its isotope, deuterium (²H). Comparatively, deuterium has a larger coherent and smaller incoherent scattering length (~2 barns) than hydrogen; thus, the SNR is significantly improved and the ability to visualise deuterium atom positions in neutron maps is improved relative to hydrogen atoms. Below, we discuss methods employed to replace hydrogen atoms with deuterium atoms in protein structures.

Traditionally, the application of neutron crystallography to proteins was limited, owing largely to the requirement for very large crystal volumes (>1 mm³) and long data collection times. Large crystal volumes are required owing to the inherent low flux of the neutron beams from nuclear reactors. However, this situation has improved dramatically with the development of protein perdeuteration techniques (Gamble et al., 1994; Shu et al., 2000), and improvements in data collection methods, instrumentation,
and detectors (Niimura et al., 1994; Tanaka et al., 2002; Wilkinson et al., 2009). These advancements have made NMC become a viable technique by allowing shorter exposure times and smaller crystal volumes.

Table 2.1. Neutron scattering lengths and X-ray scattering factors for atoms commonly observed in biological macromolecules

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<td>Neutron coherent scattering length [10^{-14} m]</td>
<td>-0.37</td>
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<td>+0.66</td>
<td>+0.94</td>
<td>+0.58</td>
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<td>0.00</td>
<td>0.49</td>
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<tr>
<td>X-ray scattering factors [10^{-14} m] sinθ/λ = 0</td>
<td>0.28</td>
<td>0.28</td>
<td>1.69</td>
<td>1.97</td>
<td>2.25</td>
<td>4.22</td>
<td>4.48</td>
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2.3.1. Neutron Sources

Neutrons are produced as a continuous beam from fission nuclear reactions at nuclear reactor sources or as a pulsed neutron beam produced from spallation sources where heavy metal targets are bombarded with accelerated particles, ejecting neutrons from the target nuclei (Carpenter, 1977). Continuous sources (such as reactors and spallation sources) produce a white beam that can be used for the application of Laue diffraction methods, providing a higher flux at the sample and allowing a large number of reflections to be stimulated simultaneously relative to monochromatic methods. In the case of pulsed sources, a single neutron pulse is made up of a spectrum of wavelengths, with the higher-energy neutrons reaching the detector faster than lower-energy neutrons. As the beam is pulsed, the wavelength of the detected neutron can be determined by measuring the difference in time that it takes for neutrons to travel a defined distance, called the time-of-flight method (TOF) (Lowde, 1956). This

* The absorption neutron cross-section of an isotope is a measure of the likelihood of interaction between an incident neutron and the target nucleus of the atom. The standard unit for measuring the cross-section is the barn where 1 barn = 10^{-28} m².
time-of-flight information can be used with the positional information to determine a 3D volume of reciprocal space in a single measurement. TOF and Laue diffraction techniques greatly improve the amount of data obtained in a single measurement and therefore dramatically decrease data collection times.

2.3.2. Detectors

There are three types of neutron detectors currently in use for NMC: the gas-proportional detectors (Niimura et al., 1995), scintillator detectors (Coates et al., 2010), and neutron image plate (NIP) detectors (Kurihara et al., 2004; Tanaka et al., 2002; Wilkinson et al., 2009). Gas-proportional detectors use a sealed tube filled with a gaseous isotope such as $^3$He. Neutrons interact with the nuclei of the gas molecules, resulting in the production of a proton and a tritium nucleus (an isotope of hydrogen containing one proton and two neutrons), both of which further ionise the gas, resulting in a cascade of ions that are gathered at an electrode. The amplification of the signal means this type of detector has high sensitivity for neutron signals but a low level of noise. Scintillation detectors similarly rely on the production of ions from incoming neutrons, except the free electrons produced from the ionisation step then go on to excite a material such as $\text{Ce}^{3+}$, resulting in the emission of photons that are then amplified by a photomultiplier. These detector types have been developed into position-sensitive-type detectors through the use of wire anode or cathode plate arrays.

The D19 diffractometer at the Institut Laue–Langevin (ILL) delivers monochromatic thermal neutrons suitable for high resolution diffraction studies of large crystals. Although most commonly used for inorganic molecules, D19 was recently used for the atomic resolution studies of a perdeuterated rubredoxin protein, which allowed identification of hydronium ions (Cuypers et al., 2013; Kovalevsky et al., 2011).
The protein crystallography station (PCS) at the Los Alamos Neutron Science Centre (LANSCE) has produced many macromolecular neutron structures since commissioning in 2002 (Langan et al., 2004). The PCS was the first station to use a spallation neutron beam and TOF techniques. Structures of amicyanin (Sukumar et al., 2010), carbonic anhydrase (Fisher et al., 2012; Fisher et al., 2009; Fisher et al., 2011), crambin (Chen et al., 2012), diisopropyl fluorophosphatase (Blum et al., 2009), dihydrofolate reductase (Bennett et al., 2006), deoxyhemoglobin (Kovalevsky et al., 2010) and xylose isomerase (Katz et al., 2006; Kovalevsky et al., 2010; Kovalevsky et al., 2008) have been determined from data collected at this beamline.

The BIX-1 diffractometer at the JRR-3M reactor, Japanese Atomic Energy Research Institute (JAERI) is an example of a $^3$He gas proportional detector, which was originally developed for NMC (Niimura et al., 1995). This diffractometer proved very effective for diffraction of smaller unit cell crystals (Ohashi et al., 1999; Ohhara et al., 2000) and utilised an elastically bent perfect silicon crystal as the monochromator, which improved the luminosity of the beam significantly (Niimura et al., 1995). However, a lack of linearity of spatial resolution (a measurement of the difference between recorded and actual position) meant that it was not suitable for large unit cell crystals such as proteins (Tanaka et al., 1997). To overcome the spatial resolution issue, Niimura and coworkers developed the first neutron imaging plate (NIP) for biological crystallography, which was first used with monochromatised neutrons on the BIX-II diffractometer at JAERI (Fujiwara et al., 1997; Karasawa et al., 1995; Niimura et al., 1994). These are position-sensitive area detectors similar to those commonly used in X-ray diffraction. They use $^6$Li or Gd mixed with photo-stimulated luminescent materials on a flexible plastic support. With a spatial resolution of 0.2 mm, this detector type has a great advantage over the position-sensitive gas proportional, and scintillator detectors, which have spatial resolutions of ~1 mm, due to the inherent mechanical
limitations of constructing close wire spacings. Additionally, as the detector material is flexible, it can be formed into a cylindrical detector, which can therefore sample a much larger area of reciprocal space in each measurement.

An NIP detector was used in conjunction with quasi-Laue diffraction techniques to form the Laue Diffractometer (LADI) instrument at the ILL (Myles et al., 1997; Wilkinson & Lehmann, 1991) and initially applied to the diffraction of tetragonal hen egg-white lysozyme crystals of 6 mm$^3$ (Niimura et al., 1997). Subsequent studies have been carried out on a wide variety of proteins including coenzyme cob(II)alamin (Langan et al., 1999), xylose isomerase (Meilleur et al., 2006; Munshi et al., 2014), endothiapepsin (Coates et al., 2001), concanavalin A (Ahmed et al., 2007; Blakeley et al., 2004; Habash et al., 2000; Habash et al., 1997), and aldose reductase (Blakeley et al., 2006; Blakeley et al., 2008; Hazemann et al., 2005). The quasi-Laue technique employed on LADI at ILL uses a multilayer bandpass filter to select a specific range of wavelengths to be incident on the sample. The use of a band pass filter (hence quasi-Laue) reduces the number of spatially overlapped reflections relative to that obtained using the full white beam. The combination of quasi-Laue diffraction, perdeuteration, and the use of a neutron imaging plate allowed the collection of a dataset to 2.2 Å resolution on a perdeuterated aldose reductase crystal of only 0.15 mm$^3$ in volume (Hazemann et al., 2005). LADI-II (later named VIVALDI) was subsequently developed as an improved version by incorporating a vertical configuration of the cylindrical drum detector and included image plates mounted and read internally within the drum, thereby improving the neutron detection efficiency by a factor of 3 (Wilkinson et al., 2002; Wilkinson et al., 2009). The current LADI-III instrument (Fig. 2.1a) further improved on LADI-I and VIVALDI by increasing the detector diameter and modification of the readout system, improving the efficiency of the detector to allow the recording of diffraction data for protein crystals with smaller crystal volumes.
(Blakeley et al., 2010; Howard et al., 2011; Petit-Haertlein et al., 2009; Weber et al., 2013). Recently, neutron data collection has been carried out under cryogenic conditions at LADI-III. The success of this approach provides opportunities to study labile crystals as well as trap transient intermediate states of a macromolecule for neutron data analysis (Myles et al., 2012).

NIP detectors are used at JAERI for the BIX-3 and BIX-4 instruments utilizing a monochromatic neutron beam from a fission reactor source (Kurihara et al., 2004; Tanaka et al., 2002). Both instruments have been used to determine a wide variety of protein structures including elastase (Tamada et al., 2009), deoxyhemoglobin (Chatake et al., 2007), insulin (Ishikawa et al., 2008), myoglobin (Ostermann et al., 2002), rubredoxin (Chatake et al., 2004; Kurihara et al., 2004) and Z-DNA (Chatake et al., 2005). Structures of Achromobacter protease (Ohnishi et al., 2013), B-DNA (Arai et al., 2005) and porcine insulin (Ishikawa et al., 2008; Iwai et al., 2009) were obtained and higher resolution structures of photoactive yellow protein (Yamaguchi et al., 2009), ribonuclease A (Yagi et al., 2009), α-thrombin bivalirudin (Yamada et al., 2013) and trypsin-BPTI complex (Kawamura et al., 2011) were studied using BIX-4. More recently the TOF i-BIX diffractometer (Fig. 2.1b), which was constructed at the pulsed neutron source, Japan Proton Accelerator Research Complex (J-PARC), has been extensively upgraded (Kusaka et al., 2013). Fourteen of the position-sensitive scintillator detectors have been upgraded and 16 new detectors installed. This upgraded position-sensitive detector provides higher spatial/time resolution, ideal for diffraction measurements on macromolecules with maximum cell dimensions of 135 Å (Hosoya et al., 2009). A comparison of the structure of ribonuclease A before and after the upgrade showed a gain in achievable resolution (1.5 Å compared to 1.7 Å before the upgrade) and a reduction of the refinement R factors (R_free of 23.6% compared to 28.0% previously) (Kusaka et al., 2013).
The first neutron protein structure for the new IMAGINE image plate diffractometer instrument at the Oak Ridge National Laboratory (ORNL) was recently reported (Meilleur et al., 2013). This quasi-Laue diffraction instrument uses the high flux isotope reactor (HFIR) as the neutron source. The detector is essentially identical to LADI-III; however, for this instrument, a series of interchangeable flat mirrors and filters are employed to select the incident quasi-Laue wavelength and the band pass characteristics best for the sample to be measured. The structure of perdeuterated rubredoxin from *Pyrococcus furiosus* was obtained from data collected using the IMAGINE instrument (Meilleur et al., 2013).

The new Macromolecular Neutron Diffractometer (MaNDI) (Fig. 2.1c) recently built at the spallation neutron source (SNS) at the ORNL is a position-sensitive scintillator-type detector (Coates et al., 2010). Photons produced by scintillation are dispersed such that they are incident on multiple photomultiplier tubes from which the centroid of the photon can be determined. This beamline was designed to allow the diffraction studies of small crystals (0.1–1 mm$^3$) with large unit cells (up to 150 Å) and is expected to be able to provide moderate-resolution data (2.0–2.5 Å) for unit cells in the range 250–300 Å.
The monochromatic Bio-DIFF diffractometer (Kleines et al., 2011) (Fig. 2.1d) located at the FRM-II neutron source has recently opened for scientific users. As well as the neutron structure of Toho-1 β-lactamase in complex with an inhibitor (Tomanicek et al., 2013), preliminary diffraction data have been collected on KDN9P phosphatase (Bryan et al., 2013) and human farnesyl pyrophosphate synthase complexed with risedronate (Yokoyama et al., 2014).

![Figure 2.1](image)

**Figure 2.1. Some diffractometers currently in use for neutron macromolecular crystallography (NMC)**


### 2.4. Visualising Hydrogen Atoms

In the neutron diffraction experiment, the positions of hydrogen atoms can be unambiguously determined, even at moderate resolutions, whereas even high resolution
X-ray crystallography cannot determine hydrogen atom positions to the same degree of accuracy (Gardberg et al., 2010). Using myoglobin, Schoenborn first showed in 1969 that neutron diffraction could feasibly reveal the positions of hydrogen atoms within protein structures (Schoenborn, 1969). The experiment was performed on D₂O-exchanged crystals 25 mm³ in volume and a dataset was collected to 2.8 Å resolution. Even in the absence of accurate phasing, the data could be used to solve the structure of the myoglobin backbone with strong agreement with the known X-ray structures, and enabled the visualisation of hydrogen or deuterium atom positions in a protein structure for the first time. Since these early studies, NMC has been used to easily visualise the protonation state of amino acid residues within numerous enzyme structures and give insight into the roles these residues play in catalysis (Adachi et al., 2009; Blakeley et al., 2008; Blum et al., 2009; Coates et al., 2001; Coates et al., 2008; Fisher et al., 2012; Fisher et al., 2011; Kawamura et al., 2011; Kovalevsky et al., 2012; Munshi et al., 2014; Ohnishi et al., 2013; Tamada et al., 2009; Tomanicek et al., 2013; Yamada et al., 2013). For example, xylose isomerase has been investigated in complex with a variety of substrates and inhibitors as well as under various pH conditions (Kovalevsky et al., 2012; Kovalevsky et al., 2010; Kovalevsky et al., 2008; Meilleur et al., 2006; Munshi et al., 2014). An active-site histidine was found to be doubly protonated and hydrogen bonded to a water molecule that binds in the same position as the substrate oxygen atom, facilitating the ring opening step of the mechanism (Katz et al., 2006; Kovalevsky et al., 2008).

Soaking protein crystals in solutions of deuterated water (D₂O) has also allowed the study of H/D exchange within several proteins. These structures have provided unique insights into the protein and microenvironment. High H/D exchange is correlated with solvent accessibility (Chatake et al., 2004) and those hydrogen atoms involved in strong hydrogen bonding interactions such as in β sheets generally do not
exhibit a high rate of exchange (Kurihara et al., 2004). However, there have been surprising examples where apparently non-exchangeable hydrogen atoms have been exchanged, lending insight into protein movement and microenvironments. For example, neutron studies of H/D-exchanged crambin revealed a fully exchanged hydroxyl group of a solvent-inaccessible threonine residue (Thr30) while the surrounding mobile regions of the structure were not deuterium exchanged (Chen et al., 2012). The threonine hydroxyl oxygen accepts a hydrogen bond from an unexchanged backbone amide of the neighbouring residue (Cys30) whereas it donates a hydrogen bond via a solvent-inaccessible, yet exchanged, deuterium atom to the main-chain oxygen of Cys26. This exchange was attributed to global protein breathing motions as well as side chain rotations of the threonine group. Similarly, the neutron structure of Z-DNA revealed a high degree of deuterium exchange for the C8–H8 group of guanine bases providing experimental evidence for the acidic nature of this aliphatic carbon (Chatake et al., 2005).

Owing to the strong positive scattering length of deuterium relative to that of hydrogen, H/D exchange of crystals is also useful for improving the SNR of the data. However, the remaining unexchanged hydrogen atoms still pose a problem of density cancellation, resulting in a decrease in the signal of both the hydrogen atom and the atom to which it is bonded, as well as a displacement of the density peak associated with the hydrogen away from the actual position of the atoms (Shu et al., 2000). Additionally, Fourier truncation artefacts visible in the nuclear scattering density maps further decrease the ability to reliably interpret negative density peaks as hydrogen atoms in neutron maps. Ostermann and coworkers developed a technique to diminish these artefacts in the maps by subtracting a map calculated from a model without hydrogen atoms, from the observed data (Ostermann et al., 2002). This resulted in
cleaner difference maps with negative density peaks for hydrogen atoms more clearly visible and not confounded by artefacts resulting from Fourier truncation of the data.

2.4.1. Perdeuteration

Soaking crystals in D$_2$O helps to circumvent problems associated with nuclear scattering density cancellation due to hydrogen atoms in neutron maps. However, many hydrogen atoms are either not solvent-accessible or not able to be exchanged (e.g. aliphatic hydrogens). This typically results in ~50% of the hydrogen atoms remaining unexchanged after extensive soaking procedures in D$_2$O. Although H/D exchange studies can provide information about the strength of hydrogen bonds, mobility of regions of the protein, and insights into protein breathing, even with the application of Ostermann’s correction procedure, visualisation of most of the hydrogen atoms in the structure requires high resolution data to overcome the cancellation effects (Gardberg et al., 2010). In contrast, deuterium atoms are easily visible in neutron maps as positive difference density peaks even at moderate resolutions.

In order to eliminate the effects of density cancellation, efforts have been made to exchange all the hydrogen atoms within the protein structure for deuterium atoms. Shu and coworkers presented the first neutron diffraction structure of a fully perdeuterated protein (Shu et al., 2000). Myoglobin was perdeuterated by expressing the protein in deuterated minimal media with deuterated succinate as the carbon source. A comparison of the neutron maps of D$_2$O-exchanged crystals and perdeuterated protein crystals clearly showed the advantage of perdeuteration for visualising the positions of deuterium atoms over that of hydrogen atoms and eliminating the effects of density cancellation due to the negative scattering of hydrogen. A comparison of the X-ray and neutron density maps for a phenylalanine residue within the myoglobin structure shows that the perdeuterated neutron maps allow unambiguous modelling of the aryl hydrogen
atoms (Fig. 2.2). Electron density for hydrogen atoms is not visible in the 1.5 Å resolution X-ray map (Fig. 2.2a). The neutron density map of the protiated protein shows the hydrogen atoms as negative contour peaks; however, they are difficult to distinguish from the noise level of the map (Fig. 2.2b). Interestingly, the neutron map calculated directly from the model of the protiated structure shows that the hydrogen atoms still lack definition and can be difficult to observe (Fig. 2.2c). In contrast, the neutron map of the perdeuterated structure shows well-defined density for the deuterium atoms as positive peaks (Fig. 2.2d).

![Figure 2.2. X-ray and neutron-scattering density maps for protiated and perdeuterated crystals of myoglobin in the vicinity of Phe43](image)

(a) 1.5 Å resolution $2F_o - F_c$ X-ray map of perdeuterated myoglobin. (b) A 2.0 Å $2F_o - F_c$ neutron map of protiated myoglobin. Magenta and green contours correspond to +1.0 σ and -1.0 σ respectively. (c) A 2.0 Å resolution $F_c$ neutron map calculated from the perdeuterated myoglobin protein model replacing D with H. The magenta map is contoured at +1.0 σ and the green map is contoured at -2.0 σ. (d) The 2.0 Å resolution $2F_o - F_c$ neutron map of perdeuterated myoglobin contoured at +1.0 σ. Figure taken from Shu et al. (2000).

One of the most difficult aspects of performing neutron diffraction studies on proteins is the ability to grow very large single crystals. This is required to overcome the low flux of the neutron beam, which is further confounded by the high level of background noise resulting from the large incoherent scattering cross-section of hydrogen atoms. However, perdeuteration of the protein alleviates this problem significantly, allowing diffraction measurements to be undertaken using smaller crystals.
than needed for protiated structures. As mentioned above, the value of this method was successfully shown through a neutron study on small crystals of perdeuterated aldose reductase (Blakeley et al., 2008; Hazemann et al., 2005). Additionally, diffraction data to 1.85 Å resolution from a 0.13-mm$^3$ type-III antifreeze protein crystal, an order of magnitude smaller than the previously required minimum crystal size of 1 mm$^3$, have been reported (Howard et al., 2011; Petit-Haertlein et al., 2009; Weber et al., 2013).

### 2.5. Recent Structural Insights

#### 2.5.1. Hydronium Ions

With the improvements in detectors, neutron sources, and crystallisation techniques, higher resolution enzyme structures have been obtained, providing valuable insights into catalytic mechanisms. Hydronium ions have been suspected to play a pivotal role in proton transfer in proton pump mechanisms (Leone et al., 2010). Owing to the poor scattering by hydrogen atoms, however, it is impossible to unambiguously identify hydronium ions in X-ray structures; H$_2$O, H$_3$O$^+$, and OH$^-$ all appear as a peak centred on the oxygen atom in X-ray structures and rarely provide sufficient scattering density for the hydrogen atom(s). As deuterium atoms are readily visible in neutron maps, neutron diffraction provides the ability to distinguish between these different forms for the first time.

D-xylose isomerase (XI) is an enzyme that catalyses the isomerisation of aldose and keto sugars. The activity of XI is strongly pH dependent, with a sharp decrease in activity under acidic conditions correlated with expulsion of bound metal ions from two binding sites. Neutron diffraction studies of the enzyme at low and high pH provided a structurally based rationale for this observed pH dependence (Kovalevsky et al., 2011). Neutron maps revealed the presence of a hydronium ion at one of the metal binding
sites at pH 7.7 (Fig. 2.3\(a\)). Interestingly, at pH 5.9, the hydronium ion is dehydrated to a proton (Fig. 2.3\(b\)) in the same binding site and the residues coordinating the hydronium at pH 7.7 are displaced towards the proton. Soaks in metal ions did not substitute the proton at this site; thus, it was concluded that rearrangement of metal binding residues due to the presence of a proton prevents metal binding, thereby decreasing the enzyme activity at low pH. This is the first structure to identify an unsolvated hydronium ion coordinated only by protein residues and lends evidence to the theory that hydronium ions play a role in proton transfer chemistry in enzyme mechanisms.

Figure 2.3. Neutron maps of H/D-exchanged D-xylose isomerase (XI) at low and high pH

\((a)\) The metal binding site of XI at pH 7.7 showing the D\(_3\)O\(^+\) ion present in the site. \((b)\) The metal binding site of XI at pH 5.9 showing the deuteron present in this site and reorganisation of coordinating residues. The \(2F_o - F_c\) neutron scattering density maps are contoured at 1.5 \(\sigma\) (magenta) and the \(F_o - F_c\) neutron density maps are contoured at 3.5 \(\sigma\) (green). The D\(_3\)O\(^+\) and D\(^+\) molecules were omitted from structure factor calculations for the \(F_o - F_c\) density map in \((a)\) and \((b)\) respectively. Maps were calculated from structure factors obtained from the Protein Data Bank (Berman et al., 2000) (PDB ID: 3KCJ and 3QZA), using the PHENIX software (Adams et al., 2010).

In 2013, Cuyper and coworkers identified four hydronium ions in the neutron structure of oxidised (1.27 Å) and reduced (1.38 Å) rubredoxin at pH 6.4 and pH 6.2,
respectively; these ions were not visible in previously determined lower resolution neutron maps (Cuypers et al., 2013). Three of the four D$_3$O$^+$ molecules visible in the structure were positioned within a 9 Å sphere from the redox Fe-S$_4$ centre. An example of a site partially occupied by a D$_3$O$^+$ molecule is shown in Figure 2.4. The identification of the hydronium ion was only possible owing to the high resolution data (1.05 Å) achieved through the perdeuteration of the sample as well as the use of improved monochromators at the D19 instrument at the ILL. It is speculated that the close proximity and strong hydrogen bonding network of these hydronium ions to the redox Fe-S$_4$ centre as well as differences in the deuterium positions between the oxidised and reduced forms play an important role in the charge transfer reaction of rubredoxin. The authors also suggest that the observed hydronium ions may help stabilise the protein through a more favourable energetic contribution of hydrogen bond formation of the hydronium group over that of H$_2$O. Furthermore, these observations suggest an explanation for the high thermostability observed for rubredoxin from *Pyrococcus furiosus*.

![Hydronium ion in neutron scattering density maps](image)

**Figure 2.4. A hydronium ion in the neutron scattering density maps of rubredoxin**

The 2$F_o$ – $F_c$ neutron scattering density map is contoured at 1.5 $\sigma$ (magenta) and the $F_o$ – $F_c$ neutron scattering density map is contoured at 3.5 $\sigma$ (green). The D$_3$O$^+$ molecule was omitted from structure factor calculations for the $F_o$ – $F_c$ density map. Maps were calculated from structure factor files obtained from the Protein Data Bank (Berman et al., 2000), (PDB ID: 4AR3), using the PHENIX software (Adams et al., 2010).
2.5.2. Oxyanion Hole and Low-Barrier Hydrogen Bonds

Serine proteases are a class of enzymes containing a catalytic triad, usually consisting of His, Asp, and Ser, which form a charge relay system (Hedstrom, 2002). A suspected ‘low-barrier hydrogen bond’ (LBHB) between the β-carboxyl group of an aspartate residue and the Nδ1 hydrogen atom of a histidine residue is hypothesised to increase the nucleophilicity of the serine Oγ for nucleophilic attack on the substrate carbonyl (Cleland et al., 1998; Cleland & Kreevoy, 1994; Frey et al., 1994). A tetrahedral intermediate is formed through a covalent bond between the Oγ atom of the catalytic serine residue and the substrate carbonyl carbon atom; the resulting negative charge on the substrate carbonyl oxygen atom is stabilised by an ‘oxyanion hole’ formed by the backbone amides of the active serine residue and a nearby residue. There is still some speculation as to the existence of the LBHB and the oxyanion hole owing to the problems with visualising density for protons and hydrogen atoms in X-ray crystal structures. By carrying out single crystal neutron diffraction studies of elastase in complex with an inhibitor (FR130180) that mimics the tetrahedral intermediate state of the enzyme, Tamada and coworkers were able to confirm the existence of the oxyanion hole (Tamada et al., 2009). Neutron data to 1.65 Å resolution and X-ray data to 1.20 Å resolution were collected from a single crystal 3.3 mm³ in volume and used for joint X-ray/neutron refinement. The nuclear scattering density maps clearly revealed deuterium atoms for the backbone amide groups of Gly193 and Ser195, which form the oxyanion hole (Fig. 2.5). Based on the lack of nuclear scattering density for a hydrogen atom in the $F_o - F_c$ omit maps near the carbonyl oxygen atom of the substrate in the intermediate, this atom was shown to exist in the anionic form rather than the hydroxyl form. These joint X-ray/neutron studies provided the first direct evidence of an oxyanion stabilised in an oxyanion hole.
Additionally, this study on elastase revealed that an LBHB is not formed between the histidine and aspartate residues in the catalytic triad of serine proteases. It has been hypothesised that an LBHB, characterised by a distance of less than 2.65 Å between the donor and acceptor atoms, is formed between the Nδ1 atom and the Oδ2 atom of the catalytic histidine and aspartate residues respectively (His57 and Asp102 in elastase) (Frey et al., 1994). However, for an LBHB, the hydrogen atom should lie equidistant between the donor and acceptor atoms. The nuclear scattering density map clearly showed that the hydrogen atom was localised nearer to the Nδ1 atom of His57 and was not shared equally with Asp102. Based on this observation, the interaction is not one of an LBHB but is defined as a short ionic hydrogen bond (SIHB).

**Figure 2.5. Neutron scattering density maps of elastase in complex with the inhibitor FR130180**

The $2F_o - F_c$ map is contoured at $2.5 \sigma$ (magenta) and the $F_o - F_c$ map is contoured at $+3.5 \sigma$ (green) and $-3.5 \sigma$ (red). Deuterium and hydrogen atoms were omitted for the calculation of the $F_o - F_c$ map. Maps were calculated from structure factor files obtained from the Protein Data Bank (PDB ID: 3HGN) using the PHENIX software (Adams et al., 2010).

Neutron diffraction maps of photoactive yellow protein (PYP) at 1.5 Å resolution revealed 87% of the hydrogen and deuterium atoms in the structure and
enabled the identification of an LBHB essential for stabilising a buried negative charge in the protein (Yamaguchi et al., 2009). Previously, two short hydrogen bonds (SHB) were identified between the PYP chromophore, $p$-coumaric acid (pCA) and Tyr42 and Glu46 (Anderson et al., 2004). However, the neutron study showed that the interactions between pCA and Glu46 were consistent with that of an LBHB with the deuterium atom located 1.37 Å from the oxygen of pCA and 1.21 Å from the oxygen of Glu46 (Fig. 2.6), thus not covalently bound to either residue. In contrast, the deuterium atom of the hydroxyl group of Tyr42 was found to be only 0.96 Å from Tyr42, suggesting that its interaction with pCA is an SIHB rather than an SHB. Furthermore, it was proposed that the LBHB is present in the relaxed state of the protein and acts to sterically restrain the phenolic ring of the chromophore. On excitation of the chromophore, the LBHB relaxes, releasing the phenolic ring of the chromophore to undergo fast isomerisation.

**Figure 2.6** The low barrier hydrogen bond in photoactive yellow protein

Positive nuclear density is observed between the carboxylic oxygen atom of Glu46 and the phenolic oxygen atom of pCA. The $F_o - F_c$ map is contoured at +3.5 $\sigma$ (green) and −3.5 $\sigma$ (red). Deuterium and hydrogen atoms were omitted for the calculation of the $F_o - F_c$ map. Maps were calculated from structure factor files obtained from the Protein Data Bank (PDB ID: 2ZOI) using the PHENIX software (Adams et al., 2010).
2.5.3. Protein–Ligand Interactions

Hydrogen bonding interactions are important for conveying specificity and affinity of ligands such as drug molecules to their protein targets. Thus, a detailed knowledge of hydrogen positions is important for correlating binding affinities of substrate and inhibitors with intermolecular ligand–protein interactions. Hydrogen bonding is inferred from X-ray structures, but without accurate visualisation of hydrogen atoms, it cannot be unambiguously determined. Single crystal neutron diffraction studies have been used to investigate the binding of several small molecules and proteins.

The increased inhibition of XI by the inhibitor xylitol at low pH was investigated by neutron diffraction using an H/D-exchanged crystal at pH 5.9 (Kovalevsky et al., 2012). As mentioned above, the activity of the enzyme was found to be inhibited at pH 5.9 owing to the collapse of the metal ion binding site. However, at low pH, the binding affinity of the enzyme for xylitol is increased. Hydrogen atoms for both the substrate and the inhibitor in complex with the enzyme were clearly visible in the neutron maps. The improved binding affinity of the inhibitor at low pH was attributed to the presence of an additional proton on the inhibitor.

Further, using different sized metals as inhibitors of the enzyme, ternary complexes containing the metal and different forms of D-glucose (cyclic and linear forms) were studied by neutron diffraction (Kovalevsky et al., 2010). The studies, as well as previous neutron studies of the enzyme without a bound ligand and the bound product form (Katz et al., 2006) represented different stages of the enzyme-catalysed reaction and enabled a detailed view of the ionisation states of key residues and ligands through the enzyme reaction mechanism. In particular, neutron maps revealed different protonation states for a lysine residue (Lys289) during the course of the reaction as well as the presence of a catalytic D2O or an activated OD−. These studies provided insights
into changes that occur to the enzyme through catalysis and particularly reveal the role of hydrogen atoms in the chemistry of the enzyme. Recent neutron studies on a mutant of XI have provided further support for the mechanistic interpretations from the above study (Munshi et al., 2014).

Human immunodeficiency virus type-1 (HIV-1) protease is a homodimeric aspartyl protease that is essential for viral survival. It cleaves newly synthesised viral polyproteins to generate functional proteins needed for establishment and replication of an infectious HIV virion (Kohl et al., 1988). As the enzyme is critical for viral infection, it is a major target for the development of small molecule inhibitors as therapeutic agents to treat acquired immune deficiency syndrome (AIDS) (Wlodawer & Vondrasek, 1998). However, many inhibitors cause adverse side effects and the rapid replication of HIV results in the development of drug resistance. The design of high potency inhibitors of the enzyme requires a detailed understanding of the catalytic mechanism. Structural characterisation of transitional state analogue complexes helps to delineate important interactions between the protein and the transition-state analogue that contribute to high-affinity inhibitor binding, thus allowing the design of more potent inhibitors. Towards these goals, Adachi and coworkers determined the 1.9 Å resolution neutron structure of HIV-1 protease in complex with the transition state mimetic inhibitor KNI-272 (Adachi et al., 2009). The active site of the enzyme contains an aspartyl dyad, one from each monomer of the homodimeric enzyme: Asp25 and Asp125 from monomer 1 and 2 respectively. The protonation state of these two residues is hypothesised to play an important role in the formation of the transition state; hence, neutron studies were aimed at visualising hydrogen or deuterium atoms within the enzyme active site. Nuclear scattering density (2Fo –Fc and Fo –Fc omit) maps showed that the carboxylate group of Asp25 was protonated whereas that of Asp125 was unprotonated (Fig. 2.7a and 2.7b). The inhibitor hydroxyl group (O2) acts
as a hydrogen bond donor to Asp125 while O4 of the inhibitor acts as a hydrogen bond acceptor from the protonated Asp25. Knowledge of these interactions along with the previously determined X-ray studies and binding experiments provide deeper insights into the catalytic mechanism and will help in the design of inhibitors with improved specificity and potency.

In addition to the above HIV-1 protease–KNI-272 complex study, a neutron structure of the enzyme in complex with a sulfonamide-containing inhibitor, amprenavir (APV) (Fig. 2.7c and 2.7d) has also been reported (Weber et al., 2013). In agreement with the enzyme–KNI-272 complex, Asp25 was found to be protonated while Asp125 was unprotonated. Interestingly, however, in this case, the deuterium atom was found on the Oδ1 atom of Asp25 (Fig. 2.7c) whereas, in the case of KNI-272, the proton was localised on Oδ2 of Asp25. Additionally, in the KNI-272 complex structure, the two Asp carboxylates interact with separate oxygen atoms of the inhibitor whereas in the APV complex structure, both Asp residues contact the same oxygen of the inhibitor (one as an hydrogen bond donor and one as an hydrogen bond acceptor). These findings suggest that the structure of the ligand’s isostere may influence the location of the proton on Asp25.
Carbonic anhydrase (CA) constitutes a class of ubiquitous enzymes catalysing the hydration of CO$_2$ to HCO$_3^-$.

They serve a variety of functions and have been widely investigated as drug targets for the development of inhibitors to treat medical conditions including glaucoma (Baldwin et al., 1989), obesity (Bray et al., 2003; Vullo et al., 2004), and cancer (Parkkila et al., 2000). However, the development of inhibitors of CA is confounded by the presence of multiple isoforms of the enzyme in diverse human
tissue (see review by Gilmour (2010)) where poor inhibitor specificity leads to side effects. In order to alleviate this problem and design more specific inhibitors targeting a single isoform of the enzyme, a detailed understanding of the hydrogen bonding interactions at the active site is needed. Using single crystal neutron diffraction methods, Fisher and coworkers investigated the hydrogen bonding interactions between human carbonic anhydrase (HCAII), an isoform found predominantly in red blood cells, and a clinically used sulfonamide inhibitor, acetazolamide (AZM) (Fisher et al., 2012). AZM exists in three physiologically relevant protonation states. High resolution X-ray studies (Sippel et al., 2009) have been unable to unambiguously determine the protonation state of the inhibitor bound to the HCAII; however, NMR studies indicate that a benzenesulfonamido anion binds to the enzyme in solution (Kanamori & Roberts, 1983).

TOF neutron diffraction data to 2.0 Å as well as X-ray diffraction data to 1.6 Å were collected from a large H/D-exchanged crystal of the enzyme and used for joint X-ray and neutron structure refinement. The $2F_o - F_c$ nuclear scattering density maps of the HCAII revealed two exchangeable deuterium atoms present on the sulfonamido and acetoamido groups of AZM. The neutron maps provide evidence that AZM binds in the anionic form, with the negatively charged sulfonamido group coordinating with the active site zinc. The identification of the hydrogen atom positions in the structure enabled elucidation of the hydrogen bonding network between the protein and the inhibitor. The negatively charged sulfonamido nitrogen atom coordinates with the zinc atom while the deuterium atom hydrogen bonds to the threonine hydroxyl oxygen atom. A detailed knowledge of the interactions between the enzyme and inhibitors will help to improve inhibitor specificity.

Urate oxidase is involved in the metabolism of purines by catalysing the oxidation of uric acid to 5-hydroxyisourate. The catalytic mechanism is not well
understood because the protonation state of uric acid is not known. Neutron structures of urate oxidase in complex with the substrate urate, as well as an inhibitor, 8-azaxanthine, show that the substrate is present in the tautomerised form 8-hydroxyxanthine, which in turns forms a hydrogen bond to a glutamine residue (Oksanen et al., 2014). These studies provided a direct visualisation of the hydrogen atoms on the acetoamidal nitrogen and on the water molecules themselves, thereby allowing elucidation of the exact hydrogen bonding network between the protein and substrate mediated through a water molecule.

β-Lactamases catalyse the hydrolysis of the β-lactam ring and play a pivotal role in bacterial resistance to β-lactam antibiotics. The design of inhibitors of this enzyme is important to circumvent the problems associated with antibiotic resistance. A longstanding question in the field has been the identification of a catalytic base in the acylation reaction. Neutron crystallography was carried out on the serine protease Toho-1 β-lactamase in complex with an acylation transition state analogue (Tomanicek et al., 2013) as well as the enzyme in the absence of a bound ligand (Tomanicek et al., 2011). Comparison of these structures showed that Glu166 was deprotonated in the ligand-free form of the protein (Fig. 2.8a) but protonated in the presence of the inhibitor, benzothiophen (BZB) (Fig. 2.8b). These studies supported previous ultra high resolution X-ray structures (Chen et al., 2007; Minasov et al., 2002; Nukaga et al., 2003) and neutron studies of a triple mutant (Tomanicek et al., 2010) which suggested that Glu166 acts as the general base for catalysis.
2.6. Conclusion

Single crystal neutron diffraction has been limited in its application to protein crystallography by the intrinsic low flux of neutron beamlines; however, advances have been made in recent years to overcome these limitations. Improved instrumentation, detectors, and perdeuteration techniques have allowed structural studies to be carried out using much smaller crystals than were previously needed. A review of the Protein Data Bank shows that these recent improvements have made NMC a more feasible technique, with more than half of the neutron structures being deposited since 2010. Recent structures in particular have provided unprecedented insights into protein structure and function, such as confirming the presence of hydronium ions, proving that LBHB can occur in proteins, and the structure of the oxyanion hole stabilising an oxyanion. The direct visualisation of hydrogen atoms in protein–ligand complexes provides important details for understanding enzyme chemistry and for the design of 

Figure 2.8. Neutron scattering density maps of the active site of Toho-1 β-lactamase

(a) In the absence of a bound ligand contoured at 1.2 σ; and (b) In the presence of the transition state analogue benzothiophen (BZB) contoured at 1.0 σ. For the complex structure, the density was removed for the BZB molecule for clarity and the orientation rotated compared to (a). Figure modified from Tomanicek et al. (Tomanicek et al., 2013; Tomanicek et al., 2011)
high potency and high specificity enzyme inhibitors as potential therapeutic agents for the treatment of various diseases. The application of neutron crystallography to proteins promises to enhance our understanding of these complex biological molecules.
CHAPTER 3

HIGH RESOLUTION STRUCTURES OF CHOLESTEROL OXIDASE IN THE REDUCED STATE PROVIDE INSIGHTS INTO REDOX STABILISATION
3.1. Authors and Contributions

This chapter was published in *Acta Crystallographica D* (Golden et al., 2014). The authors of the manuscript are Emily Golden, Amir Karton and Alice Vrielink of the School of Chemistry and Biochemistry, The University of Western Australia, Crawley, Western Australia, 6009 Australia. E.G. did all experimental work and wrote the paper. A.K. provided the computational work and analysis (DFT) and provided the text describing the computational work. A.V. provided critical revisions.

3.2. Abstract

Cholesterol oxidase (COx) is a flavoenzyme catalysing the oxidation and isomerisation of cholesterol to cholest-4-en-3-one. The reductive half-reaction occurs via a hydride transfer from the substrate to the FAD cofactor. The structures of COx reduced with dithionite under aerobic conditions and in the presence of the substrate, isopropanol, under both aerobic and anaerobic conditions, are presented. The 1.32 Å resolution structure of the dithionite-reduced enzyme reveals a sulfite molecule covalently bound to the FAD cofactor. The isoalloxazine ring system displays a bent structure relative to that of the oxidised enzyme and alternate conformations of a triad of aromatic residues near to the cofactor are evident. A 1.12 Å resolution anaerobically trapped reduced enzyme structure in the presence of isopropanol does not show a similar bending of the flavin ring system but does show alternate conformations of the aromatic triad. Additionally a significant difference electron density peak is observed within a covalent bond distance from N5 of the flavin moiety suggesting a hydride transfer event has occurred as a result of substrate oxidation trapping the flavin in the electron rich reduced state. The hydride transfer generates a tetrahedral geometry about the flavin N5 atom. High level density functional theory calculations were performed to correlate the crystallographic findings with the energetics of this unusual arrangement.
of the flavin moiety. These calculations suggest that strong H-bond interactions between Gly120 and the flavin N5 centre may play an important role in these structural features.

3.3. Introduction

Cholesterol oxidase (COx) is a flavoenzyme catalysing the oxidation and isomerisation of cholesterol to cholest-4-en-3-one (Chapter 1, Fig. 1.1). The type I COx from 
Streptomyces SA-COO (EC 1.1.3.6) is a member of the glucose-methanol-choline (GMC) oxidoreductase family and contains a single molecule of flavin adenine dinucleotide (FAD) non-covalently but tightly bound to the protein. The enzyme catalyses the oxidation and isomerisation of steroids containing a 3β hydroxyl group with the cholestane group conferring binding specificity in the active site (Pollegioni et al., 1999). COx is also able to oxidise small molecule alcohols such as isopropanol and methanol, with a preference for those containing aromatic rings, however, these molecules display a lack of specificity resulting in a much slower rate of catalysis (Pollegioni et al., 1999). Substrate oxidation results in reduction of the cofactor during the reductive half-reaction. An oxidative half-reaction occurs with re-oxidation of the flavin cofactor by molecular oxygen to form hydrogen peroxide (Chapter 1, Fig. 1.1).

High resolution X-ray structures (Chen et al., 2008; Lario et al., 2003; Lyubimov et al., 2009; Lyubimov et al., 2007; Lyubimov et al., 2006) along with mutagenesis studies (Kass & Sampson, 1998; Sampson & Kass, 1997; Yin et al., 2002; Yin et al., 2001; Yue et al., 1999) have revealed the key active site residues responsible for performing the catalytic function. His447 is important for the oxidation reaction (Kass & Sampson, 1998; Yamashita et al., 1998) with mutations of this residue significantly decreasing the rate of the oxidation of cholesterol (more than 100,000 times lower for the enzymes with mutations to lysine, glutamate, aspartate and alanine)
Atomic resolution studies of the enzyme revealed that the $\text{Ne}_2$ atom on His447 was protonated and this led to the suggestion that His447 functions to position the substrate with respect to the flavin and an active site glutamate, Glu361 (Lyubimov et al., 2006). The correct positioning of the substrate facilitates optimised orbital overlap allowing hydride transfers to occur via a trans-elimination reaction involving an anti-coplanar arrangement of the two sigma bond orbitals of the substrate. This mechanism further suggests that Glu361 functions as the base for oxidation. However Glu361 has also been proposed as the base for the isomerisation reaction (Kass & Sampson, 1995; Kass & Sampson, 1998; Sampson & Kass, 1997; Yamashita et al., 1998). The mechanism of isomerisation has been proposed to involve an intramolecular, cis diaxial transfer of the C4$\beta$ proton to the C6$\beta$ position forming an enolic intermediate (Sampson & Kass, 1997). This is supported by the multiple conformations of the Glu361 side chain in crystal structures (Lario et al., 2003; Yue et al., 1999). This mechanism implies however that, after oxidation and before isomerisation, Glu361 must be deprotonated in order that it function as the base for both steps in the overall enzyme reaction. The exact mechanism by which this would occur is not well understood. At this stage it has not been unequivocally determined whether Glu361 does act as the general base for abstraction of the substrate hydroxyl proton.

Studies have shown that Asn485 is important for the oxidation reaction by modulating the redox potential of the flavin (Yin et al., 2001). The side chain is found in two conformations, one of which makes an N-H$\cdots$π electrostatic interaction with the flavin ring system. Mutagenesis of this residue decreases both $k_{\text{cat}}$ and the redox potential. Additionally, the side-chains of Glu361, Asn485 and Met122 gate a tunnel which has been proposed to function as a conduit for oxygen access to the reduced flavin within the sequestered enzyme active site (Lario et al., 2003). This channel is gated on the bulk solvent side by Phe359 (Chen et al., 2008). These residues are found
in two conformations in the high resolution X-ray structures with their conformations correlated to each other and with an open and closed state of a hydrophobic tunnel extending from the bulk solvent to the active site. It has been proposed that the “tunnel open” conformation occurs when the flavin is in the reduced state facilitating the Asn485 N-H•••π interaction with the pyrimidine ring of the flavin ring system, thereby stabilising the reduced FAD. This initial reorientation of the side chain enables further concerted movements of the other tunnel residues thereby opening the proposed oxygen channel.

Despite the extensive structural work on this enzyme, atomic resolution structures in the presence of a substrate and trapped in the reduced cofactor state have not been characterised. In order to better understand the redox chemistry of the enzyme, and the role of the flavin and surrounding protein residues in modulating redox activity, we have determined atomic resolution crystal structures of the enzyme anaerobically trapped in the reduced state using both isopropanol and dithionite. Additionally we have carried out high level density functional theory (DFT) calculations to probe the interaction energies between the reduced flavin and nearby hydrogen bond donating groups. These studies provide important insights into the role of specific protein residues in stabilising the reduced cofactor.

3.4. Materials and Methods

3.4.1. Cloning

The wild-type *Streptomyces SA-COO* COx gene was amplified from the pCO117 plasmid (Nomura *et al.*, 1995) by polymerase chain reaction (PCR) using the forward and reverse primers, 5’-gacctcactgccactgcacaacagcatctg-3’ and 3’-gcagtgccgcagcgtggtggtggtggtgattcgaaactga-5’ respectively. The reverse primer
included codons for six histidine residues. The PCR product was cloned into the \textit{Neol} and \textit{HindIII} restriction sites of pET28a-His6-MBP-TEV-AEW (vector kindly provided by Dr. Anthony Duff, National Deuteration Facility, ANSTO, Australia). The resulting vector, pCO_P1, was verified by DNA sequencing.

\subsection*{3.4.2. Expression and Purification}

The structures of the isopropanol bound complexes were obtained using the hexa-histidine tagged protein (COx-H6). Expression of COx-H6 was carried out in \textit{E. coli} BL21 (DE3) and purification was performed by nickel-affinity chromatography. Competent \textit{E. coli} BL21 (DE3) cells were transformed with the pCO_P1 plasmid. Transformants were selected from Luria-Bertani (LB) agar plates supplemented with 50 \(\mu\)g ml\(^{-1}\) kanamycin. An overnight inoculant culture was prepared by selecting a single colony from the transformation to inoculate 250 ml LB containing the above antibiotic. The culture was used to inoculate 3 L of 2xYT media in 6 x 2 L conical flasks, which were shaken at 310 K. When the optical density (600 nm) reached 0.6, protein expression was induced by the addition of isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and the cells incubated for a further 25 hours at 293 K. The cells were harvested by centrifugation at 4,500 \(g\) for 45 minutes and the cell pellet flash cooled in liquid nitrogen at 77 K until further purification.

The cell pellet was resuspended in lysis buffer (20 mM Tris-HCl pH 7.0, 500 mM NaCl) and lysed with an Emulsiflex C5 high-pressure homogeniser (Avestin). The lysate was clarified by centrifugation at 12,600 \(g\) for 30 minutes and the supernatant applied to a 1 ml Qiagen Ni-NTA column equilibrated with 20 mM Tris-HCl pH 7.0, 500 mM NaCl and 20 mM imidazole. The protein was eluted with an imidazole gradient (0 - 500 mM over 30 column volumes) and fractions collected. Peak fractions were pooled, dialysed against 20 mM Tris-HCl (pH 7.0) and concentrated to 6 mg ml\(^{-1}\)
as determined by Bradford assay (Compton & Jones, 1985), using an Amicon centrifugal filter unit (30 kDa cutoff). Aliquots of the purified protein were snap-frozen in liquid nitrogen and stored at 193 K.

The non-histidine tagged version of the protein (COx) was used for the structure of the dithionite treated crystals. Competent *E. coli* BL21 (DE3) pLysS cells were transformed with the pCO117 plasmid (Nomura et al., 1995) and transformants selected from LB agar plates supplemented with 50 µg ml\(^{-1}\) ampicillin and 34 µg ml\(^{-1}\) chloramphenicol. 50 ml LB containing the above antibiotics was inoculated with a single colony from the transformation and grown overnight at 310 K. This starter was used to inoculate 2 L of 2xYT also containing the above antibiotics. The cells were grown at 310 K until the optical density (600 nm) reached 0.65 and protein expression induced by the addition of IPTG to a final concentration of 0.4 mM. The cells were further incubated at 293 K for 20 hours and harvested by centrifugation at 12,000 g for 30 minutes. COx was purified according to a previously reported method (Lyubimov et al., 2007). The protein was dialysed into 50 mM Hepes pH 7, aliquots snap frozen in liquid nitrogen and stored at 193 K.

### 3.4.3. Crystallisation and Crystal Soaks

Single crystals of both COx-H6 and COx were grown from conditions previously reported for the non-histidine tagged protein (Yue et al., 1999). Crystals were obtained by vapour diffusion using the hanging drop method in 24 well VDX plates (Hampton Research). The protein (7mg/ml) was mixed in a 1:1 ratio with reservoir solution consisting of 7% PEG 8000, 100 mM cacodylate pH 5.2 and 125 mM MnSO\(_4\). The drops were immediately seeded with a solution made by crushing previously obtained COx crystals in stabilising solution (12% PEG 8K, 100 mM sodium cacodylate pH 5.2 and 125 mM MnSO\(_4\)). Crystals grew to a volume of ~0.01 mm\(^3\).
Single COx crystals were soaked for 3 hours in 1 ml reservoir solutions containing varying concentrations of sodium dithionite (5 mM, 10 mM and 50 mM). The crystals were briefly transferred to a cryoprotectant solution consisting of the soak solution and 20% glycerol. Crystals were considered reduced when they changed from yellow to colourless. The crystals were placed in CryoLoops (Hampton Research Ltd) and flash cooled in liquid nitrogen. Crystals soaked in 10 and 50 mM sodium dithionite were colourless in appearance however exhibited no diffraction. Crystals soaked in 5 mM dithionite almost completely changed from yellow to colourless and maintained high resolution diffraction.

COx-H6 crystals were placed into a sealed vial containing 1 ml reservoir solution and 20% isopropanol. Oxygen was purged from the vial by gently bubbling argon gas through the liquid. Isopropanol treated crystals were allowed to soak overnight in the sealed anaerobic vial until they became colourless. A glove bag, maintained under an argon atmosphere, was used to manipulate the crystals under anaerobic conditions. The crystals were trapped in the colourless state by briefly cryoprotecting them in the soak solution containing 20% glycerol and flash frozen in liquid nitrogen. Aerobic isopropanol soaked crystals were prepared similarly but the soak solutions were not purged of oxygen, nor were the crystals manipulated in an anaerobic atmosphere, rather all crystal soaking and manipulations were carried out under aerobic conditions.

To ensure that isopropanol reduced the protein to the hydroquinone form, the absorption spectra of COx-H6 were recorded for the oxidised and isopropanol-reduced enzyme. Briefly, a cuvette containing 0.5 mg ml\(^{-1}\) COx-H6 in 50mM Tris-HCl pH 7.0 was prepared and the absorption spectra recorded on a Shimadzu UV/Vis spectrophotometer to obtain the oxidised spectrum. Then, isopropanol was added to a final concentration of 20% to the cuvette, which was sealed with a rubber septum and
purged of oxygen bubbling argon through the solution for approximately 2 minutes. The cuvette was placed in an anaerobic glove box overnight to ensure full reduction. The following day the absorption spectrum of the sample in the cuvette was measured.

### 3.4.4. X-ray Data Collection, Processing and Phasing

X-ray diffraction datasets for all soaked crystals were obtained at the Australian Synchrotron, Melbourne (AS) using an ADSC Quantum 315r CCD detector. Diffraction data for the anaerobic isopropanol-soaked crystal and for the dithionite-soaked crystal were collected at beamline MX2 while data for the aerobic isopropanol soaked crystals were collected at beamline MX1. For all datasets, 360 images were collected using a rotation angle of 1° per image. Data processing was performed using the XDS software (Kabsch, 2010) and data reduction carried out using the CCP4 suite of software (Winn et al., 2011).

For all data, crystallographic refinement was carried out starting with a previously determined high resolution (HR) ligand free structure of COx (Lario et al., 2003) (PDB accession code 1MXT). The model was edited to remove the FAD cofactor, water molecules and alternate conformations of residues. The phases was improved through iterative cycles of model building using Coot (Emsley et al., 2010) and the refinement options in PHENIX (Adams et al., 2010) and REFMAC5 (Winn et al., 2011). The side-chains of oxygen tunnel and aromatic triad residues (Tyr444, Tyr107, Phe446, Met122, Phe359 and Asn485) were modelled with partial occupancies in cases where an alternate conformation was not visible in the difference electron density but where negative density appeared and persisted after several cycles of refinement. The data processing and refinement statistics for each structure are shown in Table 3.1. The atomic coordinates have been deposited in the Protein Data Bank (accession codes 4U2L, 4U2S and 4U2T for the enzyme complexed to sulfite, the
enzyme anaerobically reduced with isopropanol and the enzyme soaked with isopropanol aerobically respectively).
Table 3.1. X-ray data processing and refinement statistics for dithionite and isopropanol structures

<table>
<thead>
<tr>
<th></th>
<th>Sulfite adduct</th>
<th>Anaerobic propan-2-ol Complex</th>
<th>Aerobic propan-2-ol Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data Processing Statistics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>19.97-1.32 (1.39-1.32)*</td>
<td>47.03-1.12 (1.18-1.12)</td>
<td>19.21-1.22 (1.29-1.22)</td>
</tr>
<tr>
<td>Total reflections</td>
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<td>808,121 (88,952)</td>
<td>526,499 (72,894)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>100,763 (9,433)</td>
<td>173,728 (25,207)</td>
<td>132,630 (18,909)</td>
</tr>
<tr>
<td>Completeness, %</td>
<td>94.2 (60.7)</td>
<td>99.9 (99.5)</td>
<td>98.3 (96.4)</td>
</tr>
<tr>
<td>Average I/σ</td>
<td>12.0 (3.0)</td>
<td>17.8 (6.5)</td>
<td>10.3 (2.0)</td>
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<tr>
<td>R_merge</td>
<td>0.38 (1.16)</td>
<td>0.05 (0.18)</td>
<td>0.07 (0.67)</td>
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<tr>
<td>CC_{1/2}</td>
<td>0.994 (0.749)</td>
<td>0.999 (0.785)</td>
<td>0.998 (0.656)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>12.0 (8.9)</td>
<td>4.7 (3.5)</td>
<td>4.0 (3.9)</td>
</tr>
<tr>
<td><strong>Refinement Statistics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
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<td>44.63-1.12</td>
<td>19.21-1.22</td>
</tr>
<tr>
<td>Total reflections used</td>
<td>98,669</td>
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<td>132,586</td>
</tr>
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<td>R factor</td>
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<td>0.0902</td>
<td>0.1201</td>
</tr>
<tr>
<td>Free R factor</td>
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<td>0.1068</td>
<td>0.1495</td>
</tr>
<tr>
<td>rms deviation bond lengths, Å</td>
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<tr>
<td>rms deviation bond angles, °</td>
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<td>1.38</td>
<td>1.50</td>
</tr>
<tr>
<td>No. of non-hydrogen atoms</td>
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<td>5142</td>
<td>4613</td>
</tr>
<tr>
<td>No. of SO₃ atoms (molecules)</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>No. of SO₄ molecules</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Average B factors, Å²</td>
<td>9.6</td>
<td>11.5</td>
<td>15.6</td>
</tr>
</tbody>
</table>

* Statistics for the highest resolution shell are shown in parentheses
3.4.5. Computational Details

The geometries and harmonic vibrational frequencies of all structures have been obtained at the B3LYP-D3/Def2-TZVPP level of theory (the optimised structures are given in Appendix 1, Table A.1) (Becke, 1993; Lee et al., 1988; Stephens et al., 1994; Weigend & Ahlrichs, 2005). Empirical D3 dispersion corrections (Grimme, 2011; Grimme et al., 2011) are included using the Becke–Johnson (Becke & Johnson, 2005; Johnson & Becke, 2005; Johnson & Becke, 2006) damping potential as recommended in Grimme et al. (2011) (denoted by the suffix D3). Zero-point vibrational energies and enthalpic temperature corrections have been obtained, within the rigid-rotor harmonic oscillator approximation, from such calculations. All geometry optimisations and frequency calculations were performed using the Gaussian 09 program suite (Frisch et al., 2009). The equilibrium structures in the unconstrained optimisations were verified to have all real harmonic frequencies, whereas the constrained structure is characterised by one imaginary frequency (of 28.324 cm⁻¹) that corresponds to the geometry constraint placed on the $\angle$N10-N5-N1 angle.

High-level double-hybrid density functional theory (DHDFT) calculations (Goerigk & Grimme, 2014) using the B2GP-PLYP procedure (Karton et al., 2008) were performed to determine the strength of the hydrogen bond interactions at the N5 centre. The B2GP-PLYP procedure involves both HF-like exchange and MP2-like correlation in the functional form and thus offers a reliable approach to treat systems that involve weak interactions (Karton et al., 2008; Tarnopolsky et al., 2008). In particular, the B2GP-PLYP functional has been found to yield excellent performance for weak interactions such as hydrogen bonds, dispersion forces, and van der Waals interactions (Karton et al., 2008). The DHDFT calculations, which inherit the slow basis-set convergence of MP2 to some degree, are carried out in conjunction with the Def2-QZVPP basis set (Weigend & Ahlrichs, 2005). All DHDFT calculations were
performed using the ORCA 3.0.1 program suite, where the resolution of the identity (RI) approximation was used in the MP2 steps (Neese, 2012).

3.5. Results

3.5.1. Bound Complexes

3.5.1.1. Sulfite – FAD Adduct Structure

The structure of the dithionite soaked crystals complex was determined to 1.32 Å resolution and revealed a single sulfite molecule as a covalent adduct to the N5 atom of FAD (Fig. 3.1a). Crystallographic refinement of the covalent adduct resulted in sulfite occupancy of 0.64. $\text{SO}_3$ is an oxidation product of dithionite and is present as a covalent adduct in several other dithionite-reduced FAD containing proteins (Emsley et al., 2010; Fritz et al., 2002; Tegoni & Cambillau, 1994). This electronegative adduct causes a significant displacement of the side-chain of Glu361 from previously observed conformations in the HR ligand free structure (Lario et al., 2003). Furthermore, the repositioning of the side chain of Glu361 restricts the orientation of the side chain of Met122. Previously, these side-chains adopted conformations that facilitated opening and closing of a channel from the exterior of the protein to the internal substrate-binding cavity (Lario et al., 2003). In the adduct bound state, Met122 is observed in the open state while the side-chains of Asn485 and Phe359, which gate the tunnel in the substrate-binding pocket and bulk solvent sides respectively, are observed only in the closed conformation.

The covalent bond formed between the sulfite molecule and N5 of the FAD causes the flavin N5 atoms to adopt a more tetrahedral-like geometry and distorts the dimethyl benzene portion of the isoalloxazine ring away from that observed by Lario et
al. (2003) in the HR ligand free structure. This bending of the cofactor ring system has been reported previously (Lyubimov et al., 2007) when a double mutant form of the enzyme was soaked in glycerol (Fig. 3.1b). However, in the current structure a smaller degree of bending along the N5-N10 plane of the isoalloxazine ring was evident relative to that observed in the mutant glycerol co-crystal structure. Furthermore the structure determined in the presence of glycerol led to two conformations of the isoalloxazine ring whereas in the current structure only a single conformation was present.

The active site of the enzyme contains a hydrophobic triad consisting of Tyr446, Tyr107 and Phe444. These residues have been observed in two discrete conformations and are proposed to play a role in substrate induced bending of the isoalloxazine ring system as the flavin is reduced (Lyubimov et al., 2007). Inspection of these residues in the sulfite adduct structure revealed similar alternate side chain conformations to those previously observed in the structure of a double mutant form of the enzyme where a flavin glyceraldehyde adduct is present (Lyubimov et al., 2007) (Fig. 3.1c).
Figure 3.1. Sulfite-adduct and aromatic triad of dithionite-soaked structure

(a) Electron density map of the FAD-sulfite adduct. The $2F_o - F_c$ electron density map is displayed as a blue mesh (contoured at 2.5 σ). (b) Superposition of the structures obtained for the sulfite adduct, the glyceraldehyde adduct and the non adduct bound cofactor (PDB accession code 3B3R for the latter two structures). The FAD cofactor in the sulfite adduct is displayed with green bonds. The glyceraldehyde bound adduct is shown with magenta bonds and the non adduct bound cofactor is shown in yellow bonds. (c) The aromatic triad residues as observed in the FAD-sulfite adduct structure. Conformation A is shown as green bonds and conformation B is shown in orange bonds. The $2F_o - F_c$ map is displayed as a blue mesh (contoured at 1.0 σ) and the $F_o - F_c$ map is displayed as green/red mesh (contoured at +/-4 σ). Density maps were calculated with the atoms of conformation B removed.
3.5.1.2. Isopropanol Bound Complex Structures

Isopropanol is a small alcohol substrate and has previously been reported to reduce FAD slowly (Pollegioni et al., 1999). Structures of the enzyme in complex with isopropanol were determined to 1.12 Å and 1.22 Å resolution for crystals soaked in solutions containing the substrate under anaerobic and aerobic conditions, respectively. In the anaerobic case, the crystals bleached, suggesting that the enzyme was trapped in the reduced state, whereas in the aerobic isopropanol case the crystals remained yellow in colour, suggesting that the enzyme had reverted back to the oxidised state. The absorption spectra of the oxidised and anaerobic isopropanol soaked enzyme in solution shows that COx is reduced to the hydroquinone form by isopropanol (Fig. 3.2a). The high resolution of the data enables near atomicity for many of the atoms in the structure.

Difference electron density was apparent in the substrate binding pocket for each of the isopropanol structures (Fig 3.2b and 3.2c) suggesting the presence of isopropanol. However, modelling of isopropanol into the density and refinement did not result in convincing $2F_o - F_c$ density therefore the substrate molecules were not included in the final structures. It is likely that the small size of the substrate relative to cholesterol, the natural substrate for the enzyme, results in multiple disordered binding modes and thus poorly defined difference electron density.

The residues comprising the aromatic triad (Tyr107, Tyr446 and Phe444) adopt two conformations (Fig. 3.2d) in the anaerobic structure whereas they are only found in a single conformation in the aerobic structure (Fig. 3.2e). In the former case, the alternate side chain conformation (B) was modelled into well-defined positive difference density but refined only to low occupancy (0.28, 0.24 and 0.24, respectively) resulting in poor $2F_o - F_c$ density for this region. However, the well-defined difference density and the low temperature factors for these residues (9.63 Å$^2$, 7.07 Å$^2$ and 11.3...
Å²) strongly suggests the presence of the alternate conformations despite poor $2F_o - F_c$ density.

Figure 3.2. Structures of oxidised and reduced COx crystals soaked in isopropanol

(a) Absorption spectra of the oxidised (red curve) and reduced (blue curve) states of cholesterol oxidase enzyme. (b) and (c) Residual difference density in the binding pocket of the reduced and oxidised structures, respectively. The $2F_o - F_c$ maps for panels (b) and (c) are displayed as a blue mesh (contoured at 1.5 σ) and the $F_o - F_c$ maps are displayed as green/red mesh (contoured at +/-3.5 σ). (d) Electron density maps for the aromatic triad amino acid residues of the reduced anaerobic isopropanol structure. The $2F_o - F_c$ map is displayed as a blue mesh (contoured at 1.5 σ) and the $F_o - F_c$ map is displayed as green/red mesh (contoured at +/-4 σ). (e) Electron density maps for the aromatic triad amino acid residues of the aerobic isopropanol structure. $2F_o - F_c$ (contoured at 1.5 σ) is displayed as blue mesh and the $F_o - F_c$ (contoured at +/-3.0 σ) is displayed as green/red mesh. Conformation A of the triad residues is shown as green bonds and conformation B residues are shown as orange bonds. Maps were calculated with the atoms of conformation B removed for panel (d).
During refinement of the anaerobic isopropanol complex structure a positive difference electron density peak became apparent at > 3.5 σ contour level positioned 1.03 Å from the isoalloxazine N5 atom in a tetrahedral geometry (C5X-N5-HN5 angle of ~111°) suggesting that a hydride transfer had occurred from the isopropanol substrate (Fig. 3.3a). Modelling of a hydrogen atom at full occupancy into this difference density removed the residual difference density. Interestingly, a similar difference electron density peak was not present in the maps of the aerobic isopropanol complex even at very low difference map contour levels (Fig. 3.3b).
Figure 3.3. COx FAD in the reduced and oxidised states

Wall-eyed stereo views of the FAD cofactor in the anaerobic \((a)\) and aerobic \((b)\) isopropanol structures. Wat541 is modelled in two conformations. The \(2F_o - F_c\) electron density map is shown as blue mesh (contoured at 2.5 \(\sigma\)) and the \(F_o - F_c\) density map is shown as a green/red mesh respectively (contoured at +/-3.5 \(\sigma\) N.B there are no peaks below -3.5 \(\sigma\)). \((c)\) The hydrogen bond formed between Gly120 amide hydrogen and the flavin N5 atom.
Hydride transfer to N5 of the flavin induced no further significant bending of the isoalloxazine moiety from that of the oxidised structure. A hydrogen bond (2.36 Å) between the flavin N5 and the backbone amide hydrogen of Gly120 completes the tetrahedral geometry about N5 (Fig. 3.3c). Theoretical calculations have been performed on free flavins and flavins within protein structures (Cavelier & Amzel, 2001; Walsh & Miller, 2003; Zheng & Ornstein, 1996); however, no experimental evidence for the position of the hydrogen atom has been obtained due to the limitations of X-ray crystallography for locating hydrogen atoms except at atomic resolutions (Elias et al., 2013). High-level DHDFT calculations using the B2GP-PLYP procedure (Karton et al., 2008) were performed in order to probe the strength of the hydrogen bond formed between Gly120 (Fig. 3.3c) and the reduced isoalloxazine system (FADH\(^-\)) and to assess the possible implications of this hydrogen bonding interaction on the FADH\(^-\) structure. For reasons of computational efficiency, the ribitol-ADP side chain on the flavin and the Gly120 residue were modelled by a methyl group and a dimethylamine moiety, respectively. Our model systems are shown in Figure 3.4 and Table 3.2 gives the hydrogen bond strengths and selected bond distances and angles for these structures. It is instructive to begin with the structure of the reduced isoalloxazine without any H-bonding interactions (Fig. 3.4a). This structure is characterised by a bending angle of the isoalloxazine of 158.3° and a shallow angle for the position of the transferred hydride atom of 165.5° (Table 3.2). Adding a dimethylamine in the calculations, to mimic the R\(_2\)N1–H1 functional group of Gly120, results in a strong hydrogen bond interaction of 7.47 kcal mol\(^{-1}\) between the negatively charged isoalloxazine (FADH\(^-\)) and the dimethylamine hydrogen bond donor (Fig. 3.4b and Table 3.2). The effectiveness of this interaction is also evident by a relatively short hydrogen bond distance (FADH\(^-\)•••H–N) of 2.252 Å. In the fully optimised structure the dimethylamine is situated directly above the N5 nitrogen such that the ∠N10-N5-H1
angle is 85.8° (Fig. 3.4b). This situation allows for an effective overlap between the lone pair on the N5 nitrogen and the N1–H1 bond of the dimethylamine. Constraining the $\angle$N10-N5-N1 angle in our model to be 125.5° allows for a less effective overlap between the lone pair on N5 and the H1–N1 bond (Fig. 3.4c). Consequently, the strength of the hydrogen bond is reduced to 5.45 kcal mol$^{-1}$ and the hydrogen bond distance is slightly elongated to 2.432 Å (Table 3.2). Constraining the H1–N1 bond to be at a similar position as in the crystal structure has two important structural consequences:

- The isoalloxazine ring system becomes less bent. In particular, the bending angle ($\alpha$) increases from a value of 157.2° (in the fully optimised model, Fig. 3.4b) to a value of 164.9° (in the constrained model, Fig. 3.4c).

- The hydrogen attached to N5 shifts further away from the plane of the central ring, in particular the $\angle$N10-N5-H5 angle decreases from a value of 164.0° (in the fully optimised model, Fig. 3.4b) to a value of 154.3° (in the constrained model, Fig. 3.4c).
Table 3.2. Selected bond lengths and angles (in Å and degrees) of the FADH– and calculated strength of the FADH–•••HNMe₂ hydrogen bond (\\(\Delta H_{298}^*\), kcal mol\(^{-1}\)).

<table>
<thead>
<tr>
<th></th>
<th>(\Delta H_{298}^*)</th>
<th>(\alpha^1)</th>
<th>(\angle N10-N5-H5)</th>
<th>(\angle N10-N5-H1)</th>
<th>N5•••H1</th>
</tr>
</thead>
<tbody>
<tr>
<td>FADH†</td>
<td>158.3</td>
<td>165.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FADH–•••HNMe₂§</td>
<td>7.47</td>
<td>157.2</td>
<td>164.0</td>
<td>85.8</td>
<td>2.252</td>
</tr>
<tr>
<td>FADH–•••HNMe₂**</td>
<td>5.45</td>
<td>164.9</td>
<td>154.3</td>
<td>124.6</td>
<td>2.432</td>
</tr>
<tr>
<td>FADH–Gly120††</td>
<td>175.8</td>
<td>129.0</td>
<td>127.4</td>
<td></td>
<td>2.374</td>
</tr>
</tbody>
</table>

* The geometries are optimised at the B3LYP-D3/TZVPP level of theory and the hydrogen-bond energy is calculated at the B2GP-PLYP/QZVPP level of theory
† Bending angle about the N\(_{10}\)•••N\(_5\) axis of the central ring of the reduced isoalloxazine, see Fig. 5a
‡ Structure shown in Fig. 5a
§ Structure shown in Fig. 5b
∥ The \(\angle N10-N5-N1\) angle is constrained to the value in the crystal structure, see Fig. 5c.
†† Crystal structure.
3.6. Discussion

In this study structures of COx were determined from crystals which were trapped in the reduced state using either an alcohol substrate or dithionite, a strong reducing agent commonly used to study redox chemistry of flavoenzymes (Ghisla & Massey, 1986). Crystals of the enzyme with an N5-sulfite adduct were prepared under aerobic conditions and the crystals bleached from yellow to colourless. Additionally crystals of the enzyme soaked in an alcohol substrate were prepared under aerobic conditions and anaerobic conditions. Only in the case of the anaerobic soak conditions did the crystals undergo a colour change from yellow to colourless, and remain colourless, indicative of a trapped reduced form of the enzyme. In this case, the absence of O$_2$ prevented the enzyme from undergoing the oxidative half-reaction. In the dithionite soaked crystals a trapped covalent flavin sulfite adduct was observed. In contrast, in the presence of isopropanol, no covalent adduct was apparent with either the aerobic or anaerobic treated crystals. Comparisons of active site residues between the two isopropanol treated crystals show differences in side chain conformations and in the structure of the isoalloxazine ring system of the cofactor that provide insights into the mechanism by which the protein accommodates the reduced state of the cofactor.

The covalent sulfite adduct structure revealed two conformations of the aromatic triad side-chains and bending of the isoalloxazine ring system of the FAD. These conformational changes have previously been observed for crystals of an enzyme double mutant (His447Gln/Glu361Gln) which were soaked in glycerol; in this case a covalent adduct was observed between the FAD-N5 atom and the reduced glycerol product, glyceraldehyde (Lyubimov et al., 2007). Additionally, a structure of a COx from Brevibacterium sterolicum (Li et al., 1993) in its reduced state with a non-covalently bound steroid molecule also showed movements of the side-chains within the aromatic triad compared to the unbound structure. Our studies, using
dithionite, further support the proposal that bending of the flavin and movement of the aromatic triad residues is linked to ligand binding, and the redox state of the cofactor (Lyubimov et al., 2007). In the previous study of the glyceraldehyde adduct structure, the high resolution of the structures enabled two conformations of the isoalloxazine ring to be observed; one conformation adopts the typical butterfly twist while the second conformation exhibits a greater degree of bending of the dimethyl benzene moiety presumably due to steric pressure exerted by the aromatic triad. The different conformations of the flavin were correlated with the amount of covalent adduct present in the model. In the present sulfite adduct structure, despite the relatively high occupancy of the adduct (0.63), the density maps did not reveal as significant a distortion in the isoalloxazine ring conformation as was observed in the double mutant/glycerol structure. This difference may be due to the lower resolution of the structure (1.3Å) relative to the double mutant structure (0.98Å). Furthermore, the degree of bending seen in the FAD/sulfite adduct was less than that observed in the glyceraldehyde adduct structure. This apparent lesser degree of bending of the flavin ring may also be a consequence of the limited resolution of the sulfite adduct structure which only provides a view of the average of the covalent flavin adduct and the unbound states. Therefore, although we cannot establish the exact extent of flavin bending in the sulfite bound structure at the current resolution, the multiple conformations of the aromatic triad provide strong supportive evidence that the presence of a ligand covalently bound to the flavin cofactor invokes changes to the active site of the enzyme that includes conformational changes to the flavin moiety.

Substrate oxidation by the enzyme is not expected to involve a covalent flavin adduct. In the case of the glyceraldehyde adduct structure, adduct formation is likely to have occurred after substrate oxidation of the primary alcohol group of glycerol to form glyceraldehyde which further reacts resulting in a covalent complex with FAD. In
contrast, isopropanol is a secondary alcohol substrate, mimicking the secondary alcohol of cholesterol. Oxidation of a secondary alcohol results in the formation of a ketone which cannot undergo further reaction with FAD to form a covalent adduct. We therefore expect the isopropanol bound forms to more accurately represent the true substrate reduced state of the enzyme.

Interestingly, the anaerobic, isopropanol bound structure showed a positive difference density peak positioned within a bond distance from the flavin-N5 atom. The position of this density, and the bleached appearance of the anaerobic crystals, suggests that the enzyme has undergone a hydride transfer from isopropanol to N5 of the flavin. This state also shows the presence of multiple conformations of the aromatic triad residues that were not observed in the aerobic isopropanol structure. High occupancy (>0.5) of the bound steroid substrate (Li et al., 1993), the glyceraldehyde adduct (Lyubimov et al., 2007) and our current sulfite adduct all correlate with a high occupancy for the alternate conformation of the aromatic triad. The isopropanol bound structures both exhibit poorly occupied ligand in the binding site. This suggests that the alternate aromatic triad conformations are due to two factors: steric pressure induced by a highly occupied ligand and reduction of the cofactor, without bending of the flavin ring system. The second conformation of the aromatic triad orients the more positively charged periphery of the Tyr446 ring (Chakrabarti & Bhattacharyya, 2007; Hunter, 1993; Hunter & Sanders, 1990; McGaughey et al., 1998), towards the dimethyl benzene moiety of the cofactor. This interaction may help to stabilise the increased electronegative state of the reduced cofactor.

FAD can exist in oxidised, semiquinone or hydroquinone states depending on the mechanism and redox state of the cofactor. Structures of these flavin states have been investigated by theoretical calculations and suggest that the isoalloxazine ring system for free oxidised flavin is planar while that of the reduced flavin is bent about
the N5-N10 axis to form a ‘butterfly’ conformation (Hall et al., 1987; Walsh & Miller, 2003; Zheng & Ornstein, 1996). Zheng and Ornstein (1996) have also suggested however, that the anionic form of flavin may be planar. Crystal structures of flavoproteins have also revealed planar ring conformations for the oxidised state (Waksman et al., 1994) as well as bent conformations for the reduced state (Abendroth et al., 2011; Gardberg et al., 2011; Porter & Voet, 1978; Singh et al., 2014). The ring conformation of the flavin is highly influenced by the local protein environment which modulates the flavin both structurally (largely through hydrogen bond interactions) and through redox activity (Li & Fu, 2008; Rohr et al., 2010; Rotello, 1999). Examples of flavoproteins are reported where the flavin changes from a planar oxidised state to a bent reduced state (Lennon et al., 1999; Senda et al., 2007; Waksman et al., 1994). In contrast other reports show reduced flavins with little or no change in the ring conformation between the oxidised and reduced states (Dobbek et al., 2002; Faust et al., 2007; Li et al., 1993; Sukumar et al., 2004; van den Hemel et al., 2006; van Straaten et al., 2012).

Comparisons of the oxidised and isopropanol reduced structures of COx indicate no significant change in the isoalloxazine ring conformations. In contrast, the formation of a covalent adduct to N5 as seen in the sulfite bound structure does result in an increase in the extent of ring bending. These finding are in further agreement with other sulfite bound flavins reported for alditol oxidase, adenosine-5’-phosphate reductase and pyranose oxidase (Emsley et al., 2010; Forneris et al., 2008; Schiffer et al., 2006). In the case of flavocytochrome B2, the sulfite adduct only moves the N5 atom to lie out of the plane of the normally planar isoalloxazine ring (Mowat et al., 2000; Mowat et al., 2004).

Density functional theory calculations on flavin derivatives showed that increasing the imposed bend angle of the flavin increased the relative stability of the
reduced hydroquinone flavin compared to the semiquinone form (Walsh & Miller, 2003). Furthermore, these studies showed that the conformational energy of the FAD was correlated to the midpoint reduction potential such that a bent flavin conformation is a better electron acceptor while a planar conformation is a better electron donor.

Our results for the covalent adduct and the anaerobic isopropanol reduced state of the enzyme provide experimental validation that the formation of the covalent adduct, and not the reduction of the flavin *per se*, causes the isoalloxazine ring system to bend further from its oxidised state. Indeed, the protein environment around the flavin stabilises the isoalloxazine ring away from planarity thereby priming the cofactor for reduction. Further bending is mediated only through formation of a covalent adduct.

We suggest that the difference density peak near the flavin N5 of the anaerobic isopropanol-reduced structure indicates the presence of a hydrogen atom. This peak is not present in the aerobic isopropanol structure where the crystals have not undergone bleaching and hence the flavin is not reduced. The difference density peak is positioned 1.03 Å from the flavin N5 consistent with an N-H bond. While a number of crystallographic structures of reduced flavins have been reported below 1.5 Å resolution (Gustafsson *et al.*, 2007; Johansson *et al.*, 2010; Khan *et al.*, 2005; Rohr *et al.*, 2010; Sedlacek *et al.*, 2014; Sukumar *et al.*, 2004), the resolution of these structures is still insufficient to establish the location of the hydride bound to the flavin. The backbone amide N-H group of Gly120 is positioned below the isoalloxazine ring system and functions as hydrogen bond donor to the flavin N5 atom. A similar hydrogen bond interaction with the hydrogen bond donor group originating from a backbone amide below the isoalloxazine ring is observed in several other flavoenzymes including glucose oxidase from *Penicillium amagasakiense* (Wohlfahrt *et al.*, 1999), S-mandelate dehydrogenase (Sukumar *et al.*, 2004) D-amino acid oxidase (Mattevi *et al.*, 1996) and
NAD(P)H:acceptor oxidoreductase (FerB) from *Paracoccus denitrificans* (Sedlacek *et al.*, 2014).

Intriguingly, the structure of a mutant form of Shewanella yellow enzyme 1 has been reported in the Protein Data Bank (Berman *et al.*, 2000) in the oxidised and reduced states (Elegheert *et al.*, 2013) at 1.0 Å and 0.98 Å resolution respectively (PDB codes 4AWS and 4AWT respectively). Inspection of the electron density maps for these structures reveals the presence of a difference density peak (3.0 σ) 1.07 Å from the flavin N5 atom only in the reduced enzyme structure suggesting a hydride transfer had occurred. This difference peak is also tetrahedrally oriented about N5. The amide N-H group of Thr26 forms a hydrogen bond with N5 from the opposite side of the isoalloxazine ring system.

The conservation of a hydrogen bond between the main-chain amide N-H group and the flavin N5 in the structures of COx, D-amino acid oxidase, glucose oxidase, choline oxidase, S-mandelate dehydrogenase, NAD(P)H:acceptor oxidoreductase and Shewanella yellow enzyme suggests an important role for this interaction in stabilising the N5 atom during catalysis. Indeed, our DFT calculations suggest that this hydrogen bond influences the position of the hydride on the N5 atom. In the fully optimised structure the strength of the hydrogen bond formed between the dimethylamine and the negatively charged isoalloxazine system is 7.47 kcal mol$^{-1}$ (Table 3.2) and the H1–N1 bond of the dimethylamine moiety is situated directly above the N5 centre. Constraining the ∠N10-N5-N1 angle in our model to be the same as we observe in the crystal structure results in a less effective overlap between the lone pair on N5 and the H1–N1 bond. Consequently, the strength of the hydrogen bond is reduced and the hydrogen bond distance is slightly elongated. Constraining the H1–N1 bond also has two important structural consequences that are consistent with what we observe in our reduced crystal structure: the isoalloxazine ring system becomes less bent and the
hydrogen attached to N5 shifts further away from the plane of the central ring compared to the free and fully optimised system. In the fully optimised model (Fig. 3.4b), the hydrogen bond interaction between the dimethylamine and the flavin N5 has little effect on the structure of the isoalloxazine system relative to the free isoalloxazine (Table 3.2). Therefore, the observed structural changes to the isoalloxazine ring can be attributed to the position of the hydrogen bond donor (Gly120), which results in a movement of the lone pair of electrons on the N5 centre from lying directly above the nitrogen atom in the fully optimised model (Fig. 3.4b) towards the N5•••H1 axis in the constrained model (Fig. 3.4c).

A conserved water molecule is observed in hydrogen bond distance from the flavin N5 and from Ne2 of His447. Lario et al. (2003) have proposed that this water molecule mimics the position of the substrate hydroxyl group prior to hydride transfer. Furthermore, a hydrogen bond interaction between HE2 of His447 and the lone pair electrons on the substrate oxygen atom orients the hydroxyl hydrogen atom towards the proposed base, Glu361. This results in an energetically favourable trans arrangement between the substrate hydroxyl hydrogen atom and the hydride atom (Lario et al., 2003). Interestingly, when dehydroisoandrosterone (DHA) is modelled into the anaerobic reduced structure with the above considerations, the substrate C3 atom lies 2.5 Å away from the flavin N5 atom and in line with the position of the hydride atom forming an angle of 128° with the N5-N10 atoms (Fig. 3.5). Rigid positioning of the substrate in this way would require a slight rearrangement of residues within the extended loop (190-228) in order to accommodate the steroid D ring and the extended C17 tail. A previous survey of flavoprotein structures with their bound substrates revealed that the carbon atom involved in oxidative attack was positioned ~3.5 Å from the flavin N5 with an angle (\(\angle\)N10-N5-C) between 96° and 117° (Fraaije & Mattevi, 2000). To achieve an angle closer to that reported by Fraaije and Mattevi would result...
in significant clashes between the substrate and protein atoms involved in catalysis or would require movement of the substrate oxygen atom away from the position of the conserved water molecule. Our structure reveals a preformed active site utilizing Gly120 to position the flavin N-5 lone pair of electrons such that they align appropriately to facilitate efficient hydride transfer from the substrate in the Michaelis complex to generate the reduced flavin during the oxidative half-reaction.

![Figure 3.5](image_url)

**Figure 3.5. Wall-eyed stereo view of the proposed binding model of the steroid substrate in the active site of COx**

Positions of all residues are those observed in the anaerobic reduced structure. Dehydroisoandrosterone (DHA) has been modelled as blue ball-and-stick representation into the protein structure (green bonds) by positioning the substrate hydroxyl oxygen atom at the location of the conserved water molecule (Wat541). The molecule was positioned to enable a hydrogen bond interaction from the substrate oxygen and Ne2-Hε2 of His447 as well as a hydrogen bond interaction between the substrate hydrogen atom and the side chain of Glu361. The observed $F_o - F_c$ electron density (3.0 σ) for the transferred hydride in the reduced structure is shown as a green mesh to compare the relative positioning of the substrate C-H in the model to the hydride location in the reduced structure.

The ability to trap crystals in the different redox states while maintaining atomic resolution diffraction has provided an unprecedented opportunity to characterise the enzyme active site of COx at an exquisite level of detail. These studies have given a unique view of a flavin in the reduced state with a hydride transferred to the cofactor.
and further allow us to delineate important interactions between the protein and the cofactor that facilitate redox chemistry. In particular, a hydrogen bonding interaction modulates the geometry of the flavin N5 centre and may play an important role in priming the flavin for redox activity.

3.7. Acknowledgments

We thank Anandhi Anandan for assistance with protein expression and purification and Professor Reto Dorta for access to an inert glove box. We also acknowledge the staff at beamlines MX1 and MX2 of the Australian Synchrotron for support with X-ray data collection.
CHAPTER 4

PRODUCTION AND CHARACTERISATION OF RECOMBINANT PERDEUTERATED CHOLESTEROL OXIDASE
4.1. Authors and Contributions

The chapter has been published in *Analytical Biochemistry* (Golden *et al.*, 2015). The authors of the manuscript are Emily Golden¹, Paul V. Attwood¹, Anthony P. Duff², Flora Meilleur³⁴ and Alice Vrielink¹. Perdeuterated enzyme was produced by A.P.D. and E.G. E.G. did all other experimental work. Determination of kinetic parameters for the enzyme assays were provided by P.V.A. E.G. wrote the paper with critical revisions provided by A.V., A.P.D. and P.V.A.

¹School of Chemistry and Biochemistry, University of Western Australia, Crawley, Western Australia, 6009 Australia

²Bragg Institute, Australian Nuclear Science and Technology Organisation, Lucas Heights NSW, Australia, 2234, Australia

³Neutron Sciences Directorate, Oak Ridge National Laboratory, Oak Ridge, TN, 37831, United States of America

⁴Structural and Molecular Biochemistry, North Carolina State University, Raleigh, NC 27695, United States of America
4.2. Abstract

Cholesterol oxidase (COx) is an FAD containing enzyme that catalyses the oxidisation and isomerisation of cholesterol. Studies directed towards elucidating the catalytic mechanism of COx will provide an important general understanding of flavin assisted redox catalysis. Hydrogen atoms play an important role in enzyme catalysis however, they are not readily visualised in protein X-ray diffraction structures. Neutron crystallography is an ideal method for directly visualising hydrogen positions at moderate resolutions because hydrogen and deuterium have comparable neutron scattering lengths to other heavy atoms present in proteins. The negative coherent and large incoherent scattering lengths of hydrogen atoms in neutron diffraction experiments can be circumvented by replacing hydrogen atoms with its isotope, deuterium. The perdeuterated form of COx was successfully expressed from minimal media, purified, and crystallised. X-ray crystallographic structures of the enzyme in the perdeuterated and protiated states confirm that there are no apparent structural differences between the two enzyme forms. Kinetic assays demonstrate that perdeuterated and protiated enzymes are functionally identical. Together, structural and functional studies indicate that the perdeuterated protein is suitable for structural studies by neutron crystallography directed at understanding the role of hydrogen atoms in enzyme catalysis.

4.3. Introduction

The Protein Data Bank (Berman et al., 2000) contains atomic coordinates for over 100,000 structures (as of 2015), the vast majority of which have been determined using X-ray crystallography. While X-ray diffraction is an excellent technique for visualisation of the more electron rich atoms commonly found in proteins (C, O, N, P, S), hydrogen atoms are only visible at very high resolutions (<1.0Å). Despite their
small size, hydrogen atoms play a dominant role in the structure and function of macromolecules. They are critical in forming hydrogen bonds, which dictate the stability and local conformations of the structure, drive the pKa of amino acid side-chains within the protein micro-environment, and thus affect the chemistry of these complex molecules in different ways. Furthermore, enzyme chemistry often involves movements of hydrogen atoms between amino acid side-chains of the protein, cofactors and bound ligands during the catalytic cycle. Thus, a detailed knowledge of the positions of hydrogen atoms is critical to fully understand the chemistry of macromolecules and especially enzymes.

Often however, even when ultra-high resolution X-ray crystallographic data are available, the hydrogen positions are still only very weakly visible in electron density maps (Blakeley et al., 2006; Chen et al., 2008; Gardberg et al., 2010; Howard et al., 2004; Koepke et al., 2002; Kuhn et al., 1998; Lario et al., 2003; Lyubimov et al., 2006). This may be due to intrinsic flexibility of the atoms to which they are bonded which would result in a diffuseness of the position of the single electron of the hydrogen atom. In addition, regions of the structure may adopt discrete alternate conformations, often providing important insights into the mobility and the flexibility of the molecule correlated with function. However, these multiple states, or occupancies, further challenge the ability to visualise hydrogen atoms that are already difficult to see at the highest resolutions obtained by X-ray crystallography. Furthermore, the physical nature of the X-ray beam during data collection can result in photoinduced reduction events (Carugo & Carugo, 2005; Hersleth & Andersson, 2011; Hersleth et al., 2008). Particularly in the case of redox specific enzymes these beam-induced events can confound interpretation of the electron density as the beam causes mixed conformational states due to the accumulation of mixed redox states of the molecule during data collection (Rohr et al., 2010).
Neutron protein crystallography (NPC) is a technique that can be used to more readily visualise hydrogen within protein molecules as these atoms scatter neutrons comparably to heavier atoms such as carbon and nitrogen and so can be visualised at moderate resolutions (1.5 Å - 2.5 Å). However, hydrogen exhibits a negative coherent scattering length, which often results in cancellation of density for the positively scattering atom it is bonded to. Additionally, hydrogen has a large incoherent scattering cross section, which decreases the signal-to-noise ratio (SNR) of data collected from hydrogen-containing samples. Deuterium, in contrast, has a small incoherent scattering cross section and has a positive coherent scattering length, nearly twice the magnitude of the hydrogen scattering length, and comparable to that of carbon, nitrogen and oxygen atoms. Exchanging the hydrogen atoms for deuterium atoms therefore improves the visibility of hydrogen (deuterium) atom positions in neutron structures. Solvent hydrogen atoms and labile protein hydrogen atoms can be exchanged by incubating protein or protein crystals in deuterated buffers. However, partial hydrogen-deuterium exchange can occur at buried or protected labile sites resulting in cancellation of density at these positions. In addition, non-labile, carbon-bound hydrogen atoms - approximately 75% of the protein hydrogen atoms, will not be exchanged by this technique and will significantly contribute to diffraction background. Full protein deuteration can alleviate the non-labile hydrogen contribution to background. Perdeuterated protein (all hydrogen atoms are exchanged for deuterium atoms) is produced by expressing proteins in fully deuterated media (Gamble et al., 1994). Shu et al. (2000) presented the first perdeuterated neutron crystal structure of myoglobin and demonstrated the power of perdeuteration in combination with neutron diffraction for visualising hydrogen atom positions. More recently, a neutron diffraction structure at 1.65 Å of perdeuterated rubredoxin was able to identify 8-fold
more deuterium atom positions than hydrogen atom positions that could be identified in an X-ray structure at 1.1 Å resolution (Gardberg et al., 2010).

Another advantage of neutron diffraction in the structural characterisation of redox proteins is that neutrons are non-ionising. Detailed and unambiguous analysis of the protonation and redox states of enzymes at defined steps along catalytic pathways can be performed by neutron crystallography. We are using a combination of high resolution X-ray and neutron crystallography to study the redox mechanism of cholesterol oxidase (COx). COx, E.C 1.1.3.6, is a flavin-containing redox enzyme catalysing the oxidation and isomerisation of cholesterol to cholest-4-en-3-one with the concomitant production of hydrogen peroxide. The oxidation reaction is catalysed by the tightly bound FAD cofactor which is usually non-covalently bound (Type I enzyme) but may also have a covalent attachment to the protein (Type II enzyme). COx is produced by a wide variety of bacterial species, however, only the 3-dimensional structures of the Type I COx from Streptomyces SA-COO (Yue et al., 1999) and Brevibacterium sterolicum (Li et al., 1993; Vrielink et al., 1991) and the Type II COxs from B. sterolicum (Coulombe et al., 2001) and Chromobacterium sp. DS-1 (Sagermann et al., 2010) have been determined by X-ray crystallography. Of these, the COx from Streptomyces SA-COO has been most extensively characterised by X-ray crystallography (Chen et al., 2008; Lario et al., 2003; Lario & Vrielink, 2003; Lyubimov et al., 2009; Lyubimov et al., 2007; Lyubimov et al., 2006; Yue et al., 1999) in combination with kinetic analysis of active site mutants (Chen et al., 2008; Lyubimov et al., 2009; Yin et al., 2001; Yue et al., 1999). Crystals of the enzyme diffract to high resolution with several atomic resolution structures obtained (Chen et al., 2008; Lario et al., 2003; Lario & Vrielink, 2003; Lyubimov et al., 2009; Lyubimov et al., 2007). Atomic resolution X-ray structures of COx at various pH values (Lyubimov et al., 2006) revealed pH dependent changes in the positions of some
hydrogen atoms that were involved in strong hydrogen bonding interactions between the flavin N3 and O4 (Chapter 1, Fig. 1.6). Additionally, these structures showed that His447 is protonated and therefore cannot be the base for deprotonation of the hydroxyl of the substrate (Chapter 1, Fig. 1.5).

Despite the very high resolution structures obtained from diffraction studies of crystals of COx, questions regarding the role of hydrogen atoms in the redox chemistry remain to be answered. As the reaction mechanism of COx involves abstraction of a hydroxyl proton and transfer of a hydride to FAD, it is of considerable interest to identify the location of hydrogen atoms of COx in various redox states. Neutron diffraction studies provide an excellent technique for elucidating the positions of hydrogen atoms through the catalytic cycle of the enzyme and will be highly complementary to X-ray diffraction studies of the enzyme.

Perdeuteration may affect the expression and purification of proteins as replacing all $^1$H atoms for the heavier $^2$H isotope in the growth media can affect the level of expression of proteins in the cell (Liu et al., 2007; Meilleur et al., 2005). Expression and purification procedures must therefore be optimised for perdeuterated proteins to ensure adequate production of highly pure protein. Perdeuteration also affects protein solubility, and crystallisation conditions need to be re-optimised. Prior to analysis of a fully perdeuterated neutron structure, it is important to establish that the protiated and perdeuterated forms of the protein are structural isomorphs and behave functionally in a comparable manner. For COx this involves a kinetic comparison of the protiated and perdeuterated forms of the enzyme as well as a structural comparison by X-ray crystallographic analysis. Towards this aim we have successfully expressed and purified perdeuterated COx. Kinetic characterisations have been undertaken for the two protein forms. Additionally the stability of both protein forms has been assessed by differential scanning fluorimetry (DSF). Finally, X-ray structure analysis has been
undertaken on single crystals of each protein form and a structural comparison carried out to establish if perdeuteration has a significant effect on the protein structure.

4.4. **Methods**

4.4.1. **Cloning**

The wild-type *Streptomyces SA-COO* COx gene was amplified from pCO117 (Nomura *et al.*, 1995) and cloned into the *Nco*I and *HindIII* restriction sites of pET28a-His6-MBP-TEV-AEW*. The forward and reverse primers, 5’-gaactccatggeactgcaacagcatctg-3’ and 3’-gcagtgccgcagcgtggtggtggtggtgattcgagaactga-5’ respectively. The reverse primer included codons for six histidine residues. The vector contains the lac repressor (LacI) and the kanamycin resistance gene.

4.4.2. **Expression and Purification**

A 1L culture of perdeuterated *E. coli* expressing COx (2H-COx) was produced using the bioreactors at the National Deuteration Facility (NDF) located at the Australian Nuclear Science Technology Organisation (ANSTO) using a similar procedure to Chen *et al.* (2012). 50 µl of OneShot BL21*(DE3)* *E. coli* (Invitrogen) were transformed with the pCO_P1 plasmid (90 ng) and rested in 250 µl SOC (super optimal broth with catabolite repression) media for 2 hours at 37°C before being used to inoculate a 50% D$_2$O (v/V) ModC1 (modified minimal media) (Middelberg *et al.*, 1991) preculture, containing deuterated glycerol (Sigma, 98%) as the sole carbon source. The bacteria were adapted to D$_2$O at 37°C, shaking at 220 RPM in sealed flasks filled to approximately 5% of capacity, by preparing successive precultures with increasing concentrations of D$_2$O (50%, 90% and 100%), the first overnight, the latter for at least

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* His6: hexahistidine tag, MBP: maltose binding protein, TEV: tobacco etch virus cleavage site, AEW: Andrew Edward Whitten
two generations (>2 hrs). The 100% D₂O preculture was obtained by gently pelleting the ice-chilled cells from the 90% D₂O preculture, followed by resuspension in 100% D₂O media. The 100% D₂O adapted preculture was subsequently used to inoculate a bioreactor containing 1L final volume of perdeuterated ModC1 media to an optical density at 600 nm (O.D₆₀₀) of 0.200. The bioreactor culture was maintained at 37°C in aerobic conditions throughout, and d-4 ammonium deuteroxide (25%, Sigma) was fed to maintain a minimum pH=6.2. When the O.D₆₀₀ reached 12.3, the temperature was dropped to 20°C, and expression induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Samples were taken periodically to ascertain the expression level of ²H-COX. The culture was grown until the carbon source was exhausted. Harvested cells were pelleted and flash frozen in liquid nitrogen for storage. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of ¹H-COX and ²H-COX partially digested by trypsin indicated that the protein was >99% deuterated. This method for production of perdeuterated COX was repeated twice more.

The cell pellet was resuspended in 20 ml g⁻¹ lysis buffer (20 mM Tris-HCl pH 7.0, 500 mM NaCl) and lysed with an Emulsiflex C5 high-pressure homogeniser (Avestin). The lysate was clarified by centrifugation (12600 g). The supernatant was applied to a 1 ml GE Healthcare Histrap nickel-affinity column equilibrated with 20 mM Tris-HCl pH 7.0, 500 mM NaCl, 20mM imidazole. Bound protein was washed with equilibration buffer (20 mM Tris-HCl pH 7.0, 500 mM NaCl, 20mM imidazole) and 50 µg ml⁻¹ trypsin was applied to the column and incubated for 45 minutes to remove the remnants of the N-terminus targeting presequence which was not properly removed during expression of the protein. The trypsin was then washed off the column with equilibration buffer and the COX protein eluted with an imidazole gradient (0 - 500 mM over 30 column volumes). Peak fractions were pooled and applied to a size
exclusion column (Superdex 200 GE Healthcare) with a buffer exchange into 20 mM Tris-HCl pH 7.0. Purified protein was concentrated to 7 mg ml\(^{-1}\) as determined by Bradford assay (Compton & Jones, 1985) using an Amicon centrifugal filter unit (30 kDa cutoff). The purified protein was aliquoted, snap-frozen in liquid nitrogen and stored at 193 K.

Protiated enzyme (\(^{1}\)H-COx) was produced similarly except transformed cells were added directly to the preculture without the need for an adaptation procedure. The main 1 L culture was inoculated to an O.D\(_{600}\) of 0.131 and induced at an O.D\(_{600}\) of 16.06. The culture was grown until the media was exhausted. \(^{1}\)H-COx protein was purified similarly except it was not applied to a size-exclusion column and was dialysed against 20 mM Tris-HCl pH 7.0 before being concentrated (7 mg ml\(^{-1}\)) and frozen.

4.4.3. **Enzyme Assay**

Enzymatic activity assays were performed on \(^{1}\)H-COx and \(^{2}\)H-COx in both deuterated and protiated buffers. An assay buffer was prepared containing 50 mM sodium phosphate buffer (pH 7.0), 0.1% Triton X-100 and 0.02% BSA. The assay buffer was prepared using D\(_2\)O, for the deuterated assay conditions, or H\(_2\)O, for the protiated assay conditions. For the deuterated assays, \(^{1}\)H-COx and \(^{2}\)H-COx protein was exchanged into deuterated buffers overnight before the assays were performed. Substrate solutions were prepared by dissolving cholesterol in propan-2-ol.

To a 1 ml quartz cuvette, cholesterol was added to 970 \(\mu\)L of assay buffer to give final substrate concentrations between 8 \(\mu\)M and 48 \(\mu\)M. 20 \(\mu\)L of 2.5 \(\mu\)M enzyme (\(^{1}\)H-COx or \(^{2}\)H-COx) was added to give a final enzyme concentration of 50 nM and the production of product, cholest-4-en-3-one, was measured by the absorbance of the solution at 240 nm. Initial velocity versus cholesterol concentration data were fitted to the Michaelis-Menten equation by non-linear least squares regression analysis. The
values of $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ obtained from the analysis of the kinetics of $^1$H-COx and $^2$H-COx were analysed for significant differences in protonated compared to deuterated buffers using t-tests. Similarly, the values of these kinetic parameters in protonated buffers were analysed for significant differences between for $^1$H-COx and $^2$H-COx.

### 4.4.4. Differential Scanning Fluorimetry

Differential scanning fluorimetry (DSF) was performed on $^1$H-COx and $^2$H-COx in both deuterated and protonated buffers. 50 mM Tris buffer, pH 7 was prepared using D$_2$O, for the deuterated assay conditions, or H$_2$O, for the protonated conditions. For the deuterated assays, $^1$H-COx and $^2$H-COx proteins were exchanged into deuterated buffers overnight before the assays were performed. 50 µl samples were prepared in the Tris buffers in triplicate containing ~50 µg/ml enzyme and SYPRO orange dye (Sigma-Aldrich). An excitation wavelength of 450 nm - 490 nm and an SYBR green emission filter (510 nm - 530 nm) was used for measurements on a CFX96 C1000 thermal cycler instrument (BIO-RAD).

Results were processed using the Mathematica software (Wolfram Research, 2014). A buffer blank measurement was subtracted from the raw fluorescence curves, which were then interpolated. The maximum of the first derivative of the interpolated function was taken as the melt temperature of the protein. Measurements were performed in triplicate and then averaged to obtain the melt temperature of each protein condition.

### 4.4.5. Crystallisation

$^1$H-COx crystals were obtained in 10% PEG 8K, 125 mM MnSO$_4$, 100 mM sodium cacodylate pH 5.2 and 7 mg ml$^{-1}$ of purified protein using the hanging drop vapour diffusion method in 24 well VDX plates (Hampton Research) as per the optimised procedure for $^1$H protein (Yue et al., 1999). A macroseeding technique was used to
optimise crystal volume. Crystals reached maximal volume after 8 rounds of macroseeding. Each seeding round involved 4 sequential 1-2 minutes washes into wash drops containing 4% PEG 8K, 125 mM MnSO₄ and 100 mM sodium cacodylate pH 5.2. The washing steps were essential to etch the crystal surface and favour the continuous growth of single crystals. Etched crystals were then transferred into freshly prepared 5 µL growth drops containing 7% PEG 8K, 125 mM MnSO₄, 100 mM sodium cacodylate pH 5.2 and 7 mg ml⁻¹ protein which were pre-equilibrated for 2 hours. Macroseeding rounds were repeated approximately once a week.

²H-COx crystals could be obtained under the same conditions as for ¹H-COx. The best ²H-COx crystals were obtained from sitting drops containing 8 - 9% PEG 8K, 125 mM MnSO₄, 100 mM cacodylate pH 5.2 and 7 mg ml⁻¹ of purified protein. A single crystal was chosen for X-ray diffraction and “back-soaked” into 100% deuterated buffers for 3 days to exchange labile hydrogen atoms for deuterium atoms. This process involved sequential transfers of the crystal into partially deuterated stabilising solutions (14% PEG 8K, 125 mM MnSO₄ and 100 mM cacodylic acid pH 5.2) with increasing deuteration level (10% increments).

4.4.6. **X-ray Data Collection and Processing**

X-ray diffraction data were collected on a single ²H-COx crystal at 100K on beamline MX2 at the Australian Synchrotron, Melbourne (AS) using an ADSC Quantum 315r CCD detector. Data collection was carried out in two stages. Firstly, 360 images were collected using a rotation angle of 1° per image and an exposure time per frame of 1 second to 1.3 Å resolution. A second high resolution sweep of 360 images was taken at a different position on the crystal using a rotation angle of 1° and an exposure time per frame of 1 second was collected to 1.1 Å resolution.
X-ray diffraction data at the AS MX2 beamline were also collected on a single $^1$H-COx crystal to 0.85 Å resolution. This dataset was collected from three sweeps of 360 images. The 1.5 resolution sweep was taken with 1° rotation and 1 second exposures. The 1.0 Å sweep was taken with 0.5° rotation and 0.5 second exposure while the 0.85 Å sweep was taken with 0.25° rotation and 0.25 second exposure.

Datasets were processed using the *XDS* software (Kabsch, 2010). Data reduction was carried out using the *CCP4* suite of software (Winn *et al.*, 2011). Crystallographic refinement was performed through iterative cycles of refinement using *PHENIX* (Adams *et al.*, 2010) and model building using *Coot* (Emsley *et al.*, 2010). Superposition of the $^1$H-COx and $^2$H-COx structure was performed using *Superpose* (Kabsch, 1976) as part of the *CCP4* suite of software (Winn *et al.*, 2011) and temperature factor analysis was performed using *BAverage* as part of the *CCP4* suite of software (Winn *et al.*, 2011).

### 4.5. Results

#### 4.5.1. Culture Growth

The rate of growth of the bacteria in perdeuterated media and protiated media for a single 1 L culture are shown in Figure 4.1. The doubling time of protiated culture prior to induction was 2.7 hours and the doubling time of the perdeuterated culture pre-induction was 4.1 hours. The perdeuterated culture exhausted the media at 35 hours post-induction with an O.D$_{600}$ of 45.0. This yielded 49.2 g of cell pellet. The protiated culture reached an O.D$_{600}$ of 53.5 after 22 hours post-induction and yielded 73.9 g of cell pellet.
Perdeuterated protein was highly expressed, representing the major protein of the cell extract, after 35 hours (Fig. 4.2a). High level protein purity was obtained after nickel affinity purification and trypsin digestion (Fig. 4.2b). The protein was estimated to be >99% deuterated by mass spectrometry. The typical yield of purified protein per gram of cell pellet was 0.6 mg g\(^{-1}\) (27 mg) which was lower than the yield of protiated protein (1 mg g\(^{-1}\)).

**Figure 4.1. Bacterial culture growth curves**

Growth curves of BL21(DE3)pCO_P1 *E. coli* in protiated (blue) and perdeuterated (red) media for the main D\(_2\)O-adapted culture. The point of induction for each culture is indicated by the arrows.

Perdeuterated protein was highly expressed, representing the major protein of the cell extract, after 35 hours (Fig. 4.2a). High level protein purity was obtained after nickel affinity purification and trypsin digestion (Fig. 4.2b). The protein was estimated to be >99% deuterated by mass spectrometry. The typical yield of purified protein per gram of cell pellet was 0.6 mg g\(^{-1}\) (27 mg) which was lower than the yield of protiated protein (1 mg g\(^{-1}\)).
4.5.2. Enzyme Activity

The results of the enzyme assays are shown in Table 4.1. No significant differences in kinetic parameters were found between $^1$H-COx and $^2$H-COx due to the effect of protein perdeuteration when assayed in protonated buffers. From a t-test analysis, the only significant difference between parameters ($p < 0.05$) which demonstrates a solvent deuterium isotope effect is between the $k_{cat}/K_m$ values for the $^1$H-COx in H$_2$O and D$_2$O, the effect is inverse with a value of 0.6. This effect is absent in the perdeuterated enzyme.

Figure 4.2. Perdeuterated COx expression, purification and crystallisation

(a) SDS-PAGE of perdeuterated protein expression in culture samples taken immediately prior to induction with IPTG ($T_0$) and at the end of the culture growth time ($T_f$). The molecular weight marker (NEB 2 - 212kDa) is in lane Mwt. (b) The purified protein before ($P_1$) and after ($P_2$) trypsin digest. The COx protein band is indicated by the red box. (c) Perdeuterated protein crystals used for X-ray diffraction.
Table 4.1. Enzymatic activity assay for $^{1}$H-COx and $^{2}$H-COx

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>$^{1}$H-COx in D$_{2}$O</th>
<th>$^{2}$H-COx D$_{2}$O</th>
<th>$^{1}$H-COx in H$_{2}$O</th>
<th>$^{2}$H-COx in H$_{2}$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$ (s$^{-1}$)$^{†}$</td>
<td>7.5 ± 0.2</td>
<td>11.1 ± 0.8</td>
<td>9.5 ± 0.7</td>
<td>11.7 ± 0.7</td>
</tr>
<tr>
<td>$k_{cat}/K_{m}$ (μM$^{-1}$ s$^{-1}$)$^{†}$</td>
<td>0.42 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>0.28 ± 0.02</td>
</tr>
</tbody>
</table>

4.5.3. Differential Scanning Fluorimetry

The melt temperature for $^{1}$H-COx and $^{2}$H-COx in H$_{2}$O buffers was 63.1°C and 63.4 °C, respectively. While for $^{1}$H-COx in D$_{2}$O buffers the melt temperature was 62.7°C and for $^{2}$H-COx the melt temperature was 63.7°C. Plots of the derivatives of the relative fluorescence for each condition are shown in Figure 4.3.

![Figure 4.3. Thermal stability of perdeuterated and protiated COx](image)

Derivative curves of DSF scans of $^{1}$H-COx and $^{2}$H-COx in H$_{2}$O and D$_{2}$O buffers. For comparison, the data was normalised and the negative of the first derivative was calculated to determine the melt temperature of the proteins.

$^{†}$ Indicated errors are standard errors of the estimates of $k_{cat}$ and $k_{cat}/K_{m}$ derived from non-linear least squares fit of the data to the Michaelis-Menten equation.
4.5.4. Crystallisation and Macroseeding

A $^1$H-COx crystal of 0.2 mm$^3$ obtained from iterative macroseeding was used for X-ray diffraction data collection. A single $^2$H-COx crystal of 0.03 mm$^3$ grown from a sitting drop volume of 100 µL (Fig. 4.2c) was selected for X-ray diffraction data collection. Perdeuterated protein crystals were obtained under the same conditions used to grow the protiated protein crystals.

4.5.5. X-ray Structure

Data collection and refinement statistics for the X-ray structures are shown in Table 4.2. $^2$H-COx crystals soaked in D$_2$O buffers, diffracted X-rays to a resolution of 1.1 Å, typical of the diffraction resolution for $^1$H-COx crystals. The macroseeded protiated crystal diffracted to 0.85 Å. The space group (P 1 2$_1$ 1) and the unit cell dimensions of the protiated and perdeuterated crystals were essentially the same as reported for other COx crystals, $a = 51.2$ Å, $b = 73.1$ Å, $c = 63.2$ Å, $\alpha = 90.0^\circ$, $\beta = 105.1^\circ$, $\gamma = 90.0^\circ$ and $a = 51.3$ Å, $b = 72.8$ Å, $c = 63.0$ Å, $\alpha = 90.0^\circ$, $\beta = 105.1^\circ$, $\gamma = 90.0^\circ$, respectively. The average mosaicity for both crystals was 0.14°.
Table 4.2. X-ray data processing and refinement statistics $^1$H-COx and $^2$H-COx

<table>
<thead>
<tr>
<th>Data Processing Statistics</th>
<th>$^1$H-COx‡</th>
<th>$^2$H-COx§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P 1 2 1 1</td>
<td>P 1 2 1 1</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>51.2, 73.1, 63.2</td>
<td>51.3, 72.8, 63.0</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90.0, 105.1, 90.0</td>
<td>90.0, 105.1, 90.0</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>46.67-0.85 (0.90-0.85)**</td>
<td>47.70-1.10 (1.16-1.10)</td>
</tr>
<tr>
<td>Total reflections</td>
<td>5,617,181 (457,672)</td>
<td>1,915,580 (159,815)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>390,495 (56,850)</td>
<td>179,607 (22,088)</td>
</tr>
<tr>
<td>Completeness, %</td>
<td>100.0 (100.0)</td>
<td>95.7 (80.7)</td>
</tr>
<tr>
<td>Average I/σ</td>
<td>16.2 (2.5)</td>
<td>19.9 (7.4)</td>
</tr>
<tr>
<td>Rmerge</td>
<td>0.38 (1.16)</td>
<td>0.08 (0.28)</td>
</tr>
<tr>
<td>CC1/2</td>
<td>0.994 (0.749)</td>
<td>0.998 (0.971)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>12.0 (8.9)</td>
<td>10.7 (7.2)</td>
</tr>
</tbody>
</table>

| Refinement Statistics       |            |            |
| Resolution range (Å)        | 34.28-0.85 | 44.96-1.10 |
| Total reflections used       | 390,626    | 177,576    |
| R factor                    | 0.1004     | 0.0976     |
| Free R factor               | 0.1209     | 0.1135     |
| rms deviation bond lengths, Å | 0.009    | 0.01       |
| rms deviation bond angles, ° | 1.60      | 1.43       |
| No. of non-hydrogen atoms   | 4911       | 5097       |
| Average B factors, Å²       | 4.9        | 6.03       |

There were no structural differences observed between the protiated and perdeuterated structures. The root mean squared deviation (RMSD) for the positions of the main-chain atoms is 0.071 Å. The maximum likelihood co-ordinate error estimation is 0.06 Å and 0.04 Å for the $^1$H-COx and $^2$H-COx structures, respectively, while the reported diffraction precision index (DPI) (Cruickshank, 1999) was 0.014 Å for $^1$H-COx and 0.028 Å for the $^2$H-COx structure. The graph of Ca atom RMSD between the $^1$H-COx and $^2$H-COx structures are shown in Figure 4.4a. 95% of the Ca atoms have an RMSD of <0.183 Å. Atoms with rmsd greater than 0.183 Å were located

‡ PDB code: 4XXG
§ PDB code: 4XWR
** Statistics for the highest resolution shell are shown in parentheses
predominantly on the surface of the protein. The isotropic B factors for the Cα atoms superimpose closely between the $^1$H-COx and $^2$H-COx structures (Fig. 4.4b).

**Figure 4.4. $^1$H-COx and $^2$H-COx structural comparison**

(a) Positional displacement between the Cα atoms of the protiated ($^1$H-COx) and perdeuterated ($^2$H-COx) X-ray protein structures by residue number. (b) Plot of the isotropic B-factors for the protiated (blue) and perdeuterated (red) X-ray structures Cα atoms. (c) Superposition of the active site residues of the perdeuterated protein (green carbons) and the protiated protein (magenta carbons).

### 4.6. Discussion

Protein perdeuteration enhances the quality of diffraction data that can be obtained from single crystal macromolecular neutron diffraction. Deuterium has a much lower incoherent scattering cross section compared to hydrogen. Furthermore,
deuterium exhibits positive neutron coherent scattering length while hydrogen has a negative neutron coherent scattering length. Thus, perdeuteration of proteins for neutron single crystal scattering experiments results in a significant improvement in the signal to noise ratio as well as eliminating density cancellation effects resulting from negative scattering which obscures neutron density maps. An assumption is made in neutron single crystal diffraction experiments, that the positions of the deuterium atoms are identical to the positions of hydrogen atoms in the protein structure. However, deuterium is not normally utilised by biological systems. Therefore, it is important to determine whether perdeuteration of the protein has an effect on the protein structure and activity. We have produced recombinant perdeuterated COx and compared the perdeuterated and protiated forms of the enzyme using enzyme kinetics, thermal stability assays and structural characterisations by X-ray crystallography and establish that no structural or functional differences exist as a result of perdeuteration.

Cells were readily adapted to deuterated media with only two adaptation precultures required beginning with 50% D_2O media. This was carried out in liquid media, similar to that described by Shu et al. (2000), rather than agar plates as was used for the adaptation of type III antifreeze protein (Petit-Haertlein et al., 2009), rat γE-crystallin (Artero et al., 2005), human arginase I (Di Costanzo et al., 2007), human carbonic anhydrase (Budayova-Spano et al., 2006), rubredoxin (Weiss et al., 2008) and cytochrome P450cam (Meilleur et al., 2004). The cell pre-induction doubling time was 33% slower for the perdeuteration media versus the hydrogenation media, and the total growth time until the media were exhausted was almost twice as long (60 hours versus 36 hours). This is not unexpected as the increased mass of deuterium can decrease the growth rate of bacteria (De Giovanni, 1961) and slow down the rate of biological reactions (Hochuli et al., 2000; Jasnin et al., 2008). As a result, a lower total cell density was obtained for expression of the perdeuterated protein compared to that of the
protiated protein (49.2g versus 73.9g). Additionally the final yield of pure perdeuterated protein was approximately 1.5 - 2 times lower than that of the protiated protein. The decreased yield is similar to that reported for production of perdeuterated cytochrome P450cam which also displayed a 2 - 3 times decrease in protein expression (Meilleur et al., 2005) and haloalkane dehalogenase which had a 1.5 times decrease in protein yield (Liu et al., 2007). Conversely, a similar yield of perdeuterated and protiated protein was obtained for human arginase I (Di Costanzo et al., 2007). Mass spectrometry analysis indicated that the deuteration level of the protein was very high (>99%) and sufficient for single crystal neutron diffraction studies. Importantly, sufficient yield and quality of the protein was produced for successful crystallisation of the perdeuterated protein. The ideal condition for crystallisation of the perdeuterated protein was slightly modified from that of the protiated protein but was in the range of conditions identified for the crystallisation of $^1$H-COx (Yue et al., 1999).

It is important to ensure that perdeuteration does not affect the overall structure of the protein. While X-ray structures of perdeuterated proteins generally show no significant differences in the structures compared to non-deuterated proteins (Artero et al., 2005; Blakeley et al., 2008; Blum et al., 2010; Di Costanzo et al., 2007; Gamble et al., 1994; Meilleur et al., 2005), Chatake et al. (2011) showed there were differences in dissimilatory sulfite reductase D crystals soaked with various deuterium concentrations although these did not translate to major structural differences. Liu et al. (2007) did find that a catalytic residue was displaced in the deuterated structure and as a consequence a water molecule which is suspected to play a role in the catalysis of haloalkane dehalogenase, was not present in the perdeuterated structure indicating that it is important to determine that perdeuteration does not affect the protein structure. Analyses of the high resolution X-ray structures obtained for both protiated and perdeuterated COx crystals indicated there are no significant differences between the
two crystal forms. The unit cell and average mosaicity of the protiated and perdeuterated crystals were essentially identical indicating that perdeuteration did not affect the crystal packing. There was little deviation of the Cα atoms due to perdeuteration with most having a deviation of <0.18 Å. Additionally, no significant differences in the isotropic B factors were observed between the structures. Furthermore, inspection of the key active site residues shows no differences between the protiated and perdeuterated proteins.

Interestingly, although the X-ray structures and kinetic activity of \(^1\)H-COx in H\(_2\)O containing buffers and \(^2\)H-COx in D\(_2\)O containing buffers show no significant differences, a reverse solvent deuterium isotope effect was observed for \(^1\)H-COx protein in deuterated buffers. From our current knowledge of the mechanism of COx (Vrielink, 2010) it is not evident how the observed inverse solvent deuterium isotope effect occurs and why it is not present when the perdeuterated enzyme is used. The investigation of this interesting phenomenon is well beyond the scope of this article and future work will be required in which a range of deuterium isotope effects are determined and analysed.

DSF revealed no change in the thermal induced melt temperature of COx due to perdeuteration in H\(_2\)O buffers. A small difference was observed between the perdeuterated and protiated protein in D\(_2\)O buffers. However, the small temperature difference would suggest there is little difference in the thermal stability of the perdeuterated and protiated protein.

These results indicate that perdeuteration and deuterated solvent has an undetectable effect on the crystal structure of COx and thus a full neutron structural analysis of the protein will provide relevant information to assess the role of hydrogen atoms in redox catalysis.
4.7. Conclusion

Perdeuterated enzyme was successfully expressed with sufficient yield and quality for crystallisation experiments. Characterisation of the protein revealed that perdeuteration does not significantly affect the structure or function of COx and therefore perdeuterated COx is suitable for further neutron diffraction studies.

4.8. Acknowledgements

The authors would like to thank the Australian Synchrotron for use of the MX2 beamline for macromolecular crystallography. The authors would also like to thank Ben Crossett of the Mass Spectrometry Core Facility, The University of Sydney, for the mass spectrometry analysis.
A Deuteron Orient the Orbitals of the Reactive Centre of FAD in Cholesterol Oxidase for Efficient Hydride Transfer: Insights From Neutron Protein Crystallography
5.1. Authors and Contributions

This chapter has been submitted for review to the *Journal of the American Chemical Society*. The authors of the manuscript are Emily Golden\(^1\), Anthony P. Duff\(^2\), Matthew P. Blakeley\(^3\), Flora Meilleur\(^4,5\) and Alice Vrielink\(^1\). E.G produced the protein crystals for neutron diffraction. F.M performed the neutron diffraction and data processing. E.G performed the analysis of the data with guidance and suggestions from A.V, F.M, A.P.D and M.P.B. E.G wrote the paper with critical revisions provided by A.V, F.M, A.P.D and M.P.B.

\(^1\)School of Chemistry and Biochemistry, University of Western Australia, Crawley, Western Australia, 6009 Australia

\(^2\)Bragg Institute, Australian Nuclear Science and Technology Organisation, Lucas Heights NSW, Australia, 2234, Australia

\(^3\)Institut Laue-Langevin, 71 Avenue des Martyrs, Grenoble, 38000, France

\(^4\)Neutron Sciences Directorate, Oak Ridge National Laboratory, Oak Ridge, TN, 37831, United States of America

\(^5\)Structural and Molecular Biochemistry, North Carolina State University, Raleigh, NC 27695, United States of America
5.2. Abstract

Cholesterol oxidase (COx) is a flavoenzyme catalysing the oxidation and isomerisation of cholesterol. The ability to visualise hydrogen atoms by X-ray diffraction is limited due to their weak scattering properties. In contrast, neutron diffraction can be used to visualise hydrogen/deuterium atoms even at moderate resolutions. We report the H/D-exchanged X-ray/neutron crystal structure of COx in the enzyme oxidised state at 2.2 Å resolution. The neutron map reveals a bridging deuterium atom equidistant between the main-chain nitrogen of Gly120 and N5 of the FAD cofactor consistent with the presence of a proton at this position. The resulting increased partial negative charge on the peptide bond of Gly120 is stabilised through a positively charged residue. Conservation of these interactions amongst a large number of flavoenzyme oxidases suggests the importance of this proton in directing the orbitals of the flavin N5 for efficient redox chemistry.

5.3. Introduction

Flavoenzymes are a diverse class of proteins containing a flavin cofactor involved in a large range of biomolecular processes (De Colibus & Mattevi, 2006). Cholesterol oxidase (COx) is a bacterial flavoenzyme catalysing the oxidation and isomerisation of cholesterol to cholest-4-en-3-one and is commonly used for quantifying human serum cholesterol levels (Richmond, 1976). The oxidative reaction is catalysed by the tightly bound flavin adenine dinucleotide (FAD) cofactor, which accepts a hydride from the steroid substrate C3 atom and becomes reduced (Chapter 1, Fig. 1.1). The reduced cofactor is re-oxidised by molecular oxygen producing H₂O₂, thus regenerating the enzyme for the next round of catalysis. A conserved histidine residue (His447) in the glucose-methanol-choline (GMC) oxidoreductase family is essential for this reaction to occur with mutations of this residue resulting in reduced or
undetectable oxidation activity (Kass & Sampson, 1998). High resolution X-ray structures reveal that His447 is protonated at position Nε2 and forms a hydrogen bond with a conserved water molecule at the active site (Lyubimov et al., 2006). This water molecule is proposed to mimic the position of the substrate hydroxyl group in the ligand free enzyme (Golden et al., 2014). These structures suggest that, in the bound enzyme form, His447 positions the substrate hydroxyl appropriately for hydride transfer to FAD through a hydrogen bond. Cleavage of the substrate C3-H bond is proposed to occur through a concerted base induced rupture of the substrate hydroxyl bond. Glu361 is the only basic residue in the vicinity of the flavin in the active site binding pocket. Mutagenesis and structural studies have suggested that Glu361 is required for both the oxidation and isomerisation activity of the enzyme (Kass & Sampson, 1998; Sampson & Kass, 1997).

Recently, the high resolution X-ray structure of an anaerobically trapped reduced enzyme was determined (Chapter 3) (Golden et al., 2014) revealing a significant difference density peak within bonding distance to N5 of the flavin and in a tetrahedral arrangement to this nitrogen atom (Fig. 3.3a). The location of this difference peak suggested hydride transfer from the substrate to the cofactor had occurred in the crystal. DFT calculations also carried out as part of this study suggest that a conserved hydrogen bond from the main-chain nitrogen atom of Gly120 to the FAD-N5 atom facilitates hydride transfer by re-arrangement of the lone pair of electrons on N5. These results suggest that the active site is pre-formed to promote hydride transfer and highlights the importance of the hydrogen bonding environment of the cofactor in the redox cycle of flavoproteins.

While high resolution X-ray structures have provided insights into the positions of some of the hydrogen atoms in the oxidised and reduced structures, neutron protein crystallography (NPC) allows hydrogen (H) and its isotope deuterium (D) to be located
at more moderate resolutions of ~1.5 and 2.5 Å, respectively (Golden & Vrielink, 2014). Therefore to further investigate the role of hydrogen atoms in stabilisation of the flavin cofactor during redox catalysis by COx we have collected neutron diffraction data to 2.2 Å resolution from a crystal of H/D-exchanged $^1$H-COx using the IMAGINE beamline at the High Flux Isotope Reactor at (HFIR) at Oak Ridge National Laboratory (ORNL) (Meilleur et al., 2013) (Table 5.1). The structure was refined in a joint refinement strategy using both X-ray and neutron diffraction data collected at room temperature. Two important deuterium atoms in the active site region were observed clearly in the neutron difference density maps and provide insight into both substrate binding and hydride transfer events in COx.

Table 5.1. X-ray and neutron data processing and refinement statistics $^1$H-COx

<table>
<thead>
<tr>
<th>Data Reduction</th>
<th>Neutron</th>
<th>X-ray</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P 1 2, 1</td>
<td>P 1 2, 1</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>51.6, 74.1, 63.8</td>
<td>51.6 Å 74.2 Å 63.9 Å</td>
</tr>
<tr>
<td>α,β,γ (°)</td>
<td>90.0, 105.2, 90.0</td>
<td>90.0, 105.2, 90.0</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>61.2-2.2 (2.32-2.2)*</td>
<td>40-1.5 (1.58-1.5)</td>
</tr>
<tr>
<td>R_{merge}</td>
<td>0.258 (0.349)</td>
<td>0.043 (0.237)</td>
</tr>
<tr>
<td>Observations</td>
<td>70194 (6681)</td>
<td>246246 (32786)</td>
</tr>
<tr>
<td>Number unique</td>
<td>17714 (2048)</td>
<td>67723 (9201)</td>
</tr>
<tr>
<td>Average I/σ</td>
<td>4 (2.6)</td>
<td>21.1 (4.23)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>76.7 (61.1)</td>
<td>88.3 (86.9)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>4 (3.3)</td>
<td>3.6 (3.6)</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R factor</td>
<td>23.93</td>
<td>13.46</td>
</tr>
<tr>
<td>Free R factor</td>
<td>28.84</td>
<td>16.87</td>
</tr>
<tr>
<td>RMSD bond lengths (Å)</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>RMSD bond angles (°)</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td>Average B factors (Å$^2$)</td>
<td>17.3</td>
<td></td>
</tr>
</tbody>
</table>

* Statistics for the highest resolution shell are shown in parentheses
5.4. Results and Discussion

The main-chain nitrogen atom of Gly120 forms an important hydrogen bond interaction with the flavin cofactor. This hydrogen bond interaction is a conserved feature in many flavoenzymes including glucose oxidase from *Penicillium amagasakiense* (Wohlfahrt *et al.*, 1999), S-mandelate dehydrogenase (Sukumar *et al.*, 2004), D-amino acid oxidase (Mattevi *et al.*, 1996) and NAD(P)H:acceptor oxidoreductase (FerB) from *Paracoccus denitrificans* (Sedlacek *et al.*, 2014) suggesting that this interaction plays an important role in the redox chemistry of flavoenzymes. Neutron maps calculated with the Gly120 amide deuterium removed results in a difference density peak visible at 3.4 σ. The peak is centred 1.65 Å from the Gly120-N atom and 1.67 Å from the FAD-N5 atom (Fig. 5.1a). An analysis of the distances between the main-chain nitrogen atoms and the centres of omit map peaks for the main-chain deuterium atoms in the structure shows that this N-to-peak distance is at the tail end of the distribution and longer than N-to-peak distances observed in D omit maps for other regions of the structure (Fig. 5.1b). This observed longer distance from the main-chain nitrogen atom to the peak may be due to a repositioning of the deuterium from Gly120 more towards the FAD-N5 centre. Alternatively, the peak may represent an average of two states: one with a normal N-D bond distance for Gly120 and a second where the deuterium has been transferred to the FAD-N5 centre. To investigate these possibilities, models were refined with either a deuteron restrained to a position half-way between the Gly120-N and FAD-N5 atoms or with partially occupied deuterium atoms modelled on the Gly120-N atom and the FAD-N5 atom such that their occupancies summed to 1. Modelling a deuteron in the centre of the peak, midway between the two nitrogen atoms, results in no residual difference density above 1.5 σ (Fig. 5.1c) (residual density is only present at 0.9 σ). When modelled as two deuterium atoms, each with occupancy of 0.5, residual density is observed at 1.5 σ in the region...
between the two modelled deuterium atoms (Fig. 5.1d). Based on these analyses we conclude that, in the oxidised state, COx reveals a deuteron atom between the protein main-chain and the reactive centre of the FAD cofactor. NPC has previously been used to identify a deuteron in the metal binding site of D-xylose isomerase at low pH (Kovalevsky et al., 2011) as well as a deuteron involved in low-barrier hydrogen bond between the oxygen atom of tyrosine residue and the chromophore of photoactive yellow protein (PYP) (Yamaguchi et al., 2009), however this is the first example where a deuteron has been observed between a peptide amide and a flavin cofactor reactive centre.
Flavoenzymes are a very large class of proteins that catalyse a diverse range of reactions. The diversity of these reactions is due to the protein microenvironment stabilising the FAD cofactor. The hydrogen bonding interaction between Gly120 and FAD-N5 in COx is conserved in several flavoprotein oxidoreductases (Mattevi et al., 1996; Sedlacek et al., 2014; Sukumar et al., 2004; Wohlfahrt et al., 1999). The carbonyl of the adjacent residue, Asn119, forms a hydrogen bond with the side chain of

Figure 5.1. Neutron density maps for the region between Gly120 and FAD.

(a) Neutron density maps calculated with Gly120-D removed. (b) A histogram of selected main-chain N-Peak distances. The bin for the Gly120-D peak is indicated by the red arrow. (c) Neutron density maps with a deuteron modelled midway between the Gly120-N and FAD-N5 atoms. (d) Neutron density maps with a deuterium atom modelled at 0.5 occupancy for Gly120-D and 0.5 occupancy on the FAD-N5. Neutron density ($2F_o - F_c$) maps are shown as magenta mesh (1.5 σ) in (a), (c) and (d). Residual difference density maps ($F_o - F_c$) maps are shown as cyan mesh contoured at 1.5 σ for (c) and (d) and 3.0 σ for (a). Residual density is only shown for a 1.6 Å sphere around the deuteron position shown in (c) and (d) for clarity. Carbon atoms are coloured green, nitrogen atoms are blue, oxygen atoms are red, hydrogen atoms are white and deuterium atoms are yellow.
a well ordered lysine residue (Lys225). The deuterium atoms of the Lys225 side chain are clearly visible in the nuclear density maps (Fig. 5.2a). Furthermore, a charged side chain interacting with the protein carbonyl appears to be a conserved feature in FAD-dependent oxidoreductases of the GMC oxidoreductase family with an arginine side chain present in glucose oxidase (1GPE) (Wohlfahrt et al., 1999), cellobiose dehydrogenase (1KDG) (Hallberg et al., 2002), aryl-alcohol dehydrogenase (3FIM) (Fernandez et al., 2009), 5-hydroxymethylfurfural oxidase (4UDP) (Dijkman et al., 2015), formate oxidase (3Q9T) (Doubayashi et al., 2011) hydroxynitrile lyase (1JU2) (Dreveny et al., 2001) and a histidine residue in pyranose-2-oxidase (1TZL) (Bannwarth et al., 2004) (Fig. 5.2b). D-amino-acid oxidase (DAAO) has a similar interaction through two highly coordinated water molecules to an arginine side chain (1C0P) (Umhau et al., 2000). This conserved positively charged residue acts to polarise the carbonyl and amide of residues Asn119 and Gly120, respectively, and thereby weakens the N-H bond of Gly120 enabling the movement of the proton from Gly120 away from the main-chain N and toward the lone pair of electrons on the FAD-N5. This interaction directs the geometry of the orbitals around the FAD-N5 to a more tetrahedral state. This pre-organises the flavin N5 to ideally position the hydride accepting orbital for flavin reduction by the substrate. Indeed this interpretation is consistent with the atomic resolution X-ray structure and computational studies of the reduced enzyme where the FAD-N5 is bonded to the hydride in a tetrahedral orientation and energetically stabilised through the interaction with Gly120 (Golden et al., 2014).
Figure 5.2. Conservation of a charged residue in the position of Lys225.

(a) Nuclear scattering density around Lys225 side chain and secondary interactions with FAD. The positively charged side chain interacts with the main-chain O atom of Gly120.

(b) Superpositions of other oxidoreductases displaying a positively charged residue interacting with the protein carbonyl corresponding to Asn119-O in COx. The N5 atoms of the FAD for each superposed structure are shown as blue spheres (cyan sphere for CO) and the rest of the FAD molecule has been omitted for clarity. Only the C, O and N atoms of the protein main-chain are displayed (corresponding to Gly120-O, Gly120-C and Asn119-O). Carbon atoms are coloured green, nitrogen atoms are blue, oxygen atoms are red and deuterium atoms are pale yellow. In (b), the COx.structure is coloured as magenta carbons and cyan nitrogen atoms. (c) A chemical representation of the interactions causing the alignment of the orbitals for accepting the hydride.
For COx, the position of the hydrogen atom may depend on the redox state of the enzyme, similarly to that observed in PYP (Yamaguchi et al., 2009), with the Gly120-D atom transitioning between a stabilised deuteron state between the two nitrogen atoms and a more conventional covalent bonded state with Gly120-N. A low-barrier hydrogen bond (LBHB) observed in PYP was proposed to be important in the relaxed state of the protein. This LBHB is proposed to sterically restrain the phenolic ring of the chromophore and is relaxed upon excitation, releasing the phenolic ring of the chromophore to undergo fast isomerisation (Yamaguchi et al., 2009). The distance between Gly120-N and FAD-N5 is 3.3 Å, which precludes this interaction from being a LBHB.

Neutron maps were also calculated to confirm the orientation of the conserved water molecule, Wat541, located in the enzyme active site (Fig. 5.3a). A difference density peak is visible, centred 2.1 Å from the Oε2 atom of Glu361 and 1.04 Å from the oxygen atom of Wat541. The oxygen atom is located 2.87 Å from His447-Nε2. Lario et al. (2003) suggested that Wat541 mimics the orientation of the substrate hydroxyl group and is stabilised by a hydrogen bond donor interaction from His447 and a hydrogen bond acceptor interaction from Glu361 (Lario et al., 2003). The second deuterium atom of the Wat541 molecule is not visible in the neutron map and is most likely due to rotation of the molecule about the O-H(D) axis. This supports the assertion that the molecule mimics the substrate hydroxyl position as the active site would have strong electrostatic interactions to stabilise the position of the hydroxyl atoms rather than a D$_2$O molecule. The position of this peak provides the first direct experimental evidence of the orientation of this water molecule, and correlates well with the proposed position of the substrate hydroxyl group in the active site of COx (Lyubimov et al., 2007). Efficient hydride transfer from the substrate C3 atom occurs when the C3 hydrogen atom and the substrate hydroxyl hydrogen atom adopt a trans
coplanar arrangement (Lario et al., 2003). When a steroid substrate, dehydroepiandrosterone (DHA), is modelled into the active site of COx such that the hydroxyl group is positioned as we observe in the X-ray/neutron structure, and the hydride is trans to the hydroxyl group (Fig. 5.3b), the C3-H atom of DHA overlaps with the position of the difference density peak that was previously observed in the reduced X-ray structure (Fig. 5.3c) (Golden et al., 2014). Thus, identification of the bound water molecule orientation provides an important model for the precise positioning of the atoms involved in catalysis and correlates well with the proposed role of Glu361 as the base needed for proton abstraction from the substrate (Lario et al., 2003). Additionally, these observations support the proposed position of the substrate C3-H atom close to the N5 of the flavin as required for efficient hydride transfer.

**Figure 5.3  Orientation of conserved Wat541 and substrate positioning**

(a) The nuclear density around conserved Wat541. (b) A superposition of DHA onto the model and density shown in (a). (c) A superposition of the DHA model in (b) onto the model and residual density of reduced COx presented in Golden et al. (2014). Nuclear density ($2F_o - F_c$) is displayed as magenta mesh contoured at 1.5 $\sigma$. Difference density ($F_o - F_c$) is displayed as cyan mesh and contoured at 3.0 $\sigma$. Electron density (blue mesh and contoured at 2.0 $\sigma$) is displayed only for Wat541 to indicate that its position is localised. Electron difference density is displayed as green mesh (3.0 $\sigma$) for the hydride near FAD-N5 in (c). Atoms are coloured as following: carbon is green, oxygen is red, nitrogen is blue, hydrogen is white, deuterium is yellow except for the DHA model for which carbon atoms are coloured magenta and the hydride which is transferred during oxidation is coloured cyan.
Efficient hydride transfer chemistry requires that the electron accepting and receiving orbitals are aligned. This neutron crystallographic study reveals how the protein acts to position the donating orbital of the substrate, through hydrogen bonding interactions with the hydroxyl group, while concurrently positioning the FAD-N5 accepting orbital through a deuteron. Thus the protein active site is pre-formed to enable efficient hydride transfer during the reductive half-reaction. The X-ray/neutron structure of COx presented here also indicates the importance of visualising hydrogen atoms within a protein structure. Despite their small size, hydrogen atoms play a key role in substrate binding, catalysis and stabilisation of enzymes. Neutron crystallography provides an excellent opportunity to experimentally obtain a complete picture of enzyme active sites as required for understanding their catalytic chemistry.

5.5. Methods

Expression, purification and crystallisation was performed as described previously (Golden et al., 2014). Macroseeding of crystals was performed as described previously (Golden et al., 2015). Crystals were H/D-exchanged by soaking in crystallisation conditions containing 100% D$_2$O for 24 hours before being mounted in a capillary for approximately 6 months prior to data collection.

Neutron diffraction data collection using a single large macroseeded crystal (0.41 mm$^3$) was performed on the IMAGINE beamline at HFIR, ORNL at room temperature using a 2.8 Å – 4.3 Å neutron wavelength range (Meilleur et al., 2013). Twenty images (24 hours per image) were collected in two crystal settings to help fill the blind region. Laue images were indexed and integrated using LAUEGEN (Campbell et al., 1998; Helliwell et al., 1989), wavelength normalised to account for the spectral distribution of the quasi-Laue beam using LSCALE (Arzt et al., 1999) and then scaled and merged using SCALA (Winn et al., 2011). An X-ray data set were collected on the
same crystal using the Rigaku HomeFlux X-ray setup equipped with a MicroMax-007 HF X-ray generator, Osmic VariMax optics and an R-AXIS IV++ image-plate detector. The diffraction data were indexed, integrated and scaled using the HKL-3000 software suite. Table 5.1 provides the data processing statistics for the neutron and X-ray datasets. An initial model of COx consisting of PDB file 1MXT (Lario et al., 2003) with all alternate conformations and water molecules removed was refined against the X-ray and neutron data using PHENIX (Afonine et al., 2010). Water molecules were placed where the $2F_o - F_c$ X-ray density was visible above 1.5 $\sigma$ and the $F_o - F_c$ was visible above 3.0 $\sigma$. Hydrogen and deuterium atoms were added to the model using ReadySet in PHENIX (Afonine et al., 2010); ReadySet allows the addition of hydrogen and deuterium atoms, each set at 0.5 occupancy, to all exchangeable sites in the model. Several cycles of joint X-ray/neutron refinement were performed after modelling in Coot (Emsley et al., 2010). DOD and OD type waters were modelled according to the presence of neutron $2F_o - F_c$ and $F_o - F_c$ density and several further cycles of joint refinement and modelling were performed. The final data refinement statistics are shown in Table 5.1.

Omit maps were calculated by removing the omit atom followed by three cycles of coordinate, ADP and occupancy refinement. Omit maps for main-chain D atoms were calculated by removing sets of 50 main-chain atoms consecutively along the protein sequence, followed by three cycles of refinement. $F_o - F_c$ density was inspected and the N-to-peak distances determined by placing an atom at the centre of the peak and optimizing the atomic position using the real-space refine option in Coot. The distances for all peaks that were centred within 20° of the ideal N-H position were tabulated and used to construct a histogram of 35 distances (Fig. 5.1b). A deuteron was modelled midway between Gly120-N and FAD-N5 and three cycles of refinement was performed using PHENIX refine using custom distance restraints of 1.66 Å from each nitrogen
atom and a standard deviation of bond lengths of 0.5 Å (compared to 0.02 Å for covalent bonds in the structure). Refinement of deuterium atoms on Gly120-N and FAD-N5 was performed using default distance and angle restraints with the occupancies of the deuterium atoms fixed at 0.5. Ten cycles of coordinate and ADP refinement was performed.
THE NEUTRON STRUCTURE OF PERDEUTERATED COX IN THE OXIDISED STATE
6.1. Introduction

The neutron structures of cholesterol oxidase (COx) in the reduced and oxidised states were pursued to determine the positions of key hydrogen atoms in the structure of COx. High resolution X-ray structures and functional studies have revealed residues and features which may be important for the mechanism of catalysis of COx (Chen et al., 2008; Lario et al., 2003; Lyubimov et al., 2009; Lyubimov et al., 2007; Lyubimov et al., 2006) such as the role of key active site residues His447 (Kass & Sampson, 1998) and Glu361 (Kass & Sampson, 1995; Kass & Sampson, 1998; Sampson & Kass, 1997) in the oxidation and isomerisation reactions, respectively, and Asn485 in stabilising the reduced cofactor (Lyubimov et al., 2009; Yin et al., 2001). His447, Glu361 and the FAD cofactor have direct interactions with the substrate during catalysis. His447 positions the substrate through a hydrogen bond to the hydroxyl oxygen (Yue et al., 1999), Glu361 transfers a proton from the C4 to C6 position of the substrate (Kass & Sampson, 1995) and FAD accepts a hydride from the substrate and becomes reduced. The redox cycle of COx primarily involves the transfer of a hydride and proton between the substrate and the protein and cofactor and so determination of the positions of hydrogen atoms in the reduced and oxidised states will provide insights into the reaction mechanism of COx and other flavoproteins.

The cofactor environment also has a substantial effect on the redox potential of the enzyme with interactions between protein and FAD providing stabilising factors for the reduced enzyme (Lyubimov et al., 2009; Lyubimov et al., 2006; Yin et al., 2001). In particular, electron difference density peaks were observed in the X-ray structure maps of COx at high resolution between the FAD-N3 atom and the Met122-O atom, and the FAD-O4 atom and Met122-H atom corresponding to the respective hydrogen atoms (Lyubimov et al., 2006) (Chapter 1, Fig. 1.6a). At pH 9.0 the single $F_o - F_c$ peak which was observed in the lower pH structures for each hydrogen atom appeared as two
peaks indicating that the hydrogen atoms in these interactions are delocalised into two positions (Chapter 1, Fig. 1.6b). These hydrogen bonding interactions are present in most flavoenzyme structures including, but not limited to, flavocytochrome C3 (Taylor et al., 1999), old yellow enzyme (Chilton et al., 2014), NrD1 (Johansson et al., 2010) and succinate:ubiquinone oxidoreductase (Sun et al., 2005) and model systems have shown they affect the redox potential of the flavin and stabilise the reduced form of the enzyme (Breinlinger et al., 1995; Niemz & Rotello, 1999) as well as affect the spin density of atoms in the isoalloxazine moiety (García et al., 2002). A CH•••π interaction occurs between the side chain of Asn119 which is situated on the si face of the isoalloxazine moiety and may play a role in the stabilisation of the cofactor during catalysis. As the high pH structure is proposed to mimic the anionic reduced form of COx, it was suggested that these hydrogen atoms may serve to stabilise the reduced cofactor by moving closer to the rings in the reduced state.

The ability to visualise hydrogen atoms in a neutron diffraction structure is limited by the negative scattering length of hydrogen which causes density cancellation (Chapter 2, Fig. 2.1). The H/D-exchanged neutron structure of $^{1}$H-COx in the oxidised state (Chapter 5) showed significant cancellation of density across the structure including most side-chains and the Cα and C atoms of the protein backbone. In fact, approximately 75% of the protein hydrogen atoms are non-exchanged resulting in the majority of the nuclear density for these atoms being cancelled or significantly decreased. Additionally, some labile hydrogen atoms are either not solvent accessible or are involved in strong hydrogen bonds and as such do not get exchanged. An example is the main-chain N-H of Met122 which exhibited only 6% deuterium occupancy and therefore could not be localised in the H/D-exchanged structure. The ability to observe these atoms is also affected by the high background noise due to the incoherent scattering of neutrons by hydrogen atoms. Even the FAD-HN3 atom which
showed a deuterium occupancy of 62% could not be easily localised in omit maps of the H/D-exchanged structure. The Cβ-H atom of Asn119 is non-labile and therefore is observed at 100% hydrogen occupancy and as such is not visible as a positive peak in the structure. Even with very high resolution neutron structures, localising hydrogen atoms in the negative contour of \(2F_o - F_c\) maps can be difficult as the peak tends to be displaced from the true position of the hydrogen nucleus.

Neutron structural studies were pursued to investigate the hydrogen atom positions in different states of the redox cycle of COx. The structure of the perdeuterated protein (\(^2\text{H-COx}\)) was pursued to improve the density maps and allow localisation of all hydrogen (deuterium) atoms of interest, including non-labile hydrogen atoms of residues such as Asn119. Perdeuteration can also allow data collection on smaller sized crystals than normally required for neutron diffraction (Hazemann et al., 2005; Petit-Haertlein et al., 2009) and thus can be advantageous in overcoming one of the biggest bottlenecks in neutron diffraction protein crystallography - crystal size. This chapter describes the production of large perdeuterated protein crystals suitable for neutron diffraction and presents the first perdeuterated protein neutron structure of COx. Improvements and deficiencies in the ability to visualise deuterium atoms in the neutron structure are also discussed.
6.2. Methods

6.2.1. Crystallisation

Perdeuterated protein ($^2$H-COx) produced at the NDF, ANSTO, was crystallised similarly to the established procedure for the protiated protein ($^1$H-COx) (Chapter 4) (Golden et al., 2015). Crystals grew to a maximum size of 0.06 mm$^3$ and were macroseeded to increase the size to at least the minimum required for neutron diffraction crystallography. The macroseeding technique used for $^1$H-COx was initially used but required modification for the perdeuterated enzyme. The largest and highest quality crystals (visually assessed) were obtained with 8 – 9% PEG 8K, 125 mM MnSO$_4$, 100 mM cacodylate pH 5.2 and 7 mg ml$^{-1}$ of purified protein. Sitting drops (100 µL in volume) were set up using this condition and crystals of 0.6 mm x 0.3 mm x 0.3 mm were obtained. Several of the largest crystals were chosen for macroseeding.

The washing solution containing 4% PEG 8K used for $^1$H-COx caused significant visible damage to the $^2$H-COx crystals therefore 7% PEG 8K (the lowest precipitant concentration which consistently produced crystals) was used for the etching step. The precipitant concentration for growth was increased to 10% PEG 8K and contained 125 mM MnSO$_4$ and 100 mM cacodylate pH 5.2. The protein concentration was decreased to 4 mg ml$^{-1}$ in order to limit growth of excess nuclei in the drops.

To limit damage to the crystals and prevent the formation of nucleation centres in the drops, the crystals were not moved between drops during the macroseeding steps of the $^2$H-COx protein crystals. Crystals were etched four times by removal of most of the surrounding liquid followed by addition of 30 µL of 7% PEG 8K, 100 mM MnSO$_4$ and 100 mM cacodylate pH 5.2. Crystals were soaked in etching solution for 10 - 15 minutes for each step to ensure the removal of the crystal surface layer. To re-establish the growth stage most of the etching solution was removed and 30 µL of 10% PEG 8K
solution was added to each drop, and removed immediately. This was repeated twice to ensure the solution around the crystal had changed completely to 10% PEG 8K. Finally, most of this solution liquid was removed and 10 µL of precipitant solution (10% PEG 8K, 100 mM MnSO₄ and 100 mM cacodylate pH 5.2) containing fresh protein (4 mg ml⁻¹) was added. Macroseeding cycles were performed ~10 - 12 times with incubations periods of approximately 2 weeks per cycle.

Perdeuterated protein was purified and crystallised in protiated buffers. Crystals were “back-soaked” into deuterated buffers to exchange hydrogen atoms for deuterium atoms. Crystals were sequentially transferred into partially deuterated stabilising solutions (14% PEG 8K, 125 mM MnSO4 and 100 mM cacodylic acid pH 5.2) increasing in deuteration level by 10% increments for each step over a period of 3 days. Crystals were left in 100% D₂O buffers for a further 3 weeks with several exchanges of the surrounding solution.

6.2.2. Neutron Data Collections

Neutron diffraction data were collected at the IMAGINE beamline at the Oak Ridge National Laboratory (ORNL) (Meilleur et al., 2013). Previously, ²H-COx crystals with volumes ranging from 0.32 to 0.42 mm³ were screened using a 2.8 Å – 10 Å wavelength bandpass. Quasi-Laue neutron images were collected using a narrow band-pass 2.8 Å – 4.3 Å (λ_peak ~ 3.6 Å, δλ/λ~40%). The best diffracting crystal (with a crystal volume of 0.37 mm³) was chosen for data collection. 21 Laue images of 24 hr exposures were measured and indexed using the LAUEGEN suite of programs from CCP4 (Campbell et al., 1998; Helliwell et al., 1989). Data reduction and scaling was performed with LSCALE (Arzt et al., 1999) and then scaled and merged using SCALA (Winn et al., 2011).
6.2.3. **X-ray Data Collection**

An X-ray data set to 1.5 Å resolution was collected using the Rigaku HomeFlux X-ray setup equipped with a MicroMax-007 HF X-ray generator, Osmic VariMax optics and an R-AXIS IV++ image-plate detector. 360 images were obtained from exposures of 1 second with 1 degree rotation of the crystal. The diffraction data were indexed, integrated and scaled using the *HKL-3000* software suite. X-ray data were collected on the same crystal as the neutron data were collected from. This ensures the datasets are completely isomorphous and most suitable for joint X-ray/Neutron refinement.

6.2.4. **Refinement**

Joint X-ray neutron refinement (Afonine *et al.*, 2010) was performed using the *PHENIX* suite of software (Adams *et al.*, 2010). Hydrogen and deuterium atoms were placed using *ReadySet* such that labile hydrogen atoms were placed as both a deuterium atom and hydrogen atom at 50% occupancy which could be refined. All other hydrogen atoms were placed as deuterium atoms.

6.2.5. **H/D Atoms Involved in Protein-FAD Interactions**

Omit maps were calculated for several of the hydrogen atoms which are involved in interactions with the isoalloxazine moiety. Each omit map was calculated with the removal of only one atom each. The atoms that were removed were Gly120-D, Asn485-Dβ2 and Dβ3, and FAD-DN3. Additionally, an omit map of the conserved Wat541-D was also calculated. Maps were calculated using the *PHENIX* software (Adams *et al.*, 2010).
6.3. Results

6.3.1. Macroseeding

A large crystal (0.37 mm³) was achieved through a modified macroseeding procedure (Fig. 6.1). Several factors were found to be important for the macroseeding procedure of the perdeuterated protein. Firstly, macroseeding of $^2$H-COx crystals was more successful with a higher precipitant concentration and lower protein concentration than was required for $^1$H-COx crystals (Chapter 4). Secondly, a significantly increased etching time was required for growth to occur particularly as the crystal aged (up to 15 minutes per wash for the perdeuterated crystals versus ~1 minute for protiated crystals). This increased etching time may be due to the age of the crystal and also the higher precipitant concentration used for etching. Finally, adding an additional purification step using size exclusion chromatography improved the growth rate of perdeuterated crystals. This additional purification step was not required for macroseeding of the $^1$H-COx protein crystals. Another important modification to the macroseeding procedure was to minimise crystal movement between etching steps, rather the liquid was removed and replaced from around the crystal.

![Figure 6.1. Perdeuterated COx crystals](image)

(a) Crystals of perdeuterated COx grown initially grown in sitting drops prior to macroseeding. (b) The 0.37 mm³ crystal obtained by macroseeding and used for neutron diffraction data collection.
6.3.2. Data Collection and Refinement

Data reduction and refinement statistics for the neutron and X-ray refinement are shown in Table 6.1. The neutron diffraction experiment yielded a completion of 71% at 2.1 Å resolution. A quasi-Laue diffraction image from the data collection is shown in Figure 6.2. Close inspection of the diffraction spots reveals that the crystal was twinned (red box enlarged).

Table 6.1. X-ray and neutron data processing and refinement statistics for $^2$H-COx

<table>
<thead>
<tr>
<th></th>
<th>Neutron</th>
<th>X-ray</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data Processing Statistics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P 1 2, 1</td>
<td>P 1 2, 1</td>
</tr>
<tr>
<td><strong>Cell dimensions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>51.2, 73.1, 63.2</td>
<td>51.3, 72.8, 63.0</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90.0, 105.1, 90.0</td>
<td>90.0, 105.1, 90.0</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>41.1-2.1 (2.21-2.1)</td>
<td>50-1.5 (1.59-1.55)</td>
</tr>
<tr>
<td>R$_{merge}$</td>
<td>0.313 (0.412)</td>
<td>0.045 (0.224)</td>
</tr>
<tr>
<td>Observations</td>
<td>55,193 (6,800)</td>
<td>212,187 (12,406)</td>
</tr>
<tr>
<td>Number unique</td>
<td>19,217 (2,426)</td>
<td>67,309 (3,877)</td>
</tr>
<tr>
<td>Average I/σ</td>
<td>3.9 (2.2)</td>
<td>20.9 (4.6)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>71.9 (61.9)</td>
<td>94.1 (86.9)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>2.9 (2.8)</td>
<td>3.4 (3.2)</td>
</tr>
<tr>
<td><strong>Refinement Statistics</strong></td>
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<td></td>
</tr>
<tr>
<td>R factor</td>
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<td>0.1274</td>
</tr>
<tr>
<td>Free R factor</td>
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<td>0.1576</td>
</tr>
<tr>
<td>RMSD bond lengths (Å)</td>
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<td>0.018</td>
</tr>
<tr>
<td>RMSD bond angles (°)</td>
<td></td>
<td>2.088</td>
</tr>
</tbody>
</table>
6.3.3. Map Quality

Our neutron maps of the $^2$H-COx protein show the clear advantage of using perdeuterated protein over H/D-exchanged protiated protein. Figures 6.3a and 6.3b show the $2F_o - F_c$ nuclear density maps for the aromatic residues Phe45 and Trp55 which contain mostly non-labile hydrogen atoms. The maps of the H/D-exchanged $^1$H-COx residues clearly show density cancellation over the hydrogen atoms as well as the carbon atoms they are bonded to. In comparison, the deuterium atoms in the $^2$H-COx are clearly visible.

Figure 6.2. Quasi-Laue neutron diffraction image

Neutron diffraction image taken from a 12 hr exposure of the crystal using the IMAGINE beamline, HFIR. The red box is blown up in the right panel to show a magnified view of the doubling of the diffraction spots.
However, a major drawback for our neutron maps is the lack of density over the oxygen atoms and sulphur and phosphate atoms. This is most clearly illustrated with the density for the FAD cofactor. Figures 6.4a and 6.4b show the nuclear FAD H/D omit maps for the perdeuterated and protiated protein, respectively. The $2F_o - F_c$ density is continuous for most of the FAD atoms in the H/D-exchanged maps and the deuterium atoms of the hydroxyl groups are clearly observed as positive $F_o - F_c$ peaks in the omit maps (Fig. 6.4a). The perdeuterated maps show discontinuity and lack of density over all the oxygen and phosphorous atoms of the FAD (Fig. 6.4b). Density is also not visible for the C4X, C5X and C6 carbon atoms (numbering shown in Fig. 6.4c) of the isoalloxazine ring system of FAD and only a small $2F_o - F_c$ peak is visible for the important N5 atom. In particular, the deuterium atom peaks which are observed in the H/D-exchanged omit maps for all hydroxyl groups are not observed at all in the perdeuterated omit maps. Conversely, the non-labile deuterium atoms of FAD are clearly shown in the omit maps of the perdeuterated structure and are not seen as negative peaks in the H/D exchange structure.
6.3.4. FAD-Protein Interactions

Omit maps were calculated for several deuterium atoms which form hydrogen bonding interactions to FAD (FAD-DN3, Asn119-CβD, Gly120-D and Met122-D). Additionally, an omit map for the conserved water (Wat541) was also calculated. Omit maps failed to show clearly the positions of the FAD-DN3 atom and the Asn119-CβD atoms. $F_o - F_c$ peaks were observed for these atoms however, they were largely displaced from the expected positions and showed poorly formed density (not shown). Similarly, the Gly120-D omit map showed a strong $F_o - F_c$ density, however, this density was displaced toward the O4 atom of FAD (Fig. 6.5a). Figure 6.5b shows the omit map for the deuterium atom on the conserved Wat541 and this orientation...
corresponds with the position previously observed in the H/D-exchanged structures (Chapter 5). Clear $F_o - F_c$ density was also visible for the Met122-D atom which is a hydrogen bond donor to the FAD-O4 atom (Fig. 6.5c). This peak is 1.0 Å from the Met122-N atom.

6.3.5. H/D Exchange

Main-chain N-H(D) atoms which have a high (>0.8) deuterium occupancy are shown in Figure 6.6a. Most of the main-chain N-H(D) atom positions have a high deuterium occupancy although 174 atoms had less than 0.8 occupancy. Regions of high deuterium occupancy for main-chain N-H(D) atoms either indicate a high rate of H/D exchange (where D is easily exchanged for hydrogen during purification crystal growth

**Figure 6.5. Neutron maps of important FAD-protein interactions**

Nuclear density omit maps for (a) the Gly120-D omit map, (b) Wat541-D omit map and (c) Met122-D omit map. $2F_o - F_c$ nuclear density is contoured at 1.5 σ (magenta) and the $F_o - F_c$ nuclear density is contoured at 3.0 σ (cyan). $2F_o - F_c$ electron density is shown for Wat541 in (b) and contoured at 2.0 σ (blue).
and then exchanged back to D during “back-soaking”) or indicate regions of poor exchange (where D did not get exchanged at all and remained D throughout the process). It is difficult to assess the H/D exchange of those positions that show low deuterium occupancy as the absence of nuclear density could either be interpreted as the presence of hydrogen atom or simply a lack of sufficient map quality to observe the deuterium adequately. Figures 6.6b and 6.6c offer a comparison of H/D occupancies in the H/D-exchanged structure and the perdeuterated structure. Figure 6.6b shows those main-chain deuterium atoms that displayed >0.8 occupancy in both the H/D-exchanged and perdeuterated structures and are therefore atoms that exhibit a high exchange rate and are also well ordered enough to observe the density. Interestingly, some high exchange deuterium atoms were observed inside α-helices (Fig. 6.6b, red box). Figure 6.6c shows those main-chain deuterium atoms which refined to an occupancy of <0.2 in the H/D-exchanged structure and >0.8 in the perdeuterated structure i.e. those atoms that are likely to have very low levels of exchange. These atoms appear predominantly in the buried α-helices and β-sheets in the region that binds the FAD cofactor (Fig. 6.6c, red box).
Figure 6.6. H/D exchange of perdeuterated and protiated protein

Main-chain deuterium atoms with greater than 0.8 occupancy are shown as green spheres in (a). Main-chain deuterium atoms with an occupancy greater than 0.8 in both the H/D exchanged and perdeuterated structure are shown as green spheres in (b). Main-chain deuterium atoms with occupancy greater than 0.8 in the perdeuterated structure and occupancy less than 0.2 in the H/D-exchanged structure are shown as cyan spheres in (c). The red box in (b) shows a rotated and enlarged image of an alpha helix with exchanged deuterium atoms while the red box in (c) shows a rotated and enlarged image of alpha helices and beta sheets near the FAD cofactor.
6.4. Discussion

Perdeuteration of COx was pursued to improve the visibility of deuterium atoms in the nuclear density maps of the neutron diffraction experiment. Perdeuteration resolves the issue of cancellation of density by hydrogen atoms and improves the signal to noise ratio by decreasing the noise in the experiment due to incoherent scattering by hydrogen atoms. Crystals of a sufficient size for neutron diffraction were obtained by a macroseeding method and a neutron diffraction dataset to 2.1 Å resolution was obtained. The neutron maps show a clear advantage in using perdeuterated protein for neutron diffraction experiments but also highlight some difficulties and the need for further optimisation of crystal quality in order to visualise all the deuterium atoms of interest in the structure.

Chapter 4 showed that sufficient yield and quality of perdeuterated COx could be obtained for successful crystallisation of the enzyme (Golden et al., 2015). While functional and structural characterisation of the enzyme showed no significant differences due to perdeuteration, it was necessary to modify the macroseeding technique described in Chapter 4 in order to obtain large crystals of perdeuterated COx. Multiple cycles of macroseeding were required to achieve the size suitable for neutron diffraction crystallography. The ideal condition for crystallisation of the perdeuterated protein was slightly modified from that of the protiated protein but still within the range of conditions in which protiated COx is crystallised. Perdeuterated protein crystals were smaller than protiated protein crystals, similarly to that observed in the crystallisation of perdeuterated HIV-1 protease (Weber et al., 2013). Additionally the macroseeding technique was varied throughout the growth such as using protein that had been additionally purified by size-exclusion chromatography and using longer etching times as macroseeding progressed. Similar observations were made for the crystallisation of perdeuterated proteins in other crystallisation studies of protiated vs.
perdeuterated proteins (Chatake et al., 2003; Di Costanzo et al., 2007; Petit-Haertlein et al., 2009). Interestingly, perdeuterated human arginase I crystallised more readily using a lower protein concentration (3.5 mg ml\(^{-1}\)) (Di Costanzo et al., 2007) than for the protiated protein (5 mg ml\(^{-1}\)) (Di Costanzo et al., 2005). Ultimately, 12 cycles of macroseeding of the perdeuterated crystals was successful in producing crystals of suitable size (0.37 mm\(^3\)) for neutron diffraction.

Neutron diffraction data to 2.1 Å resolution of perdeuterated COx was collected on a crystal of 0.37 mm\(^3\). This is in comparison to the 2.2 Å resolution structure obtained on a 0.43 mm\(^3\) H/D-exchanged crystal (Chapter 5), indicating that smaller perdeuterated crystals compared to H/D-exchanged crystals could be used for neutron diffraction to similar resolutions. Hazemann et al. (2005) were able to improve the diffraction resolution of human aldose reductase crystals of 0.15 mm\(^3\) from 4.5 Å to 2.2 Å to by using perdeuterated protein. Similarly, diffraction to 1.85 Å was obtained on perdeuterated crystals of the Type III antifreeze protein of only 0.13 mm\(^3\) (Howard et al., 2011; Petit-Haertlein et al., 2009). These studies represent an order of magnitude reduction in the size of crystals typically required for neutron diffraction experiments. While perdeuteration of COx did not have such dramatic effects on the size of crystals required for neutron diffraction, a perdeuterated crystal that was 20% smaller than protiated crystal, diffracted to similar resolution. As will be discussed in the following sections, the improvement due to perdeuteration may have been limited by the crystal quality and further optimisation of crystallisation and macroseeding may yield greater increases in diffraction resolution.

The neutron maps for the perdeuterated structure were far superior to the H/D-exchanged structure for the non-labile deuterium atoms even at 2.1 Å resolution showing the great advantage of perdeuteration for visualising deuterium atoms in protein structures using neutron protein crystallography. Almost all non-labile
deuterium atoms were clearly visualised in the nuclear density maps. Furthermore, as most hydrogen atoms had been exchanged for deuterium atoms, the density cancellation issue shown in the H/D-exchanged structure was minimised and continuous density observed for protein backbone atoms. However, there were significant problems with the neutron maps for the perdeuterated protein that was evident on atoms with smaller neutron scattering lengths than deuterium and hydrogen atoms i.e. oxygen, sulphur and phosphorous atoms (Chapter 2, Table 2.1). While X-ray maps clearly localise these heavy atoms, the lack of nuclear density for these atoms does affect the ability to localise nearby deuterium atoms. In particular, some hydroxyl groups did not show density for either the oxygen atom or the deuterium atom. This could reflect that the hydrogen atom was unexchanged for these hydroxyl groups or it could simply reflect the poor density in this region due to the smaller coherent scattering cross-section of oxygen compared to carbon and deuterium. The crystals did spend more time in H$_2$O buffers than in D$_2$O buffers and were “back-soaked” into D$_2$O buffers for a shorter time than the protiated crystals (Chapter 5). However, deuterated crystals were “back-soaked” in a larger volume (sitting drops rather than in a capillary) that would help to maintain a D$_2$O gradient between the crystal and surrounding solution and therefore maximise the exchange rate of labile protein hydrogen atoms.

Of most concern is whether the deuterium atoms of interest are clearly visible in the nuclear density maps. While some deuterium atoms were clearly visible such as Met122-D (Fig. 6.5c) and the deuterium of Wat541 (Fig 6.5b), others were not visible or could not be reliably localised such as Asn119-CβD, FAD-DN3 and Gly120-D. Interestingly, the latter atoms did appear as $F_o - F_c$ peaks in the omit maps, but their positions were displaced from any reasonably expected position. This probably reflects the close interaction of these atoms with the FAD cofactor which itself showed very poor density. In contrast the deuterium atom of Met122 was clearly visible with a
well-shaped $F_o - F_c$ peak which was 1.0 Å from the main-chain nitrogen atom suggesting it is well localised and the density is not distorted by its interaction with FAD as was observed for Gly120-D and Asn119-CβH. Interestingly, this atom showed low levels of exchange evidenced by the low deuterium occupancy in the H/D-exchanged structure (6%) and the high deuterium occupancy in the perdeuterated structure (99%) indicating that this atom is involved in a strong hydrogen bond to FAD-O4. The negative charge of the reduced FAD may be stabilised at several positions on FAD including the O4 atom (Chapter 1, Fig. 1.4) so a strong hydrogen bonding interaction may serve to stabilise the reduced cofactor. Additionally, the position of the Wat541-D atom observed in the H/D-exchanged structure is confirmed in the perdeuterated maps. The perdeuterated neutron maps were sufficient for determining some deuterium atom locations but there were deficiencies in determining others, particularly the density surrounding FAD. This indicates that higher resolution structures and/or better quality crystals will be required to obtain all the desired information to elucidate the reaction mechanism of COx but also shows the viability of neutron studies of the perdeuterated COx.

It is interesting to compare the H/D exchange between the H/D exchange neutron structure and the perdeuterated neutron structure. By comparing the main-chain hydrogen atoms that exhibit high deuterium exchange in both structures, we can identify regions which are most easily exchanged. Interestingly, several main-chain deuterium atoms that exhibited a high exchange are found in α-helical regions of the structure (Fig. 6.5b). These helices are on the surface of the molecule and solvent exposed. In contrast the α- helices and β-sheets involved in interactions with the ribityl and nucleotide portions of the FAD cofactor show very low exchange behaviour (Fig. 6.5c) (as determined by high deuterium occupancy in the perdeuterated map and low deuterium occupancy in the H/D-exchanged map). A criticism could be that the lack of nuclear
density for these atoms in the H/D-exchanged map is not due to the lack of a deuterium atom but rather to the map quality; however, taking the information together with the perdeuterated structure does provide stronger evidence for a low exchange rate in this region. Furthermore, the FAD cofactor itself clearly shows exchange of the hydroxyl hydrogen atoms which form hydrogen bonding interactions to the protein in the H/D-exchanged structure.

The perdeuterated neutron maps were successful for the visualisation of deuterium atoms on hydrophobic residues however, there was not complete exchange of all labile atoms indicating that a longer H/D exchange is required prior to the neutron experiment. Also, the completeness of the data and twinning of the crystal has affected the final quality of the map making inferences about the localization of deuterium atoms difficult. Overall, the perdeuterated neutron structure was a successful preliminary study, indicating the viability of further neutron work with COx. Higher resolution data and longer H/D exchange times will significantly improve the visibility of atoms of interest particularly near the FAD cofactor.
CHAPTER 7

GENERAL DISCUSSION
7.1. Discussion

Neutron diffraction studies of cholesterol oxidase (COx) were pursued to determine the positions of hydrogen atoms in the reduced and oxidised enzyme states in order to elucidate the detailed redox chemistry of the enzyme. Neutron diffraction has the potential to definitively locate hydrogen atoms in crystal structures due to the larger scattering characteristic of hydrogen and deuterium atoms compared to X-ray crystallography. X-ray crystallography has provided unprecedented understanding of enzymes, in particular, that the enzyme structure is integral to its function. The structure creates the substrate binding specificity and also brings the chemically reactive elements into close proximity: thus creating a catalyst. Hydrogen atoms constitute ~50% of the atoms in a protein and are involved in the local interactions that determine the structure of the enzyme such as hydrogen bonds, salt bridges, hydrophobic interactions and pi-bonding. While many hydrogen atom positions can be inferred from the positions of heavier atoms in the proteins, there are several situations where the hydrogen atom must be determined experimentally. The protonation states of some residues determine whether they can act as a base or acid in catalysis or as hydrogen bond donors or acceptors such as in the case of β-lactamase (Tomanicek et al., 2011) and HIV-I protease (Adachi et al., 2009) and inferring the wrong protonation states can radically alter the interpretation of the catalytic mechanisms of proteins. NPC has also been used to identify hydrogen atoms involved in LBHBs (Yamaguchi et al., 2009) which are not located at the expected covalent bonding distance and are instead found at a longer distance, and to discriminate between short hydrogen bonds and LBHBs (Tamada et al., 2009). The hydrogen atom positions cannot be inferred from X-ray structures in these cases and must be determined experimentally. One other key advantage of NPC is that it can identify protons, which do not have any electrons to scatter X-rays and also to discriminate between D₂O and D₃O⁺ molecules (Cuypers et
al., 2013; Kovalevsky et al., 2011). Many advances have improved the viability of the technique for proteins and, in the future, further interesting insights are expected to be gained which are unique to NPC.

The chemical reactions catalysed by COx involve the transfer of hydrogen atoms in the form of protons and hydrides, therefore, the ability to visualise their positions is essential to fully understand the catalytic mechanism. Additionally, previous X-ray diffraction pH studies of COx indicated that some hydrogen atoms involved in interactions with the FAD cofactor may assume multiple positions (Chapter 1, Fig. 1.6) in the reduced state, which may play a role in stabilisation of the reduced cofactor. It is not known where the hydrogen atoms from the substrate are transferred in the reduced state. Furthermore, the exact identity of the active site base for deprotonation of the substrate hydroxyl group in the oxidation reaction is also not unequivocally known. By pursuing neutron diffraction studies of COx we hoped to gain insights into these questions and gain a more detailed understanding of the redox chemistry of COx. Specifically the aim of this project was to compare the positions of hydrogen atoms in the reduced and oxidised states of COx through the use of neutron diffraction crystallography.

In order to determine single crystal neutron structures it is necessary to obtain crystals much larger than required for X-ray crystallography. In the case of COx, it was also important to develop techniques for reduction of the crystals in capillaries. These techniques were first developed using the protiated protein (\(^{1}H\)-COx) and were then adapted to perdeuterated (\(^{2}H\)-COx) protein. The initial step to enable these studies including the design of an expression clone of the protein containing a hexa-histidine tag and to establish that the protein would still be expressed and purified sufficiently for crystallisation. The tag could not be removed before crystallisation as it was added to the C-terminus of the protein to ensure that it would not be removed with the N-terminal
targeting presequence during expression. Successful crystallisation and structure determination of COx-H6 indicated that the tag did not interfere with crystallisation or structure of the protein and so it was reasonable to apply the technique to $^2$H-COx. Expression and purification of the perdeuterated protein was successful and we were able to produce protein in milligram quantities. The macroseeding technique produced crystals which were large enough for neutron diffraction for both $^1$H-COx and $^2$H-COx protein. Functional and structural characterisation of the protein showed that the $^1$H-COx and $^2$H-COx proteins are essentially identical for our purposes. Crystallised enzyme could be reduced using a small molecule secondary alcohol under an anaerobic atmosphere. NPC of COx is therefore a viable technique and we have the ability to complete all of the technical aspects of the neutron diffraction study of both perdeuterated and protiated enzyme in the reduced and oxidised states.

The reduced-enzyme X-ray structure gave insights into the conformation of the FAD as well as movement of the aromatic triad residues. Previously, a structure of COx with a glyceraldehyde adduct suggested that the FAD is bent in the reduced state and the aromatic triad residues adopt the second conformation upon substrate binding (Lyubimov et al., 2007). A dithionite reduced structure with an FAD-N5 sulfite adduct, and an isopropanol reduced structure without the covalent FAD-N5 adduct were presented in Chapter 3 (Golden et al., 2014) and indicated that bending of the FAD most likely occurs due to the formation of the covalent adduct at the N5 position rather than the reduction of the FAD per se. These studies also showed that the aromatic triad exists in two conformations in the reduced structures with and without an FAD-N5 covalent adduct suggesting that the triad conformation may be influenced by both the redox state of the enzyme as well as the presence of N5 adducts. The pH studies of COx identified the FAD-N3H atom and the Asn119-CβH atom as difference density peaks in the X-ray diffraction maps (Chapter 1, Fig. 1.6) (Lyubimov et al., 2006) which
appeared to be split into two smaller peaks at pH 9.0. The structure determined at pH 9.0 was suggested to mimic a reduced anionic state of the cofactor. Our X-ray structure of the reduced COx could not identify these hydrogen atom positions well which is unsurprising as the pH study was on crystals which diffracted to sub-Angstrom resolution (0.98 Å) while the reduced structure had a maximum resolution of 1.12 Å. The neutron structures of the oxidised enzyme at 2.1 Å (\(^{2}\)H-COx) and 2.2 Å (\(^{1}\)H-COx) resolution showed the locations of many hydrogen (deuterium) atoms that could not be identified in the X-ray structures however, some atoms of interest (such as Asn119-CβH) were not clearly identifiable in the neutron structures either due to the presence of hydrogen rather than deuterium (\(^{1}\)H-COx) or due to poorer quality maps (\(^{2}\)H-COx). Moderately higher resolution (~1.8 Å - 2.0 Å) neutron structures should significantly improve the visibility of hydrogen atoms in the neutron maps.

The most interesting findings came from the analysis of the H/D-exchanged \(^{1}\)H-COx oxidised neutron structure in combination with the reduced X-ray structure. The neutron and X-ray scattering density maps both showed the positions of some hydrogen atoms with a resolution of 1.12 Å for the reduced X-ray structure compared to 2.2 Å and 2.1 Å for the neutron structures, demonstrating the power of neutron diffraction for visualising hydrogen atoms. In the X-ray electron density maps the hydrogen atoms were only localised by difference density (\(F_o - F_c\)) peaks and were not seen in the observed density (\(2F_o - F_c\)) at a significant level but were visible in both the neutron observed density (\(2F_o - F_c\)) and difference density (\(F_o - F_c\)). The reduced X-ray structure (Golden et al., 2014) showed the position of the transferred hydride to FAD-N5 atom while the neutron structures showed the orientation of a conserved water 541. Furthermore, an unusual deuteron was observed between the FAD-N5 atom and the protein main-chain. Theoretical calculations indicated that a hydrogen bond at this position serves to tetrahedralise the N5 centre. Taking all this information together, and
modelling the substrate in the active site in a way inferred from the electron and neutron density and also taking into account theoretically proposed restraints, we showed that the protein acts to position the substrate donating orbital and modulates the position of the FAD receiving orbital such that they almost perfectly align. This would be the most efficient way for the hydride transfer to occur and indicates how the protein is able to act as an efficient catalyst.

Furthermore, a conserved positively charged residue (Lys225) was found interacting with the Asn119-O atom of the peptide bond of Gly120/Asn119 suggesting a role in stabilising a negative charge over this bond which would be present if the main-chain amide hydrogen atom of Gly120 exists as a proton. DFT calculations were performed using an amide with an unrestrained N10-N5-H1 angle (Chapter 3, Fig. 3.4b and Table 3.2) as well as an amide with this angle restricted to the angle observed in the crystal structure (Fig. 3.4c and Table 3.2). The unrestrained amide localised at a position with an N10-N5-H1 angle which was ~86 degrees and had the shortest and strongest hydrogen bond compared to other cases. Restraining the amide to the crystal structure resulted in a slightly longer and weaker hydrogen bonding interaction but significantly altered the N10-N5-H5 angle to be more tetrahedral, similar to that observed in the crystal structure. The presence of a positive charge from Lys225 may serve to polarise the peptide bond resulting in movement of the amide hydrogen atom of Gly120 towards N5. This would result in a stronger and closer hydrogen bond which would orient the lone pair of electrons on N5 and thus also more strongly localise the position of the accepting orbital on this nitrogen atom. This specific interaction between a charged side chain, the peptide of Gly120/Asn119 and FAD-N5 is only conserved in the GMC oxidoreductases; some other oxidoreductases have an interaction between Gly120-N and FAD-N5 but do not have a positively charged residue suggesting that this specific set of interactions with a proton between the two nitrogen
atoms serves an important and unique function. While an interaction between the Gly120-N and FAD-N5 atoms has long been known for FAD-dependent oxidases (Fraaije & Mattevi, 2000), it is not possible to probe its role using mutagenesis as this interaction is with a backbone nitrogen atom. However, identification of the conserved positively charged side chain offers a mutagenic target for investigation of the Gly120-N/FAD-N5 interaction in GMC oxidoreductases.

This model of substrate binding and hydride transfer supports the assertion that Glu361 is the base for abstraction of the hydroxyl proton. Other members of the GMC family use the conserved histidine residue as the base for deprotonation of the substrate hydroxyl prior to hydride transfer. Initially it was assumed that His447 would serve this same function in COx based on both the conservation of active site topology amongst COx and the GMC oxidoreductases, and also on the basis of mutagenesis studies. However, His447 was subsequently ruled out as the active site base required for hydride transfer as the imidazole nitrogen facing the substrate is protonated and thus, could not act as a base. This was first seen in the omit map peaks of the high resolution X-ray structure (Lyubimov et al., 2006). The neuron structures confirm this with the nuclear density clearly showing a deuterium on Ne2 of His447. Mutation of His447 reduces oxidation activity (Kass & Sampson, 1998) more than mutations of other residues (Kass & Sampson, 1998; Sampson & Kass, 1997; Yin et al., 2002; Yin et al., 2001) despite not acting as a base. Even the Glu361Gln/His447Gln double mutant has residual oxidative activity indicating that even without an active site base, the hydride transfer can still occur (Yin et al., 2002). Along with the current work presented in this thesis, this suggests that positioning of the substrate (through hydrogen bonding interactions with Asn485 and His447) is critical for substrate oxidation, more so than hydroxyl deprotonation by an active site base and that with correct positioning of the substrate,
other active site features may be able to compensate for the lack of an active site base for deprotonation.

Overall, the neutron diffraction studies of COx have resulted in a number of unique findings about the mechanism of COx, which can also be used to better understand the mechanisms of other GMC-oxidoreductases. With less than 40 neutron diffraction structures of unique proteins currently deposited in the Protein Data Bank, the studies of COx expand the repository of neutron diffraction structures and add to the understanding of enzyme mechanism through a knowledge of hydrogen atom positions. These studies further demonstrate that NPC is a useful technique for understanding the relationship between protein structure and function.


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Table A.1. Atomic coordinates of the B3LYP-D3/Def2-TZVPP optimised geometries (Å) for all the species considered in the present work (shown in Fig. 3.4 of the main text).

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