X-ray Crystallographic Studies of Complexes of Human Myeloperoxidase with Hydroxamic Acids and Nitrite

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X-RAY CRYSTALLOGRAPHIC STUDIES OF COMPLEXES OF HUMAN MYELOPEROXIDASE WITH HYDROXAMIC ACIDS AND NITRITE

By

Corneliu Sologon

A DISSERTATION

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X-RAY CRYSTALLOGRAPHIC STUDIES OF COMPLEXES OF HUMAN
MYELOPEROXIDASE WITH HYDROXAMIC ACIDS AND NITRITE

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Compound I of myeloperoxidase is capable of both one-electron oxidation and two-electron oxidation reactions. Halides and pseudohalides are the substrates for the two-electron oxidation and other compounds including a large variety of aromatic alcohols and amines can be oxidized via the single electron oxidation pathway. To investigate the catalytic mechanism of myeloperoxidase four structures of complexes of myeloperoxidase were solved. Two of them are complexes with hydroxamic acids and the other two are complexes with nitrite.

Hydroxamic acids (salicylhydroxamic acid and benzylhydroxamic acid) can function as structural analogues for the aromatic alcohol and amine substrates of myeloperoxidase. The crystal structures of complexes of MPO with both hydroxamic acids have been solved at 1.85 Å resolution and their binding to myeloperoxidase is compared. The models show similar binding of their hydroxamic acid moieties but different orientations of their aromatic rings. The absence of the hydroxyl group covalently bound to the benzyl group in benzylhydroxamic acid creates an environment that does not permit the same favorable interactions with MPO when compared to salicylhydroxamic acid.
These findings could explain the three orders of magnitude difference in the
tvalue of the dissociation constants of the two complexes.

Nitrite has been shown to bind myeloperoxidase and also to reduce
Compound I and Compound II. Crystal structures of the complex between
myeloperoxidase and nitrite confirmed the binding of nitrite to the native enzyme
both in the distal cavity and the chloride-binding site. The binding in the distal
cavity occurred to the heme iron in the nitro mode. In the MPO-cyanide-nitrite
ternary complex, nitrite had been shown to bind only at the chloride-binding site.
No secondary site for nitrite binding had been seen in the distal cavity when
cyanide was liganded to the iron.

Overall, this study is the first to show from a crystallographic point of view
a comparison in the mode of binding of the two hydroxamic acids to a
mammalian peroxidase and also the binding of nitrite to a heme peroxidase.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

APX, ascorbate peroxidase
ARP, *Arthromyces Ramosus* peroxidase
BHA, benzylhydroxamic acid
CCP, cytochrome c peroxidase
CMPO, canine MPO
CN, cyanide
CTAB, cetyltrimethyl ammonium bromide
EPO, eosinophil peroxidase
hEPO, human eosinophil peroxidase
hLPO, human lactoperoxidase
hMPO, human myeloperoxidase
HP, hydrogen peroxide
HRP, horseradish peroxidase
HRPC, horseradish peroxidase peroxidase C
IPO, intestinal peroxidase
kd, dissociation constant
LPO, lactoperoxidase
MPO, myeloperoxidase
NMR, nuclear magnetic resonance
NO, nitrogen monoxide
NO², nitrite
PBA, perbenzoic acid
PDB, Protein Database
PEG, polyethylene glycol
PGHS, prostaglandin H synthase
PMN, polymorphonuclear
rmsd, root mean squared deviation
rsAPX, recombinant soybean cytosolic ascorbate peroxidase
Rz, Reinheitzahl
SCN, thiocyanate
SHA, salicylhydroxamic acid
TPO, thyroid peroxidase
CHAPTER 1
INTRODUCTION AND BACKGROUND

1.1 HEME PEROXIDASES

Heme-peroxidases are a large group of proteins that share one common function: to oxidize organic or inorganic substrates using hydroperoxides. The reaction can be a one-electron oxidation or a two-electron oxidation.

Currently the consensus is that heme peroxidases are classified into two superfamilies. The first superfamily is the plant, fungal, bacterial and archaeal peroxidase superfamily and the second is the mammalian peroxidase superfamily (of which myeloperoxidase is a member).

The first group, also called the “non-animal” superfamily, includes catalase-peroxidases, ascorbate peroxidases, cytochrome c peroxidases, manganese and lignin peroxidases and plant secretory peroxidases.

Cytochrome c peroxidase (CCP) is a heme-containing enzyme that can be found in yeast mitochondria. A particularity of CCP that distinguishes it from other peroxidases is that its substrate is also a protein, ferrocytochrome c. It catalyzes two successive one-electron oxidations of the substrate, decaying to the native reduced state in the process (Yonetani and Ray, 1966).

CCP has been thoroughly investigated and it was the first heme-peroxidase for which the crystal structure was elucidated (Poulos et al., 1978, Poulos et al., 1980). Later, refined at 1.7 Å resolution (Finzel et al., 1984), the structure shows a 294 residue monomer, organized into two domains. The
secondary structure is approximately 50% α helical and less than 12% β sheet. The heme lies in a crevice between two helices (Fig. 1.1), each being part of one of the two domains. The heme separates this crevice into two cavities. In each of the cavities, a conserved histidine residue is present: one is coordinated to the heme iron (His 175) (in the literature this is referred as the proximal histidine) and the other is usually too far for a coordination bond and it is known in the literature as the distal histidine. There is a series of other conserved residues for this superfamily: Arg 48, Asn 82, Asp 106, Gly 129, Arg 130, Val 169, and Asp 235 (Table 1.1). Many of these residues have analogs in human myeloperoxidase (hMPO): Arg 48 (distal arginine) in CCP is analogous to Arg 239 in MPO, His 52 (distal histidine) in CCP is analogous to His 95 in MPO, His 175 (proximal histidine) in CCP is analogous to His 336 in MPO (Fig. 1.2).

The catalase-peroxidases perform the catalatic reactions, represented by the breaking down of hydrogen peroxide (H$_2$O$_2$) to H$_2$O and O$_2$ (Nicholls and Schonbaum, 1963) but also have substantial peroxidase activity. The first three-dimensional structure of a catalase-peroxidase was determined for *Haloarcula marismortui* catalase-peroxidase (Yamada et al., 2002).

Several other structures for members of this superfamily of peroxidases have been determined including lignin peroxidase (Poulos et al., 1993), manganese peroxidase (Sundaramoorthy et al., 1994), *Arthromyces Ramosus* peroxidase (ARP) (Kunishima et al., 1994), ascorbate peroxidase (APX) (Patterson and Poulos, 1995), horseradish peroxidase (HRP) (Gajhede et al., 1997).
Fig. 1.1: Structure of Cytochrome C Peroxidase. The primarily helical structure can be observed (green) with the heme (highlighted in black) positioned between the helices pointed by arrows. The diagram was obtained in Pymol from the PDB entry 2CYP.
Fig. 1.2: Schematic view parallel with the heme plane of hMPO. For clarity, only important residues in relation to the heme are present in the picture. The five water molecules present in the distal cavity are also shown (W1-W5). The diagram was prepared using the PDB entry 1CXP representing the 1.8 Å resolution structure of native human myeloperoxidase.
Table 1.1: Important structural or functional conserved residues in peroxidases*

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>Conserved residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCP</td>
<td>R48    H52    N82   D106   G129  R130  V169  H175  D235</td>
</tr>
<tr>
<td>HRP</td>
<td>R38    H42    N70   D99    G122  R123  V164  H170  D247</td>
</tr>
<tr>
<td>ARP</td>
<td>R52    H56    N93   D116   G140  R141  V178  H184  D246</td>
</tr>
<tr>
<td>APX</td>
<td>R38    H42    N71   D95    G118  R119  V166  H163  D208</td>
</tr>
<tr>
<td>hMPO</td>
<td>R239   H95    H336</td>
</tr>
</tbody>
</table>

*The perfect conservation among the plant, fungal and bacterial peroxidase family members (CCP, HRP, ARP, APX) for the residues shown can be noted. Residues conserved in human myeloperoxidase (hMPO) are illustrated at the bottom of the table.

The second superfamily, the mammalian peroxidase superfamily takes its name from the fact that its members were initially discovered in mammalian species, although it is now known that closely related proteins occur throughout the rest of the animal kingdom (Davies et al., 2008). This family includes myeloperoxidase (MPO), eosinophil peroxidase (EPO), prostaglandin H synthase (PGHS) and other peroxidases present in milk (lactoperoxidase, LPO), thyroid gland (thyroid peroxidase, TPO), saliva, intestine and uterus. They have a variety of biological functions including defense against microorganisms, hormone synthesis and roles in pathogenesis. Mammalian peroxidases also have important differences when compared to the first superfamily including the longer polypeptide chains and the covalent bonds to the heme (O’Brien, 2000).

TPO and PGHS have pure biosynthetic functions. TPO is involved in the synthesis of thyroxine and triiodothyronine (thyroid hormones) by iodination and coupling of monoiodotyrosyne and diiodotyrosine (Rawitch et al., 1992). PGHS is a homodimeric membrane bound glycoprotein, each monomer binding a heme. Its double function as cyclooxygenase and peroxidase contributes to the synthesis of prostagandin H2 from arachidonic acid. The two activities occur at
separate sites with the arachidonate being bound in a hydrophobic pocket and the hydroperoxide and other small reducing agents being bound in the distal cavity as expected for a peroxidase (Dunford, 1999).

EPO is very similar to MPO in terms of localization and function since it is the major constituent of eosinophilic granules (approximately 40% of their total mass) in blood granulocytes and it oxidizes halides and pseudohalides to their corresponding hypohalous or pseudohypohalous acids (Abu-Ghazaleh et al., 1992). In contrast to MPO, EPO is a monomer, however it shares other characteristics with MPO: the EPO monomer contains two polypeptide chains which are the result of a proteolytic cleavage; the level of sequence identity with MPO is high (70%); and EPO is also a highly cationic protein (as is MPO), with a modified iron protoporphyrin IX prosthetic group (Ten et al., 1989).

LPO is a heme-containing glycoprotein that has the ability to inactivate a wide range of microorganisms. Bovine LPO is a single chain enzyme that has around 50% sequence identity with human MPO, EPO and TPO (Dunford, 1999). Human LPO is very similar or identical with the human peroxidase present in saliva since they are encoded by the same gene. (Ueda et al., 1997).

Table 1.2 shows the conservation of the most important residues in the mammalian peroxidase catalytic site.
Table 1.2: Important conserved residues in mammalian peroxidases*

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>Heme bound</th>
<th>Distal His</th>
<th>Distal Arg</th>
<th>Heme bound</th>
<th>Proximal His</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO</td>
<td>D94</td>
<td>H95</td>
<td>R239</td>
<td>E242</td>
<td>M243**</td>
</tr>
<tr>
<td>LPO</td>
<td>D108</td>
<td>H109</td>
<td>R255</td>
<td>E258</td>
<td>H351</td>
</tr>
<tr>
<td>EPO</td>
<td>D93</td>
<td>H94</td>
<td>R238</td>
<td>E241</td>
<td>H335</td>
</tr>
<tr>
<td>TPO</td>
<td>D238</td>
<td>H239</td>
<td>R396</td>
<td>E399</td>
<td>H494</td>
</tr>
</tbody>
</table>

*The distal histidine, distal arginine, proximal histidine and heme covalently bound residues are named at the top of each table column. The residues are of sequences of human peroxidases excepting LPO, which is from caprine peroxidase. The numbering from the sequences is as follows: for MPO and LPO according to their crystal published structure, for EPO after the cleavage of the signal peptide (residues 1-17) and propeptide (residues 18-139) - so residue 1 in the mature form is residue 140 in the precursor, and for TPO according to the precursor sequence for isoform 1 (UniProtKB/Swiss-Prot identifier P07202-1).

**It can be observed that there are no homologs for myeloperoxidase Met 243 in any of the other mammalian peroxidases.

1.2 MYELOPEROXIDASE

1.2.1 Molecular Biology

The neutrophils, also named polymorphonuclear leukocytes (PMN) because of their irregular, polylobate nuclei, represent the first line of defense of the innate immune system. Their rapid response is possible due to their ability to engulf and ingest foreign organisms, with the consequent formation of phagolysosomes in which they release the content of their intracellular granules (Klebanoff, 1967). There are at least three types of intracellular granules with one of them staining positively for peroxidase activity (Davies et al, 2008).

Human myeloperoxidase (hMPO; donor: hydrogen-peroxide oxidoreductase, E.C. 1.11.1.7) is a green heme protein (Agner, 1941) and one of the principal constituents of azurophil granules of mammalian neutrophils and, to a lesser extent, of monocytes. Degranulation makes MPO available to act on its physiological substrate (Cl−) and the product of the reaction, hypochlorous acid (HOCl), kills the phagocytised bacteria, viruses and fungi (Klebanoff and Clark...
1978; Belding et al., 1970; Lehrer, 1969). In vitro studies also demonstrated that purified MPO has a strong anti-bacterial activity in the presence of H$_2$O$_2$ and halides (Klebanoff, 1967).

The oxidative products of MPO - H$_2$O$_2$ - Cl$^-$ system have also been shown to be responsible for cellular damage, occurring with clinical significance in diseases like emphysema - by means of inactivation of alpha-1-anti-proteinase inhibitor which will induce in turn proteolysis in lung (Clark et al., 1981), atherosclerosis - because of cholesterol deposition and foam cell formation following the ingestion by macrophages of oxidatively modified low density lipoprotein (Hazen et al., 1999), and other harmful effects.

Chromosome 17 hosts the gene that encodes for MPO on its long arm (17q22-q23) (Ruano et al., 1988) in a cluster with those for hEPO and hLPO (Sakamaki et al., 2000). According to the gene sequence, a 745 residue unprocessed primary transcript is translated from 12 introns (Morishita et al., 1987). After being cotranslationally N-glycosylated and cleaved of the signal peptide between amino acids 48 and 49 (Yamada et al., 1990), a proMPO is formed via binding of the ferriprotoporphyrin IX heme. Subsequently, an N-terminal prosequence of 116 residues is removed and excisions of 6 additional internal residues and of C-terminal serine give rise to the heavy and light chains present in the mature enzyme (Iwamoto et al., 1988); The final dimeric form (Fig. 1.3) exists only after localization within the azurophil granules (Taylor et al., 1990). Inside the azurophil granules, MPO is tightly compacted and due to its highly cationic structure, it binds to a matrix-like material consisting of polyanionic
glycosaminoglycans. It is believed that MPO is present inside the granules in an inactive form, and it is activated upon neutrophil activation and the subsequent release into the phagolysosome (Egesten et al., 1984).

Recombinant systems were incapable of reproducing the natural hMPO, because one or more than one of the following processes did not occur: (1) removal of the propeptide, (2) excision of the internal residues that result in the formation of the 2 chains in the hemi-enzyme, (3) proper processing of the extra manose carbohydrate and (4) dimerization (Jacquet et al., 1991, Moguilesky et al., 1991, Poulos and Fenna, 1994, Shin et al., 2000). A non-recombinant method using the promyelocytic HL 60 cell line yielded highly purified protein that was indistinguishable from human neutrophil MPO when characterized by size exclusion chromatography, SDS-PAGE, Western blot analysis, NH$_2$-terminal sequence analysis and sensitivity to inhibitors of the peroxidation or chlorination activities (Hope et al., 2000). Monomeric hemi-MPO has spectral properties and catalytic activity indistinguishable from the native dimeric enzyme (Andrews and Krinsky, 1981; Andrews et al., 1984).

### 1.2.2 Function

In order to achieve its primary physiological role, killing of phagocytised microorganisms, the native ferric enzyme reacts with H$_2$O$_2$ to generate Compound I [1] which is an unstable oxyferryl Fe (IV) π-cation radical (Dolphins et al., 1971; Harrison et al., 1980). Compound I formation is a characteristic shared by all heme-peroxidases. In MPO, EPO and LPO, oxidation of the ferric heme
yields two electrons that are used to reduce \( \text{H}_2\text{O}_2 \) to water. MPO Compound I oxidizes its physiological substate, \( \text{Cl}^- \), or other halide and pseudohalide (SCN-) anions except \( \text{F}^- \) to hypochlorous acid (or other (pseudo)hypohalous acids) [2].

\[
\begin{align*}
[1] \text{Peroxidase} + \text{HOOH} & \rightarrow \text{Compound I} + \text{H}_2\text{O} \\
[2] \text{Compound I} + \text{X}^- + \text{H}^+ & \rightarrow \text{Peroxidase} + \text{HOX} \\
\text{(where X}^- \text{can be represented by Cl}^-, \text{Br}^-, \text{I}^-, \text{or SCN}^-) &
\end{align*}
\]

An alternative reaction among heme-peroxidases (MPO included) is the one-electron oxidation of substrate molecules [3] and [4]:

\[
\begin{align*}
[1] \text{Peroxidase} + \text{HOOH} & \rightarrow \text{Compound I} + \text{H}_2\text{O} \\
[3] \text{Compound I} + \text{AH} & \rightarrow \text{Compound II} + \text{A}^- \\
[4] \text{Compound II} + \text{AH} & \rightarrow \text{Peroxidase} + \text{A}^- + \text{H}_2\text{O} \\
\text{(AH can be a large variety of aromatic alcohols and amines)} &
\end{align*}
\]

1.2.3 Mammalian heme peroxidases studied by x-ray crystallography

Myeloperoxidase was the first mammalian peroxidase for which the three-dimensional structure was determined, first for the canine MPO (CMPO) (Zeng and Fenna, 1992) and later for the human homologue (Fenna et al., 1995 and Fiedler et al., 2000). To investigate the catalytic mechanism of hMPO a series of complexes were also probed by x-ray crystallography: the complex with bromide (Fiedler et al., 2000) the complex with cyanide, with thiocyanate and the complex
with both cyanide and thiocyanate (Blair-Johnson et al., 2001), the mode of binding of bromide to the hMPO-cyanide complex (Blair-Johnson et al., 2001), the complex with salicylhydroxamic acid (Davey and Fenna, 1996). Later, structures – with or without ligands – for the most distant relative of MPO among mammalian heme-peroxidases, prostaglandin H2 synthase were reported (Picot et al., 1994; Luong et al., 1996; Kurumbail et al., 1996; Gupta et al., 2004). These two heme-peroxidases were the only ones probed by x-ray crystallography until recently, when the crystal structure of lactoperoxidase was also determined (Singh et al., 2008; Singh et al., 2009).

The latest and highest resolution (1.8 Å) crystal structure of native hMPO (Fiedler et al., 2000) showed a model composed of 1140 amino acids, 2 hemes, 16 sugars, 2 calcium ions, 2 chloride ions, 4 sulfates, 6 acetates and 838 water molecules. This structure confirmed that human myeloperoxidase (Fig. 1.3) is a 140 kDa homodimer. Each monomer has two polypeptide chains of 108 and 466 amino acids respectively, which result from post-translational cleavage of a single initial polypeptide chain from which 6 residues are excised (Akin and Kinkade Jr., 1986). The two halves are linked together by a disulfide bridge at Cys 153 (Andrews and Krinsky, 1981; Zeng and Fenna, 1992). Each monomer contains a covalently bound heme, bound calcium and chloride ions and three sites of asparagine-linked glycosylation (Zeng and Fenna, 1992, Fenna et al., 1995).
Fig. 1.3 View along local dyad axis of the myeloperoxidase dimer. For each monomer (red and blue), the large polypeptides are shown in dark shades and the small ones in light shades. Other colors are used to highlight: hemes (green), carbohydrates (orange), calcium (purple) and chloride (yellow) (Fenna, 2001).
As first established in the canine myeloperoxidase structure at 3 Å resolution (Zeng and Fenna, 1992), the heme is situated in a crevice deep within the molecule and has access to solvent through an open channel. The heme separates this crevice into two sides known as the proximal side facing His 336 (proximal histidine) and the distal side facing His 95 (distal histidine) (Poulos et al., 1980).

The heme moiety of human MPO was determined to be a derivative of protoporphyrin IX with the methyl groups on pyrrole rings A and C modified so that they can form ester bonds with the carboxyl groups of Glu 242 and Asp 94 respectively (Fig. 1.4). It was proposed that similar linkages between the polypeptide chains and the prosthetic group take place in EPO, LPO and TPO since their sequences show a high degree of conservation (Fenna et al., 1995). In addition to these two ester bonds, MPO has a sulfonium ion linkage between the vinyl group β-carbon of the pyrrole ring A and the sulfur atom of the non-conserved Met 243 (Fig. 1.4). MPO is unique among peroxidases in its ability to oxidize chloride to hypochlorite and in its modified spectral properties. These characteristics are the consequence of the electron withdrawing effects of this sulfonium ion linkage and the porphyrin ring distortion (Jacquet et al., 1994; Kooter et al., 1999). The x-ray crystal structure first revealed this distortion: pyrrole rings A and C are tilted toward the distal side, giving the heme a bow-like shape.
The heme moiety of MPO and its linkages to the apoprotein (corrected after Kooter et al., 1999). Heme pyrrole rings A, B, C, D are named and the exact bonds between Asp 94, Glu 242 and Met 243 with the heme can be observed.

The native enzyme encloses 5 water molecules (W1-W5) in its distal heme cavity. The hydrogen bonding pattern of these five water molecules (Fig. 1.5) is as follows: W1 with Nε of the distal His 95, W2 with NH2 of Gln91 and W3
with guanidinium group of the distal Arg 239. W4 is hydrogen bonded to the heme ring D propionate and W5 is indirectly linked to the protein through hydrogen bonding with W2 (Fenna, 2001).

Fig. 1.5: Diagram showing the positions occupied by the five water molecules (W1-W5) in the distal cavity (Fenna, 2001). The four heme pyrrole rings are named and the dashed lines symbolize the hydrogen bonds. Q91, H95 and R239 stand for the N\_\_ of the distal His 95, NH\_2 of Gln91 and guanidinium group of the distal Arg 239 respectively. W4 also makes a hydrogen bond with propionate attached to heme pyrrole ring C.

Another well-described region in the molecule is the halide-binding site, present one per monomer (Fiedler et al., 2000). This site is composed of two short parallel \( \beta \)-strands (amino acids 30-33 and 324-327) connected by two hydrogen bonds. Here, in the native enzyme, a chloride ion functions as the
ligand in a trigonal planar geometrical arrangement made by the peptide NH group of Val 327, the peptide NH group of Trp 32 and a water molecule (Fig. 1.6).

Fig. 1.6: The proximal helix chloride binding site. Important residues that are part of the two short parallel $\beta$-strands (amino acids 30-33 and 324-327) are shown together with the chloride ion functioning as the ligand in a trigonal planar geometry arrangement (dashed lines) made by the peptide NH group of Val 327, the peptide NH group of Trp 32 and a water molecule (designated "water" in the picture).
1.2.4 Ligand Binding

Determination of the crystal structure of a myeloperoxidase-bromide complex (Fiedler et al., 2000) showed that a bromide ion can replace the chloride ion bound at the amino terminus of the helix containing the proximal histidine, His336 (the halide-binding site mentioned above). Another bromide anion also bound at partial occupancy in the distal cavity, where it replaced the water molecule hydrogen bonded to Gln 91 (W2). This site is believed to be the location where halide binding inhibits the enzyme by competing with H\textsubscript{2}O\textsubscript{2} and probably also the location at which the halides bind to Compound I as substrates.

In the MPO-thiocyanite complex (Blair-Johnson et al., 2001) SCN\textsuperscript{-} was located parallel to the heme plane, replacing two water molecules (W2 and W5) with the nitrogen atom being closer to the iron than the carbon atom and making the interaction of W2 in the native enzyme. The sulfur atom was shown to possess van der Waals contacts with C\textsubscript{Y} of Glu242, C\textsubscript{D} of Arg 239 and the methyl carbon of heme pyrrole ring D.

The complex of MPO with cyanide was proposed to be a good model for MPO Compound I, as a compound containing a six-coordinated low spin iron center. The structure of this complex was characterized at 1.9 Å resolution (Blair-Johnson et al., 2001). Cyanide bound to the heme iron, forming Fe-C-N angle of \(\sim 157^\circ\) with a slight movement of 0.2 Å of the iron atom in the heme plane. The bent orientation of the cyanide relative to the heme can be explained by the tight environment in the distal cavity (a very short distance of 5.6 Å between heme iron and N\textsubscript{E} of His 95) and other hydrogen bonding restrictions.
The interactions of the MPO-cyanide complex with bromide and thiocyanite were also investigated (Blair-Johnson et al., 2001). The bound cyanide appeared to influence the binding of the bromide since the position occupied in this complex by the bromide was not the one seen in the MPO-bromide (corresponding to W2 in the native enzyme) but the one occupied by W5 in the native enzyme. In turn, the bromide anion also influences the cyanide binding, making the Fe-C-N angle sharper. The bound cyanide does not have the same effect on the thiocyanite in the distal cavity since thiocyanite occupies the same position as the one in the MPO-thiocyanite complex.

The complex between hMPO and salicylhydroxamic acid will be discussed at length in the chapter 3 of my thesis.
CHAPTER 2
GENERAL METHODS EMPLOYED IN X-RAY CRYSTALLOGRAPHY

2.1 PROTEIN CRYSTALLIZATION

Protein crystallization was originally employed as a method of purification. Using x-ray diffraction on a crystal of purified protein is one of the two important methods used in structural biology to determine the three dimensional structures of proteins, the other being NMR spectroscopy. Crystallization per se of a purified protein is not sufficient for this type of analysis; the crystal must also achieve certain dimensions because the intensity of the diffraction pattern is approximately proportional to the crystal size and inversely proportional to the unit cell volume (Blundell and Johnson, 1976). Supersaturation of the protein solution can lead to crystallization. Supersaturation is dependent on solubility, protein concentration, ionic strength, temperature, solvent, pH, and the presence of counter ions. Salting-in or salting-out methods can be employed to induce crystallization of proteins using precipitants like salts (ammonium sulfate, calcium chloride, sodium acetate, etc.) and even polymers like PEG (polyethylene glycol) with varying molecular masses. Modifying the pH changes the protonation state of the surface aminoacids and this renders different charge-charge interactions between the protein molecules that can help or hinder crystallization. Another factor that can be varied in trying to crystallize proteins is the temperature that
affects the free entropic term in the free energy equation as well as the dielectric constant (Blundell and Johnson, 1976).

Once a supersaturation concentration is achieved (concentration that is dependent on the factors mentioned above), one can hope that a metastable supersaturated state will attain an equilibrium with the saturated condition and result in removal of protein molecules from solution to a crystalline state. It is important to have only a small number of sites of nucleation (initial centers from which the whole crystals will grow) in order to get large enough crystals suitable for data collection. The supersaturation state must be carefully controlled since the number of nuclei is direct proportional to the degree of saturation. Moreover, these sites of nucleation can occur where foreign surfaces are present (dust, air bubbles, container walls) and in order to minimize their number careful attention should be paid to the purity of the protein solutions used in the crystallization trials and the cleanliness of the containers (Blundell and Johnson, 1976). Some proteins are very difficult to form nucleation sites, and this problem can be overcome by seeding techniques (microseeding using bits of crushed crystals already formed or macroseeding using one single crystal). Since myeloperoxidase is one of the proteins very difficult to crystallize de novo, microseeding was used to obtain crystals for data collection.

There are many techniques that are used in crystallization to get to a point of supersaturation of proteins solutions (batch crystallization, hot box technique equilibrium dialysis, evaporation, vapor diffusion) (Blundell and Johnson, 1976). The hanging droplet vapor diffusion method is one the most commonly used
because of its ease in terms of set-up, follow-up, seeding and variation of different diffusion rates between the droplet and the reservoir. The reservoir is the container in which a crystallization solution is placed. A little quantity of this solution is combined with a similar quantity of protein solution and the resulting mix is placed onto a cover slip, which is sealed upside down above the reservoir solution. Through an equilibration process, the solvent in the droplet is transferred to the reservoir because the reservoir solution has a higher concentration. In this manner both the protein and precipitant concentrations in the droplet will rise gradually favoring the crystallization process.

2.2 X-RAY DIFFRACTION

Radiation scattered in a certain direction by a crystal (in which the atoms are arranged in a periodic manner) has an amplitude and phase, corresponding to the sum of the amplitudes and phases of the radiation scattered from every atom in the crystal. The following equation describes the above relationship:

\[ F(S) = \sum_{j} f_j \exp(2\pi i r_j \cdot S) \]

where:

(S) is the direction of scattering
F(S) is the amplitude and phase as a complex number of a diffraction position expressed as a function of the direction of scattering (S)
f_j is the atomic scattering factor for atom j
r_j is the vector from the same atom to the unit cell origin, making the right hand side of the equation 1 the vectorial sum of scattered radiation with amplitude f_j and phase angle 2πr_j • S.

This equation can be re-written after replacing the vector product r_j as a\(x_j\)+b\(y_j\)+c\(z_j\) where a, b, c are the cell unit dimensions and \(x_j, y_j, z_j\) are the fractional coordinates for atom \(j\) as

$$[2] \quad F(hkl) = \sum_{j=1}^{N} f_j \exp(2\pi i(hx_j + ky_j + lz_j))$$

where:

F(hkl) is known as the structure factor

h, k, l are integers and represent the positions in diffraction space where the phase difference between waves is 2π (the condition for scattering to be observed for a crystal).

This equation is in fact a Fourier transform sampled at points \(hkl\) in the reciprocal lattice (Blundell and Johnson, 1976).

If one knows the type of atoms and their locations in the unit cell, one can calculate the diffraction pattern. Usually this information is not known and, moreover, the information about all the phases are lacking after data collection. The intensities of the reflections are known and they are the squares of the structure factors F(hkl).

Since the diffraction pattern is a Fourier transform of the structure, then the structure can be also a Fourier transform of the diffraction pattern and the following equation can be written:
\[ \rho(xyz) = \frac{1}{V} \sum_{l=-\infty}^{\infty} \sum_{k=-\infty}^{\infty} \sum_{h=-\infty}^{\infty} F(hkl) \exp(-2\pi i (hx + ky + lz)) \]

That means that the electron density \( \rho \) at fractional coordinates \( x, y, z \) in each unit cell can be calculated knowing the structure factors \( F(hkl) \) for all the reflections (Blundell and Johnson, 1976). Unfortunately, the phase information prevents solving this equation easily, but there are methods that can be used to circumvent this impediment such as: molecular replacement (MR), multiple isomorphous replacement (MIR), multiple anomalous dispersion (MAD) and direct methods. The initial phases for the structures in this dissertation project were determined using a previously determined starting model in which the data came from crystals of the same space group with very similar unit cell dimensions.

With acceptable phase information in hand, one can interpret the initial electron density map and a starting model can be created. This model usually contains a lot of errors and needs to be corrected gradually in order to achieve as best as possible consensus between the observed and calculated structure factors, a process known as refinement. Checking the agreement between the observed and calculated structure factors amplitudes controls the accuracy of the refinement. The percentage of the following fraction

\[ \sum |F(hkl)_o| - |F(hkl)_c| / \sum |F(hkl)_o| \]

represents the crystallographic R factor.
In order to minimize overfitting of the diffraction data, another statistical quantity is introduced, $R_{\text{free}}$ (Brünger, 1992a). It also measures the agreement between the observed and calculated structure factors amplitudes, but this time for a test set of reflections (usually 5% of the total number of reflections) that are not included in the refinement and modeling of the structure.

Two types of processes are usually employed in structural protein refinement in an alternative fashion: the manual and automated processes. A manual process is a real space refinement where atomic positions are modified with the help of a graphic software (O in this case). An automated process consists of minimizing the differences between the model and the data using computational methods.

In this study the principal method of automated refinement was the simulated annealing (SA) method using the XPLOR v.3.8 software package (Brünger, 1992b). The principle on which it is based is the physical process of annealing in which heating is used to increase the temperature of a solid in a bath to a value that allows the particles of the solid to randomly arrange themselves in liquid phase. What follows is a slow cooling by a smoother decrease in the temperature of the bath. Simulated annealing can reach more optimal solutions since, in contrast to other methods like gradient descent, search directions are not restricted to downhill. For going uphill, a control parameter is used in order to facilitate a large degree of freedom for the alternate conformations of the atoms. The higher the value of the control parameter (in this
case a temperature of 2000 K - although this temperature has no physical meaning) the higher the likelihood overcoming energy barriers (Brünger, 1991).
3.1 OVERVIEW

It has been known for a very long time that aromatic peracids are very efficient in oxidizing peroxidases (Schonbaum and Lo, 1972). In original studies done on horseradish peroxidase (HRP), aromatic hydroxamic acids were classified along with hydrazides (R-CO-NHNH$_2$), $\alpha$-hydroxyketones (R-CO-CH$_2$OH) or amides (R-CO-NH$_2$) as being part of subclass I of R-CO-XY compounds that have structures very similar to aromatic peracids (Schonbaum, 1973). This type of compound is not hydrolyzed by peroxidases, thus forming stable, reversible and spectroscopically different complexes with them. The other group of R-CO-XY compounds, subclass II, comprises N- and O-substitued hydroxamic acids, benzaldehyde, O-benzoyl hydroxylamine, N-hydroxybenzene sulfonamide and phenacyl halides which also bind to the enzyme but which differ from the members of subclass I by not changing the characteristic HRP spectrum upon binding.

A wide variety of aromatic alcohols and amines (that behave with respect to the native peroxidases as the subclass II of R-CO-XY compounds) have been described as being capable of reducing peroxidase derivatives (compound I or compound II) (Everse et al., 1991). Aromatic hydroxamic acids (Fig. 3.1) which are structurally related to both of these subclasses (Schonbaum, 1973), bind to and act as strong inhibitors of heme peroxidases, including MPO (Davies and
Edwards, 1989; Ikeda-Saito et al., 1991). The dissociation constants of MPO-hydroxamic acid complexes were reported to be 2 μM for the MPO–salicylhydroxamic acid (SHA) complex and 5 mM for the MPO–benzylhydroxamic acid (BHA) complex (Ikeda-Saito et al., 1991).

The first reported structure of a complex between a hydroxamic acid and a peroxidase was the 2.3 Å resolution structure of the complex between hMPO and SHA (Davey and Fenna, 1996). A refined version to 1.85 Å will be presented in this chapter along with the structure of an hMPO-BHA complex.

The MPO-SHA complex structure showed the SHA aromatic ring at the entrance of the distal cavity, sandwiched in a hydrophobic region between the β, δ and γ carbons and ε nitrogen of Arg239 and pyrrole ring D of the heme (Fig. 3.2). The hydroxamic acid part of the ligand displaced three of the four water molecules described at the time in the distal cavity and slightly moved the remaining one. The result of this displacement was a nearly perfect duplication of the hydrogen bonding performed by the water molecules in the distal cavity with the oxygen atoms of the SHA molecule playing the part of the oxygen atoms of the water molecules displaced.

The SHA molecule appeared almost planar with an angle between the hydroxamic group and the benzene ring of 8°. This planarity has been mostly explained by the fact that an internal hydrogen bond could be formed in the molecule between the salicyl hydroxyl group and the NH group of the hydroxamic acid, and this hydrogen bond must restrict rotation about the C-C bond between the aromatic ring and the hydroxamic acid moiety.
Fig. 3.1: A side by side view of the structures of benzylhydroxamic acid (BHA), salicylhydroxamic acid (SHA), perbenzoic acid (PBA) and hydrogen peroxide (HP). The atom nomenclature is consistent with the initial nomenclature used in the previous article that described the structure of the MPO-SHA complex (Davey and Fenna, 1996). The BHA atom nomenclature follows the model of the SHA nomenclature and it is the same with the one that will be deposited in the Protein Data Bank.

Fig. 3.2: SHA mode of binding in the distal cavity of human MPO as was determined by the 2.3 Å resolution structure of the hMPO-SHA (Davey and Fenna, 1996). The four water molecules present in the native enzyme (W1-W4) are shown superimposed. (Davey and Fenna, 1996).
Apart from the 2.3 Å resolution MPO-SHA structure already mentioned, to date there are only two other structures of complexes between SHA and a peroxidase: the complex with *Arthromyces ramosus* peroxidase (ARP) (Tsukamoto *et al*., 1999), and the complex with recombinant soybean cytosolic ascorbate peroxidase (Sharp *et al*., 2004).

The binding of SHA to ARP (Fig. 3.3) is similar to the binding of BHA to ARP (Itakura *et al*., 1997) and differs from the binding reported in the 2.3 Å resolution structure of SHA-MPO complex first by the fact that the SHA molecule is rotated by approximately 180° about the bond between the benzene ring and the hydroxamic acid moiety and second by the distances from the heme iron to the atoms forming the benzene ring of SHA which are reported as 1-2 Å shorter for the SHA-MPO complex. This suggests that SHA is deeper situated in the distal cavity in the MPO complex which is also consistent with the tighter binding of SHA to MPO. There is also a striking difference in terms of dissociation constants between the MPO-SHA and the ARP-SHA complexes. While the $k_d$ for MPO-SHA complex is on the order of micromolar, the $k_d$ for ARP-SHA complex is 1.7 mM (Indiani *et al*., 2003). The dissociation constant for ARP-SHA complex is on the same order of magnitude with the one for the ARP-BHA complex (2.9 mM) (Indiani *et al*., 2003). This difference will be discussed later in the thesis.

The structure at 1.46 Å resolution of the complex between recombinant soybean cytosolic ascorbate peroxidase (rsAPX) and SHA (Fig.3.4) (Sharp *et al*., 2004) also revealed some interesting facts: (1) The binding of SHA is at a different site than the binding of the enzyme substrate, ascorbate (Sharp *et al*.,
The binding of SHA to rsAPX is similar to the binding that occurs to ARP, but in the former complex, SHA is directly coordinated to the heme iron as opposed to the latter in which the interaction occurs via a water molecule. Within the SHA molecule, the position of the hydroxamic acid moiety relative to the benzene ring is similar that seen in the MPO-SHA complex. Overall, SHA appears deeper situated in the binding pocket and the dissociation constant for SHA-rsAPX complex is on the order of micromolar (8 μM). This combination of binding deeper in the pocket and micromolar $k_d$ is similar to what is observed for the MPO-SHA complex.

In parallel with the higher resolution structure (1.85 Å) of the MPO-SHA complex I will describe in this dissertation thesis the 1.85 Å resolution structure of the MPO-BHA complex. The absence of the hydroxyl group on the benzene ring of the benzyl hydroxamic acid makes this molecule a more suitable analogue of perbenzoic acid (Fig 3.1) which is one of the substrates for the native enzyme which it can oxidize it to produce Compound I. Interestingly, the dissociation constant of this complex is on the order of millimolar. The most important rationale for carrying out this parallel crystallographic experiment is the attempt to obtain an explanation from a structural point of view of the large difference between the values of the dissociation constants for the two MPO-hydroxamic acid complexes.

Like SHA, BHA was also investigated by x-ray crystallographic methods. So far, the following structures of peroxidases with BHA have been determined:
Arthromyces ramosus peroxidase (Itakura et al., 1997) and horseradish peroxidase peroxidase C (HRPC) (Henriksen et al., 1998).

Fig. 3.3: Mode of binding of SHA (shown in blue) in the distal pocket of Arthromyces ramosus peroxidase (Tsukamoto et al., 1999). The different conformation relative to the SHA bound to hMPO can be observed (a relative rotation of approximately 180° about the C6-C7 bond). An important water molecule is also shown in green (W365) near the heme iron at a distance close to a coordination bond (2.8 Å). Hydrogen bonds are depicted as broken lines: O7-Arg52 Nε, N8-Pro154 O, O8-His56 Nε, O8-W365. The diagram was prepared using the PDB entry 1CK6 and the numbering of the SHA atoms is the one in Fig. 3.1.
Fig. 3.4: Mode of binding of SHA (shown in blue) in the distal pocket of recombinant soybean cytosolic ascorbate peroxidase (Sharp et al., 2004). In this complex O8 of SHA interacts directly with the heme iron (unlike the SHA-ARP complex shown in Fig. 3.2). Hydrogen bonds are depicted as broken lines: O7-Arg38 Nε, O1-Pro132 O, O8-Trp41 Nε. The diagram was prepared using the PDB entry 1V0H and the numbering of the SHA atoms is the one in Fig. 3.1.
The ARP-BHA complex solved at 1.6 Å resolution reveals that BHA binds similarly to the mode of SHA binding to ARP (Fig. 3.5). The aromatic rings have similar distances from the heme iron (7-9 Å), are surrounded at the cavity entrance by hydrophobic residues and are nearly parallel with the heme plane (Tsukamoto et al., 1999). The binding of the hydroxamic acid moieties occurs also in the same manner for the two compounds: hydrogen bonds are made with His56, Arg52 and Pro154.

The other peroxidase-BHA complex for which the three dimensional structure is known, HRPC-BHA, shows similarities to ARP-BHA (Fig. 3.6) (Henriksen et al., 1998). The BHA hydroxamic acid moiety forms hydrogen bonds with the corresponding conserved residues His42, Arg38 and Pro139, a water molecule is present in the distal cavity and situated at a matching distance from the heme iron. The differences arise in terms of the identities of the atoms that serve as hydrogen bonding partners for the water molecule in the distal cavity (carbonyl oxygen atom in the HRPC complex, hydroxyl group oxygen in ARP complex) and for the the carbonyl oxygen of BHA (N₁ for HRPC Nₑ in ARP complex of the conserved arginine, Arg38 an Arg52 respectively).

I will present in this chapter the MPO-SHA and MPO-BHA structures at 1.85 Å resolution and I will describe how each of the hydroxamic acids binds in the distal cavity emphasizing the differences between the two. In chapter 5 of my thesis I will underline the implication of these differences on the very disparate $k_d$ values reported for these two complexes.
Fig. 3.5: Mode of binding of BHA (shown in blue) in the distal pocket of *Arthromyces ramosus* peroxidase (Itakura et al., 1997). The similarity with the SHA binding to ARP can be easily observed. The water molecule close to the heme iron is shown in green (W415). Hydrogen bonds are depicted as broken lines: O7-Arg52 Nε, N8-Pro154 O, O8-His56 Nε, O8-W415. The diagram was prepared using the PDB entry 1HSR and the numbering of the BHA atoms is the one in Fig. 3.1.
Fig. 3.6: Mode of binding of BHA (shown in blue) in the distal pocket of recombinant horseradish peroxidase C (Henriksen et al., 1998). A water molecule close to the heme iron (2.6 Å) is shown in green (W). Hydrogen bonds are depicted as broken lines: O7-Arg38 N$_{H_2}$, N8-Pro139 O, O8-His42 N$_e$, O7-W, O8-W, His42 N$_e$-W, Arg38 N$_c$-W. The diagram was prepared using the PDB entry 2ATJ and the numbering of the BHA atoms is the one in Fig. 3.1.

3.2 METHODS

The leukocyte enriched human blood samples used as a source of hMPO were donated by Dr. Walter Scott’s laboratory, University of Miami Miller School of Medicine.

A hypo-osmotic shock was performed by combining 24 tubes of blood cell pellets (about 300 ml) with 8 volumes of H$_2$O. The mix was centrifuged for 15 min
at 8000 rpm. After the supernatant was decanted the new pellets were combined, 300 ml of H$_2$O was added and the new mix was centrifuged for 20 min at 9000 rpm. The pellet was resuspended in 0.05 M potassium phosphate pH 8.8 and solid CTAB detergent was added to 1% (w/v). After stirring for 1h and homogenizing, the sample was centrifuged for 20 min at 8000 rpm. The supernatant was applied to a CG50 cation exchange column equilibrated with 50 mM potassium phosphate pH 8.8 and then the column was washed with the same buffer. Elution of the sample from the resin was carried out using a gradient of 100ml 0.1 M potassium phosphate/100ml 0.6 M potassium phosphate. The collected fractions were checked for enzyme activity. This was carried out with a colorimetric assay monitoring guaiacol oxidation at 470 nm (Chance and Maehly, 1955). 1 ml of guaiacol buffer (0.22 ml guaiacol in 100 ml 10mM potassium phosphate pH 7.5) was mixed with 10 μL 33mM H$_2$O$_2$ solution for the negative control. For each fraction, 10 μL of enzyme were added to the above-described mix, and the guaiacol oxidation curve was recorded for 60s. The samples that were positive for enzyme activity were collected, concentrated and dialyzed against 25 mM HEPES pH 7.5.

Next the sample was applied to a phenyl sepharose column in 1.5 M Ammonium sulfate, 25 mM HEPES pH 7.5, washed with the same buffer and eluted with a 1.5 M to 0 M AMS elution gradient (Fig 3.7). At this point, purity of the sample was monitored by measuring the ratio of absorbance at 430 nm to absorbance at 280 nm (reinheitzal, Rz). After pooling the fractions that showed an Rz value larger than 0.7 the final purification step – obtaining the isoform C of
hMPO – was performed using a cation exchange column (Mono S) on a Pharmacia FPLC system. Loading and washing buffers consisted of 50 mm Bicine pH 8.0. The gradient used was 0.3 M to 0.7 M NaCl in 50 mM Bicine pH 8.0 (Fig. 3.8).

The average yield of isoform C of hMPO that could be obtained from 24 tubes of blood cell pellet was 0.8 mg. After concentrating to a concentration suitable for crystallization (~24 mg/ml), about 37 μl of pure isoform C was obtained (Rz value greater that 0.75) for use in 6 crystallization droplets.

Since it was known that de novo nucleation of these crystals rarely occurs and that the crystals crack when exposed to even micromolar concentrations of hydroxamic acids (Davey and Fenna, 1996), seeding and cocry stallization were performed. The seeding was performed using seeds of crystals of hMPO that we already had in the laboratory. A droplet of 6 μl containing hMPO (~20 mg/ml) was combined with 2 μl of precipitant (50 mM ammonium sulfate, 2mM calcium chloride, 20-24% PEG 8K, 50 mM sodium acetate pH 5.5 and 2 mM SHA or 20 mM BHA) and allowed to equilibrate with a reservoir containing the same precipitant. After 48 hrs the droplets were seeded and the seeds were allowed to grow at room temperature.
Fig. 3.7: Phenyl sepharose hydrophobic interaction column chromatography. The linear gradient described in the text is absent in the picture. The black curves represent individual fraction absorbance at 280 nm (the double humped curve) and 430 nm. The red curve represents individual fractions Rz value. The shaded red box at the bottom of the picture indicates the pooled fractions.

Fig. 3.8: FPLC Mono S cation exchange chromatography. The linear gradient described also in the text is 0.3 M to 0.7 M NaCl in 50 mM bicine pH 8.0. The black curve represents individual fraction absorbance at 280 nm and the blue curve represents individual fraction absorbance at 430 nm. The shaded red box at the bottom of the picture indicates the pooled fractions that showed a final Rz value of 0.77.
3.3 X-RAY DATA COLLECTION

The crystals were washed in a substitute mother liquor containing cryoprotectant (20% (v/v) 2-methyl-2,4-pentanediol) and frozen in a stream of dry nitrogen gas at −190°C. Diffraction data were collected with a Rigaku RU300 X-ray generator equipped with a 30 cm MAR image plate detector. Data were indexed, integrated, scaled, and merged using the HKL2000 data processing software (Otwinowski and Minor, 1997). The total number of reflections, the number of unique reflections, completeness and other statistical data for the two complexes are shown in tables 3.1 and 3.2.

3.4 SOLUTION AND REFINEMENT OF THE STRUCTURES

Frozen crystals of the MPO–SHA and MPO–BHA complexes belonged to the P2₁ space group and were closely isomorphous with the native ones (a = 110.9, b = 63.4, c = 92.0, β = 97.2° for MPO-SHA, a = 111.2, b = 63.5, c = 92.2, β = 97.3°, for MPO- BHA complex compared with a = 110.0, b = 63.4, c = 92.2, β = 97° for the native). The 1.8 Å resolution native human MPO model (Fiedler et al., 2000) and the difference Fourier method were used to calculate difference maps for both complexes using \( F_o(MPO-SHA) - F_c(MPO) \) or \( F_o(MPO-BHA) - F_c(MPO) \) respectively as coefficients and the native MPO phases. Seven water molecules (including the five water molecules present in the distal cavity) were removed from the model before calculation of the native MPO structure factors. Models of
SHA and BHA obtained from Hetero-compound Information Centre – Uppsala (Hic-Up) website were manually fitted to the observed densities (Figs. 3.9 and 3.10). Water molecules were reinstated close to the original positions occupied in the native state according to the observed densities as follows: for the MPO-SHA complex, one water molecule (W4 in Fig. 3.9 and Table 3.3) and for the MPO-BHA complex, two water molecules (W3 and W4 in Fig. 3.10 and Table 3.3). No other features above $3\sigma$ were observed in the distal cavities of either of the two halves of the molecule.

The models were refined at 1.85 Å resolution using the X-PLOR program (Brünger, 1988). Between stages of visualization and minor rebuilding in the modeling program O, cycles of simulated annealing, energy minimization and isotropic temperature factor refinement were performed. A subset of 5% of reflections was kept out of the refinement in order to be used for calculation of a free $R$-factor (Tables 3.1 and 3.2). Refining the MPO-SHA at this better resolution offered better insight into the complex and also gave a good model of comparison with the MPO-BHA complex presented in this thesis.
Table 3.1: Data collection and refinement statistics for the MPO-SHA complex

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<th>(A) Data Collection*</th>
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<td>Wavelength (Å)</td>
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<tr>
<td>Unit cell dimensions (Å)</td>
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<tr>
<td>Resolution Range (Å)</td>
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<td>( R_{\text{merge}} )</td>
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<tr>
<td>Reflections</td>
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<tr>
<td>Unique</td>
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</tr>
<tr>
<td>Completeness (%)</td>
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</tr>
<tr>
<td>( I/\sigma(I) )</td>
<td>19.00 (5.97)</td>
</tr>
</tbody>
</table>

*The numbers in parentheses are values for the highest resolution shell (1.92 Å – 1.85 Å)

<table>
<thead>
<tr>
<th>(B) Refinement statistics**</th>
<th></th>
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</thead>
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<td>R factors</td>
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<td>( R_{\text{work}} )</td>
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<tr>
<td>( R_{\text{free}} )</td>
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**The numbers in parentheses are values for the highest resolution shell (1.93 Å – 1.85 Å)
Table 3.2: Data collection and refinement statistics for the MPO-BHA complex

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<th>(B) Refinement statistics**</th>
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<td><strong>Acetate</strong></td>
<td>24</td>
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<td><strong>Ligand</strong></td>
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<td><strong>Solvent</strong></td>
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<td><strong>Ramachandran plot statistics (%)</strong></td>
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<td><strong>Residues in most favored regions</strong></td>
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</table>
Fig. 3.9: Initial Fo-Fc difference Fourier map superimposed on the refined model of the bound SHA molecule. The contour level is $3.8 \sigma$. One of the water molecules that were omitted from the phasing model also shows as positive density (W4).

Fig. 3.10: Initial Fo-Fc difference Fourier map superimposed on the refined model of the bound BHA molecule. The contour level of is $3.4 \sigma$. Two of the water molecules that were omitted from the phasing model also show as positive densities (W3 and W4).
3.5 RESULTS

3.5.1 Displacement of water molecules in the distal cavity

Since the 2.3 Å resolution X-ray crystal structure of the MPO-SHA complex was published, a higher resolution (1.8 Å) structure for the native enzyme has allowed more accurate modeling of the water molecules in the distal cavity. As shown previously in the structure at 2.3 Å, the SHA molecule displaces all but one of the five water molecules present in the distal pocket of the native enzyme (Fig. 1.5). The only water molecule that is still present in the distal pocket is W4 that makes a hydrogen bond with the salicyl ring hydroxyl oxygen and is slightly moved in the two halves from the original position (0.14 Å and 0.19 Å respectively) (Fig. 3.11 and Table 3.4). Distances between pairs of atoms that are involved in hydrogen bonds are presented in Table 3.3.

The absence of the hydroxyl group on the ring of the benzylhydroxamic acid resulted in the displacement of only three water molecules in its complex with MPO (Fig. 3.12). The water molecule that is present only in the MPO-BHA complex (W3) makes a hydrogen bond with W4 (Table 3.3, Fig. 3.12). Both these water molecules moved from their original positions in the native enzyme (Table 3.4) The hydrogen bonding pattern of the hydroxamic acid part of the molecule is similar with that seen in the MPO-SHA complex (Table 3.4 and Fig. 3.13).

Table 3.3: Distal cavity hydrogen bond distances (in Angstroms)

<table>
<thead>
<tr>
<th>native MPO</th>
<th>MPO-SHA complex</th>
<th>MPO-BHA complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>from to A B</td>
<td>A B</td>
</tr>
<tr>
<td>H95 Nε</td>
<td>W1 2.87 2.82 SHA-O8 2.61 2.61 BHA-O8 2.74 2.69</td>
<td></td>
</tr>
<tr>
<td>Q91 Nε</td>
<td>W2 3.15 3.10 SHA-O7 3.17 3.00 BHA-O7 3.07 3.11</td>
<td></td>
</tr>
<tr>
<td>R239 NH2</td>
<td>W3 2.99 2.92 SHA-O1 3.14 3.12 W3 3.05 2.86</td>
<td></td>
</tr>
<tr>
<td>W4</td>
<td>W3 2.89 2.90 SHA-O1 2.69 2.71 W3 2.67 2.72</td>
<td></td>
</tr>
<tr>
<td>HEM(O)</td>
<td>W4 2.58 2.61 W4 2.60 2.63 W4 2.60 2.71</td>
<td></td>
</tr>
</tbody>
</table>

Note: A and B refer to the crystallographically independent halves of the hMPO molecule HEM(O) is the carboxyl oxygen of pyrrole ring C propionate.
In each of the two complexes, apart from the perturbations shown in the distal cavity, two other water molecules near the hydroxamic acid aromatic ring appear to be moved (Table 3.4).

Table 3.4: Movement of water molecules (in Ångstroms) in the distal cavity and near hydroxamic acid aromatic ring upon ligand binding

<table>
<thead>
<tr>
<th></th>
<th>MPO-SHA complex</th>
<th>MPO-BHA complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>W3</td>
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<td>N/A</td>
</tr>
<tr>
<td>W4</td>
<td>0.14</td>
<td>0.21</td>
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<tr>
<td>W946</td>
<td>1.82</td>
<td>3.47</td>
</tr>
<tr>
<td>W947*</td>
<td>0.27</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Note: A and B refer to the crystallographically independent halves of the hMPO molecule
*the movement was not away from the aromatic ring

3.5.2 Conformations of the bound hydroxamic acid molecules and relative positions to the surrounding environment

The bound SHA and BHA molecules are almost planar (the angles between the plane of the aromatic ring and the plane of the hydroxamic acid part are $22^\circ$ and $15^\circ$ for SHA and of $-20^\circ$ and $-13^\circ$ for BHA in the two halves respectively). I used negative numbers to describe the angles for the BHA molecule to underline the fact that the relative rotation occurs in the opposite sense if one would start with a perfect planar molecule (no angle between the hydroxamic acid moiety plane and the benzene ring plane). The SHA aromatic ring is almost parallel to the heme plane (it forms a $7^\circ$ angle with the heme pyrrole ring D in both halves). In contrast, the angles between the aromatic ring of BHA and the plane of pyrrole ring D are much larger ($44^\circ$ and $40^\circ$ in the two halves respectively). The positions of the hydroxamic acid groups of the two molecules show a very close match when the two structures are superimposed.
Fig. 3.11: SHA mode of binding in the distal cavity of human MPO. The five water molecules present in the native enzyme (W1-W5) are shown superimposed in red. It can be seen that three of them (W1, W2 and W3) are replaced respectively by O8, O7 and O1 of SHA (shown in blue) as it was previously shown (Davey and Fenna, 1996). W4 is still present in the MPO-SHA complex, albeit slightly moved and W5 is displaced by the aromatic ring of SHA.
Fig. 3.12: BHA mode of binding in the distal cavity of human MPO with the five water molecules present in the native enzyme (W1-W5) shown superimposed in red. Due to the absence of the hydroxyl group attached to the aromatic ring, one more water molecule (W3) is still present when compared with the MPO-SHA complex (Fig. 3.11). Two of them (W1 and W2) are replaced respectively by O8 and O7 of BHA (shown in green) similar to SHA and W5 is displaced by the aromatic ring of BHA.
Fig. 3.13: Superposition of the binding of the two hydroxamic acids (SHA in blue and BHA in green) in the distal cavity of human MPO. A third superposition on the five water molecules (W1-W5) present in the native enzyme is also shown in red. Because W4 and W3 are present in more than one model, they are seen in triplicate and duplicate respectively. The difference in the positions of the aromatic rings and the similarity in the positions of the hydroxamic acid counterparts can also be noted.
3.5.3 Hydrogen bonding

The oxygen atoms of the hydroxamic acid groups are involved in hydrogen bonding with residues of the distal cavity, replacing almost perfectly the hydrogen bonding pattern of the displaced water molecules. In the MPO-SHA complex the O8 atom forms a hydrogen bond with Nε of atom of His95, replacing W1. When compared to the position of W1 in the native structure O8 appears closer to the Nε of His95 and farther from the heme iron, so coordination of O8 to the heme iron seems unlikely. O7, replacing W2, forms a hydrogen bond with Nε of Gln91. O1 forms a hydrogen bond with the remaining water molecule (W4) and probably with Nε of Arg239 since the distances between the two atoms appear smaller than the ones showed by the 2.3 Å resolution structure of MPO-SHA (average of the two halves of 3.13 Å for the current structure and 3.52 Å for the older structure). Likewise, the corresponding O8 and O7 of the BHA molecule are involved in the same hydrogen bonds as their SHA counterparts. O8 is also farther from the heme iron than the corresponding W1 in the native enzyme. Because of the lack of a hydroxyl group on the benzene ring of BHA there is room for the corresponding W3 that is not present in the MPO-SHA complex. This water molecule makes a hydrogen bond with W4 as in the native structure although the geometry is slightly reconfigured (Fig. 3.12, Tables 3.3 and 3.4).
CHAPTER 4
COMPLEXES WITH NITRITE

4.1 OVERVIEW

Nitrite (NO$_2^-$) (Fig.4.1) forms by oxidation of nitrogen monoxide (NO•), which is produced in cells by nitric oxide synthase. As is the case with the myeloperoxidase product HOCl, synthesis of NO• has beneficial effects for the organism during the inflammatory processes, but excessive production can lead to tissue injury (Moncada and Higgs, 1993).

Reaction of MPO with nitrite became a focus of study when it was shown that myeloperoxidase was implicated in nitration of tyrosine and tyrosyl residues in peptides and proteins (Eiserich et al., 1998). Further research suggested that nitrite can function as a substrate for myeloperoxidase, although it is a poor one at concentrations less than 100 µM (van Dalen et al., 2000). However, at physiological concentrations, tyrosine acting as a co-substrate can enhance the nitration reaction. The same study showed that by converting MPO to compound II, nitrite inhibits the chlorination activity of purified MPO.

Another study showed that NO$_2^-$ can form complexes with the parent enzyme and both Compound I and II (Burner et al., 2000). The dissociation constant of the native MPO-nitrite complex varied with pH and was found to be 2.3 mM at pH 7 and 31.3 µM at pH 5.
Many structures between peroxidases and nitrogen compounds have been solved through the years. Among them, I will mention the crystal structures between CCP and nitric oxide and between hMPO and cyanide.

The crystal structure between CCP and nitric oxide (Edwards and Kraut, 1988) revealed that NO• bound to the heme iron at the sixth coordination site via its nitrogen atom. The Fe-N-O angle was estimated to be $143^\circ$. The structure also showed perturbations near the proximal histidine, particularly a movement of its imidazole ring away of the indole ring of Trp191 and a slight movement (0.2 Å) of the iron into the heme plane. The perturbations in this complex were not limited to the proximal side of the heme, the well conserved distal arginine (Arg48 in this case) was also shown to move away from the ligand because of the ligand’s tilted configuration toward this residue.

The crystal structure of the hMPO-CN complex (Blair-Johnson et al., 2001) showed binding of CN in a bent configuration and the Fe-C-N angle was determined to be approximately $157^\circ$. This permits the nitrogen atom of the cyanide molecule to be involved in hydrogen bonds with two water molecules (W2 and W3) (Fig. 4.2) and the distal histidine. The cyanide carbon-heme iron distance was approximately 2.06 Å - less than the 3 Å distance from the displaced water molecule, W1 (Fig. 1.5) to the heme iron in the native enzyme. W2 was also slightly moved and consequently W5 shifted its position to preserve the hydrogen bond with W2. This complex is considered a good analogue of compound I as a low spin complex with a diatomic heme ligand. For this reason two ternary complexes (MPO-CN-Br and MPO-CN-SCN) were previously studied.
by X-ray crystallography in Dr Fenna’s laboratory and compared with the MPO-Br and MPO-SCN structures respectively.

![Resonance hybrid of nitrite. Each bond has a bond order of 1.5 and the oxygen atoms share the single negative charge.](image)

**Fig 4.1:** Resonance hybrid of nitrite. Each bond has a bond order of 1.5 and the oxygen atoms share the single negative charge.

![Diagram of the distal cavity environment of hMPO upon cyanide binding. The cyanide anion bound to the heme iron replacing W1 is shown as a linear diatomic compound (C-N). The positions of the remaining water molecules (W2-W5) are also shown. The diagram was prepared using the PDB entry 1D5L representing the 1.9 Å resolution structure of the myeloperoxidase-cyanide complex.](image)

**Fig 4.2:** Diagram of the distal cavity environment of hMPO upon cyanide binding. The cyanide anion bound to the heme iron replacing W1 is shown as a linear diatomic compound (C-N). The positions of the remaining water molecules (W2-W5) are also shown. The diagram was prepared using the PDB entry 1D5L representing the 1.9 Å resolution structure of the myeloperoxidase-cyanide complex.
There are two possible modes of nitrite binding to a heme protein: the nitro binding mode with the bond occurring between the heme metal center and the nitrite nitrogen, and the nitrito binding mode, in which one nitrite oxygen is involved in binding to the metal center (Hitchman and Rowbottom, 1982). In the majority of the complexes between heme proteins and nitrite, the nitrite is liganded to the heme metal center in the nitro binding (Williams et al., 1997; Crane et al., 1997; Wyllie and Scheidt, 2002). Copeland et al reported the first structure in which the nitrite was bound in the nitrito binding mode in 2006. An illustration of each of the two modes of binding based on crystal structures is shown in Figs 4.3 and 4.4.

Fig 4.3: Diagram showing the nitro binding mode of nitrite to the heme iron of the *Escherichia coli* sulfite reductase hemoprotein (SiRHP). The diagram was prepared using the PDB entry 3GEO representing the 2.1 Å resolution structure of SiRHP-nitrite complex.
During my studies I determined the crystal structure of the MPO-nitrite complex and investigated the binding of nitrite to the MPO-CN complex.
4.2 METHODS

Purification of human myeloperoxidase for these studies was described in chapter 3.

Crystals of native MPO were equilibrated for 24h with a solution of 50 mM ammonium sulfate, 2mM calcium chloride, 24% PEG 8K, 50 mM sodium acetate pH 5.1 and 50 mM sodium nitrite, in order to obtain crystals of the MPO-nitrite complex. To prepare crystals of the MPO-cyanide-nitrite complex, first cocrystallization of MPO and CN was performed under the same conditions used to obtain crystals of native enzyme, but with the addition of 2 mM sodium cyanide. The second step involved soaking these crystals in a solution having the following composition: 50 mM ammonium sulfate, 2mM calcium chloride, 24% PEG 8K, 50 mM sodium acetate pH 6, 50 mM sodium nitrite and 2mM sodium cyanide.

X-ray data collection was performed in the same manner as described in chapter 3. The total number of reflections, the number of unique reflections, completeness and other statistical data for the two complexes are shown in tables 4.1 and 4.2.
Table 4.1: Data collection and refinement statistics for the MPO-nitrite complex

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<th>(A) Data Collection*</th>
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<tbody>
<tr>
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*The numbers in parentheses are values for the highest resolution shell (1.86 Å –1.80 Å)

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<tr>
<td>angles (deg)</td>
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**The numbers in parentheses are values for the highest resolution shell (1.88 Å –1.80 Å)
### Table 4.2: Data collection and refinement statistics for the MPO-CN-nitrite complex

#### (A) Data Collection*

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<tr>
<td>( I/\sigma(I) )</td>
<td>29.95 (17.45)</td>
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</tbody>
</table>

*The numbers in parentheses are values for the highest resolution shell (1.86 Å – 1.80 Å)

#### (B) Refinement statistics**

<table>
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<th>Value</th>
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</thead>
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<tr>
<td>( R_{\text{free}} )</td>
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<td>angles (deg)</td>
<td>1.182</td>
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</tbody>
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**The numbers in parentheses are values for the highest resolution shell (1.88 Å – 1.80 Å)
4.3 SOLUTION AND REFINEMENT OF THE STRUCTURES

Frozen crystals of the MPO–nitrite and MPO–CN-nitrite complexes belonged to the \( P2_1 \) space group and were closely isomorphous with the native ones (\( a = 111.4, b = 63.6, c = 92.7, \beta = 97.3^\circ \) for MPO-nitrite complex and \( a = 111.3, b = 63.5, c = 92.4, \beta = 97.3^\circ \), for MPO-CN-nitrite complex). The 1.8 Å resolution native human MPO model (Fiedler et al., 2000) and the difference Fourier method were used to calculate difference maps for both complexes using \( F_o(MPO-nitrite) - F_c(MPO) \) or \( F_o(MPO-CN-nitrite) - F_c(MPO) \) respectively as coefficients and the native MPO phases. Seven water molecules in the distal cavity and close to it plus the chloride anion and the water molecule hydrogen bonded to it in the chloride-binding site (Fig. 1.6) were removed from the model before calculation of the native MPO structure factors. Models of nitrite and cyanide obtained from Hetero-compound Information Centre – Uppsala (Hic-Up) website were manually fitted to the observed densities. Water molecules were reinstated gradually during refinement according to the observed densities. The models were refined and visualized between cycles of refinement as described in Chapter 3.

4.4 RESULTS

4.4.1 MPO-nitrite complex

The preliminary difference Fourier map using \( F_o(MPO-nitrite) - F_c(MPO) \) showed elongated positive peaks consistent with a triatomic molecule in the distal cavity
at the position occupied in the native enzyme by W1 and at the position occupied by the chloride anion in the chloride binding site (Figs. 4.3 and 4.4). The distal cavity positive peak was in the vicinity of a positive peak near the iron. On the other side of the iron center (in the proximal cavity), a negative peak was observed. This combination of positive-negative peaks on the opposite sides of the iron usually suggests binding of the ligand to the iron and a concurrent movement of the iron into the heme plane (Blair-Johnson et al., 2001). Positive spherical peaks at the positions of the other water molecules that had been removed from the phasing model were also observed. After the nitrite and water molecules had been added to the model, refinement was carried out and the consequent residual maps did not show any significant features (Figs. 4.3 and 4.4).

The refined model showed a nitrite molecule in the distal cavity bound in the nitro-binding mode. The heme iron moved into the heme plane with an average distance between the two halves of 0.22 Å (it is impossible to accurately define the heme plane due to the bow-shaped configuration of the heme; this movement was measured relative to lines that connects the nitrogen atoms of pyrrole rings A and C or the pyrrole rings B and D: the calculated distance is an average of four measurements relative to the two lines in the two halves). The nitrogen atom is coordinated to the heme iron, the bond length being approximately 2.10 Å in both halves. Consequently the nitrogen is farther from Nε of the distal histidine than W1 in the native enzyme. The closest nitrite atom to Nε of His95 is O1 (within hydrogen bond distance) and this might be the only
interaction of O1 with the environment since it is positioned too far from any other atoms. Nitrite O2 replaces W1 in the hydrogen bond with W2, but seems not to be involved in a hydrogen bond with W3. However, it can make another hydrogen bond with Glu91 Nε replacing W2 in this interaction when compared to the native enzyme distal cavity hydrogen bond network (Fig. 1.5). All the important distances between atoms in the distal cavity are presented in Table 4.3 and the nitrite binding is depicted in figure 4.5.

At the chloride binding site, as suggested by the elongated positive density observed in the initial difference Fourier map, a nitrite molecule occupies the position of the bound chloride in the native enzyme. The water hydrogen bonded to the chloride ion in the native enzyme is situated in the same plane with the nitrite atoms (Fig 4.6). All the atoms in the nitrite ion are within hydrogen bonding distance from the atoms that serve as ligands for the chloride anion in the native enzyme (Table 4.4).
Fig. 4.5: Diagram showing the refined model of nitrite superimposed on the $F_{o(MPO-nitrite)} - F_{c(MPO)}$ difference Fourier map in the distal cavity. The positive and negative maps are countered at $+5\sigma$ (green) and $-5\sigma$ (red) respectively. Positive and negative densities flanking the heme iron were interpreted as movement of the iron into the heme plane. The nitrite atom that binds to the iron is the nitrogen. Positive densities are also shown for the water molecules that were initially removed.
Fig. 4.6: Diagram showing the refined model of nitrite superimposed on the $F_{o(MPO-nitrite)} - F_{c(MPO)}$ difference Fourier map at the chloride-binding site. The positive and negative maps are countered at +5σ (green) and -5σ (red) respectively (no negative density was seen in the region at this contour level). A spherical positive peak is also shown for the associated water molecule that was also removed in the initial model (designated “wat”).
### Table 4.3: Interactions in the distal cavity of the MPO-nitrite complex

<table>
<thead>
<tr>
<th>from</th>
<th>to</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₂⁻ [N]</td>
<td>Fe</td>
<td>2.10</td>
<td>2.09</td>
</tr>
<tr>
<td>NO₂⁻ [N]</td>
<td>H95 Nε</td>
<td>3.25</td>
<td>3.31</td>
</tr>
<tr>
<td>NO₂⁻ [O1]</td>
<td>H95 Nε</td>
<td>2.73</td>
<td>2.83</td>
</tr>
<tr>
<td>NO₂⁻ [O2]</td>
<td>H95 Nε</td>
<td>2.98</td>
<td>2.90</td>
</tr>
<tr>
<td>NO₂⁻ [O2]</td>
<td>Q91 Nε</td>
<td>2.97</td>
<td>2.92</td>
</tr>
<tr>
<td>NO₂⁻ [O2]</td>
<td>W2</td>
<td>2.55</td>
<td>2.46</td>
</tr>
<tr>
<td>NO₂⁻ [O2]</td>
<td>W3</td>
<td>3.63</td>
<td>3.48</td>
</tr>
<tr>
<td>W2</td>
<td>W5</td>
<td>3.25</td>
<td>3.34</td>
</tr>
<tr>
<td>W2</td>
<td>Q91 Nε</td>
<td>3.66</td>
<td>3.35</td>
</tr>
<tr>
<td>W3</td>
<td>R239 NH2</td>
<td>2.98</td>
<td>2.86</td>
</tr>
<tr>
<td>W3</td>
<td>W4</td>
<td>3.00</td>
<td>3.20</td>
</tr>
<tr>
<td>W4</td>
<td>HEM(O)</td>
<td>2.57</td>
<td>2.68</td>
</tr>
</tbody>
</table>

Note: A and B refer to the crystallographically independent halves of the hMPO molecule; HEM(O) is the carboxyl oxygen of pyrrole ring C propionate.

### Table 4.4: Interactions at the chloride-binding site of the MPO-nitrite complex

<table>
<thead>
<tr>
<th>from</th>
<th>to</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₂⁻ [O1]</td>
<td>wat</td>
<td>2.55</td>
<td>2.57</td>
</tr>
<tr>
<td>NO₂⁻ [O1]</td>
<td>W32 peptide N</td>
<td>3.28</td>
<td>3.30</td>
</tr>
<tr>
<td>NO₂⁻ [O2]</td>
<td>W32 peptide N</td>
<td>3.05</td>
<td>3.01</td>
</tr>
<tr>
<td>NO₂⁻ [O2]</td>
<td>V327 peptide N</td>
<td>2.99</td>
<td>3.06</td>
</tr>
<tr>
<td>NO₂⁻ [N]</td>
<td>V327 peptide N</td>
<td>3.11</td>
<td>3.12</td>
</tr>
</tbody>
</table>

Note: A and B refer to the crystallographically independent halves of the hMPO molecule.
Fig 4.7: Nitrite mode of binding in the distal cavity of human MPO. The nitrite nitrogen is coordinated to the heme iron, which moved into the heme plane to facilitate the interaction (The N-Fe distance is approximately 2.10 Å). One nitrite oxygen is hydrogen bonded to Nε of the distal histidine and the other interacts with Nε of Gln91 and W2.
Fig 4.8: Nitrite mode of binding at the chloride-binding site of human MPO. The nitrite oxygens atoms make the corresponding ligands of the replaced chloride anion as follows: O1 with the associated water molecule and O2 with peptide NH groups of Trp32 and Val327.

### 4.4.2 MPO-CN-nitrite complex

The preliminary difference Fourier map using $F_{o}^{\text{MPO-CN-nitrite}} - F_{c}^{\text{MPO}}$ showed positive-peaks consistent with only one new molecule besides the water molecules W2-W5. After separate rounds of refinement with a cyanide or nitrite modeled at the position of W1 the compound present in distal cavity proved to be cyanide (Fig. 4.7). No additional elongated positive density has been found in the
distal cavity that would have suggested the binding of nitrite to another position when cyanide is already bound. In the chloride-binding site an elongated positive density proved to be a nitrite anion bound at the position of the chloride anion as occurred in the MPO-nitrite complex.

Fig. 4.9: Diagram showing the refined model of the cyanide anion superimposed on the \( F_o(MPO-CN-nitrite) - F_c(MPO) \) difference Fourier map in the distal cavity. The positive and negative maps are countered at +4.5\( \sigma \) (green) and -5\( \sigma \) (red) respectively. Positive and negative densities flanking the heme iron were interpreted as movement of the iron into the heme plane. Positive densities are also shown for the water molecules that were initially removed. No nitrite molecule was present in the distal cavity.
Summary

In this thesis I have presented the structures of 4 complexes of human myeloperoxidase with ligands. Two of these structures involve hydroxamic acids: salicylhydroxamic acid and benzylhydroxamic acid. They are discussed side by side and I will highlight the differences and similarities in binding and their implications for the affinities of these ligands for MPO. The resolution at which these structures were determined permits a very good visualization of the distal cavity and comparison with the native state of the enzyme. A comparison will be made with the known structures of complexes of these two hydroxamic acids with other peroxidases.

The other part of this dissertation project focused on studying complexes with nitrite. Among the nitrogen compounds that were tried, I obtained good resolution structure data with two: the complex with nitrite and the complex with cyanide and nitrite. I will discuss the binding that occurred in each of the complexes and the implication of this binding for deciphering the catalytic mechanism of myeloperoxidase.

Complexes with hydroxamic acids

The 1.85 Å resolution structure of the MPO-SHA complex has confirmed the findings of the previous lower resolution structure solved in Dr. Fenna’s laboratory. As shown before, the SHA molecule displaced all but one water
molecule observed in the distal cavity in the 1.8 Å resolution structure of the native enzyme. The two crystallographically independent halves of hMPO showed roughly the same distal cavity architecture. The 1.85 Å x-ray crystal structure for the MPO-BHA complex revealed the same correspondence between the two halves and, when compared with the MPO-SHA complex structure, some similarities but also differences in the mode of binding in the distal cavity.

The similarities occur in the mode of binding of the hydroxamic acid moiety of each of the two molecules. There is no gross difference in the orientation of this moiety and in the mode of replacement of the water molecules that were present in the native enzyme structure. O8 and O7 of both compounds occupy very close positions to the ones formerly occupied by W1 and W2 respectively. It can be concluded that these oxygen atoms are involved in their respective complexes in hydrogen bonds with Nε of H95 and of Gln91. The closest atom to the heme iron for both ligands is O8 but the Fe-O8 distance for each is too large for a coordination bond (an average of 3.13 Å and 3.26 Å in the MPO-SHA and MPO-BHA complexes respectively).

The differences arise first from the fact that the SHA molecule has an extra hydroxyl group attached to the benzene ring. The oxygen of this hydroxyl group (O1) occupies the position of W3 in the native enzyme and makes a hydrogen bond with W4 and probably with an NH2 of Arg239. This hydroxyl group is involved also in an intramolecular hydrogen bond with the NH group of the hydroxamic acid. This internal hydrogen bond was suspected as one of the main causes for the nearly coplanar configuration of the SHA molecule in the
previously determined structure. In the analogous complex with MPO, the configuration of the BHA molecule (lacking this hydroxyl group and hence the internal hydrogen bond) has been one of the questions answered by this study. There were at least two pieces of information that made the answer to this question interesting: First, the crystal structure of Fe(III) (benzohydroxamate)$_3$ trihydrate (Lindner and Gottlicher, 1969) showed mobility about the C6-C7 bond (3 different angles were described with values of 9.4°, 20.7° and 39.1°). Second, a crystal structure of a complex between HRPC and ferulic acid - another compound with aromatic and aliphatic moieties - did not show strong density of the ferulic acid as proof for an unequivocal binding at a certain site, in a certain orientation or configuration (Henriksen et al., 1999). In the MPO-BHA structure the position, orientation and configuration of the ligand is unambiguous and consistent in the two halves. There is a nearly planar configuration of the molecule, although the very sharp angle between the hydroxamic acid moiety and the benzene ring is in a different sense when compared to the angle measured for the bound SHA molecule.

The mode of binding of the aromatic acid moieties of the two ligands differs significantly, although they occupy roughly the same hydrophobic pocket at the entrance in the distal cavity. The relative orientation to the heme plane in the MPO-SHA complex is nearly parallel. It is important to note that since the heme covalently bound to the apoprotein in MPO is bow shaped with the concavity facing the distal cavity, it is impossible to define a plane for it. However, for consistency, the orientation relative to the closest of the pyrrole rings of the
heme, ring D, to the aromatic rings of both hydroxamic acids was measured. In both halves of the molecule, the plane of the aromatic ring of SHA forms an angle of only 7° with the plane of pyrrole ring D. This allows a good stacking interaction between SHA aromatic ring and pyrrole ring D of the heme. In the MPO-BHA complex the plane of the aromatic ring of BHA forms an angle averaging 42° with the plane of pyrrole ring D. It is obvious that this orientation is not the most energetically favorable since it would not permit the same good stacking interaction between the aromatic ring and pyrrole ring D.

The reason for this very different orientation of the aromatic ring relative to the heme pyrrole ring D seems to be the presence of the extra water molecule in the MPO-BHA complex. SHA has at this position the oxygen of the hydroxyl group bound to the benzene ring. This allows for hydrogen bonds to be formed between this oxygen (O1) and W4 and also between O1 and the NH group of SHA (the internal hydrogen bond) and O1 and NH₂ of Arg239. Along with the hydrogen bonds made by the hydroxamic acid moiety with Nε of His95 and Nε of Gln91 these interactions seem to stabilize the binding of the SHA in the distal cavity and very likely explain the low $k_d$ for this complex. On the other hand, the lack of a hydroxyl group on the aromatic ring leaves enough space for the presence of W3 in the MPO-BHA complex. Not only does this difference prevent BHA from making some good hydrogen bonding interactions, but it also creates steric hindrance between W3 and the neighboring hydrophobic aromatic ring. The hydrogen bonds formed by the hydroxamic acid moiety of BHA seem unaffected by the repulsion of BHA by W3 but since rotation is not restricted
about the C6-C7 bond, the aromatic ring is repelled by W3, forcing it to rotate to an orientation less favorable for optimal interaction with the heme pyrolle ring D. To summarize, in the MPO-BHA complex: (1) W3 repels the BHA aromatic ring forcing it to rotate about the C6-C7 bond and to adopt a conformation that still keeps the hydroxamic acid moiety in an energetically favorable position and (2) The “tilted” orientation of the BHA aromatic ring relative to the heme pyrolle D prevents an energetically favorable stacking interaction between the two rings. It is highly likely that these two differences can explain the difference in affinity reported for the two complexes.

The same differences in affinity seem to persist among the mammalian peroxidases (Table 5.1). This is not surprising knowing the large degree of conservation of the residues in the distal cavity. Crystal structures for other mammalian peroxidase-hydroxamic complexes would probably confirm this finding. ARP is the only peroxidase among the plant, fungal, bacterial and archaeal peroxidase superfamily for which structures for both ARP-SHA and ARP-BHA complexes have been determined. Unlike the structures of the SHA and BHA complexes with MPO, there is no gross difference in the mode of binding to ARP. The hydroxamic acid moieties of both ligands form hydrogen bonds in a similar fashion and both aromatic rings are surrounded by hydrophobic residues at the entrance to the distal cavity and are nearly parallel with the heme ring (Itakura et al., 1997; Tsukamoto et al., 1999). Not surprisingly, the dissociation constants are in the same order of magnitude (Table 4.1). An explanation for the fact that these dissociation constants are on the order of
millimolar and not in the order of micromolar like the dissociation constant for the MPO-SHA complex would be the deeper situated SHA in the distal cavity for the MPO-SHA complex. A better comparison between the interactions in the distal cavity is prevented by the different arrangement of the residues that form the active site in the two enzymes.

Table 5.1 Dissociation constants \( (k_d, \text{M}) \) of peroxidase-hydroxamic acid complexes

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>SHA</th>
<th>BHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO</td>
<td>( 2 \times 10^{-6} ) (1)</td>
<td>( 5 \times 10^{-3} ) (1)</td>
</tr>
<tr>
<td>LPO</td>
<td>( 7 \times 10^{-5} ) (1)</td>
<td>( 3 \times 10^{-2} ) (1)</td>
</tr>
<tr>
<td>IPO</td>
<td>( 2 \times 10^{-6} ) (1)</td>
<td>( 3.2 \times 10^{-3} ) (2)</td>
</tr>
<tr>
<td>ARP</td>
<td>( 1.7 \times 10^{-3} ) (3)</td>
<td>( 2.9 \times 10^{-3} ) (3)</td>
</tr>
<tr>
<td>HRPC</td>
<td>( 7.7 \times 10^{-6} ) (4)</td>
<td>( 2.3 \times 10^{-6} ) (4)</td>
</tr>
<tr>
<td>APX</td>
<td>( 8 \times 10^{-6} ) (5)</td>
<td></td>
</tr>
</tbody>
</table>

MPO-myeloperoxidase; LPO-lactoperoxidase; IPO-intestinal peroxidase; ARP-\textit{Arthromyces ramosus} peroxidase; HRPC-horseradish peroxidase isoform C; APX-ascorbate peroxidase

\(^a\)All the dissociation constant values have been determined at pH 6 except the values for ARP-hydroxamic acid complexes which have been determined at pH 5.5 and the value for APX-SHA complex which has been determined at pH 8.3

(1) From Ikeda-Saito et al (1991)
(2) From Kimura and Yamazaki (1979)
(3) From Indiani et al (2003)
(4) From Aitken et al (2001)

For HRPC, only the structure of the complex with BHA has been determined. There are differences in the mode of binding of BHA when compared to the ARP-BHA structure: (1) O7 of BHA makes a hydrogen bond with N\( \eta \) of Arg38 in HRPC-BHA and with N\( \varepsilon \) of Arg52 in ARP-BHA. (2) The water molecule present in the distal cavity in the two complexes is hydrogen bonded to O7 of BHA in HRPC-BHA in and to O8 in ARP-BHA (Henriksen \textit{et al.}, 1998, Itakura \textit{et al.}, 1997). The dissociation constants of the HRPC-hydroxamic acid
Complexes are again in the same order of magnitude, but the values are this time on the order of micromolar. The large difference when compared with the $k_d$’s for the ARP-hydroxamic acid complexes have been speculated to be the result of extra hydrophobic contacts that occur in the HRPC complexes (Phe68, Phe179, Phe142) (Henriksen et al., 1998; Indiani et al., 2003).

The last member of this peroxidase superfamily for which a structure was determined with a hydroxamic acid is the recombinant soybean cytosolic ascorbate peroxidase (rsAPX). The structure of the rsAPX-SHA complex revealed some differences when compared with the ARP and HRPC complexes: (1) There is no hydrogen bond between SHA and distal histidine in APX-SHA (2) The interaction with the heme iron is not through a water molecule but through its own O8. (3) The proline carbonyl group is hydrogen bonded to the salicyl hydroxyl group (O1) and not to the hydroxamic NH group (Sharp et al., 2004). The dissociation constant is known only for the ARP-SHA complex and is on the order of micromolar (Table 4.1). It would be interesting to investigate how BHA would bind to APX and, if there is a difference in affinity when compared to SHA binding and how this would be reflected in the crystal structure of the APX-BHA complex.

Complexes with nitrite

It has been known that nitric oxide and nitrite interact with iron-containing proteins. EPR spectroscopy studies showed that nitrite and MPO form a typical low spin iron complex (Cooper and Odell, 1992). Kinetic studies performed in the
pH range 4-8 showed that the dissociation constant of the complex between nitrite and the native state of the enzyme increases by about 3 orders of magnitude over this range (Burner et al., 2000). This variation suggests that either a protonated form of a residue with an approximately $pK_a$ of 4.3 or $\text{HNO}_2$ should occur in the distal cavity. The residue with the $pK_a$ around 4.3 is believed to be the distal histidine (Bolscher and Wever, 1984; Ikeda-Saito, 1987). Since the $pK_a$ value of $\text{HNO}_2$ is 3.3 only 1.6% of the nitrite and about 15.6% of the distal histidine should be protonated at the pH of the structure determination reported here ($i.e.$ 5.1). However the observed binding of nitrite at full occupancy in the distal cavity is consistent with the reported dissociation constant at pH 5 (approximately 31.3 $\mu$M (Burner et al., 2000)) and the nitrite concentration in the soaking solution used for crystallization (50 mM).

Reports stating that nitrite binds to the heme iron and causes spectral changes typical of a low spin iron complex (Cooper and Odell, 1992; Burner et al., 2000) are in agreement with the nitrite mode of binding to the native MPO reported in this thesis. The crystal structure of the MPO-nitrite complex showed the nitrite nitrogen coordinated to the heme iron at a distance of approximately 2.10 Å. The iron movement into the heme plane facilitated this interaction. The two nitrite oxygens interact with the distal histidine and both W2 and Gln91 respectively.

Results on the MPO-CN-nitrite ternary complex, are also consistent with the proposed mechanism that nitrite is oxidized by Compound I in a single electron oxidation (Burner et al., 2000; van Dalen et al., 2000). It is very likely
that the site of two-electron oxidation is the one occupied by W2 in the native enzyme since this was the site at which known substrates in a two-electron oxidation reaction like bromide and thiocyanate have been shown to bind in their respective complexes with MPO. Since nitrite did not displace W2, the single electron oxidation of nitrite could take place in a manner similar to the single electron oxidation of aromatic alcohols and amines, although the exact site at which the single electron oxidation occurs has not been established by structural studies so far.

The rationale for x-ray crystallographic studies of a ternary complex of MPO-CN-nitrite was to investigate if there is a second site in the distal cavity at which nitrite would bind as a substrate since it had been shown that nitrite reacts with both compounds I and II and also that nitrite reversibly inhibits the chlorination activity of myeloperoxidase (Burner et al., 2000; van Dalen et al., 2000). The claim made by Burner et al. (2000) that the site at which nitrite can function as a substrate for compound I is the same site at which it binds to the native enzyme cannot be true since it is physically impossible to have both nitrite and the oxyferryl oxygen liganded to the heme iron at the same time. To explain the data of the two studies cited in this paragraph, which showed reduction of the compound I by nitrite one must search for a secondary site at which nitrite acts as a substrate. As in the ternary complexes of MPO-CN-SCN and MPO-CN-Br, cyanide coordinated to the heme iron would resemble the compound I as a low spin diatomic structure. Both SCN\(^-\) and Br\(^-\) showed binding in the distal cavity when cyanide was bound directly to the heme iron displacing W1. It is worth
mentioning that the MPO-Br and MPO-SCN complexes did not show binding near the heme iron (as was the case of the MPO-nitrite complex). Moreover, the crystal structure of the MPO-CN-nitrite complex was performed at pH 6, pH at which the nitrite is almost completely deprotonated. The distal histidine, whose protonation was shown to favor reactivity toward nitrite and halides, would be also mostly deprotonated creating electrostatic repulsion towards deprotonated nitrite. Burner et al (2000) also showed that oxidation of nitrite by MPO and hydrogen peroxide is also dependent on pH and there is minimal activity above pH 6. All these pieces of information could explain that, at pH 6, nitrite was not able to bind at a detectable occupancy at another site (which would be its substrate site) in the distal cavity, although it is possible that binding could occur at a lower pH.

Since in the ternary complex, nitrite showed to bind only at the chloride-binding site, one must ask if this could be the site in which nitrite could act as a substrate. There are strong arguments against this possibility. Hydroxamic acids that function as analogues of aromatic alcohols and amines as substrates for single electron oxidation bind only in the distal cavity. Since nitrite is also a substrate for single electron oxidation, it should also bind as a substrate in the distal cavity. Moreover, there have been no reports of the involvement of amino acid free radical intermediates in the catalytic mechanism of MPO, although there are two tryptophan residues in the vicinity.

The structure of the hMPO-nitrite complex is the first structure that showed a nitrogen atom coordinated directly to the heme iron center in a mammalian
peroxidase. The only other structure with a ligand that showed direct heme binding was the MPO-CN complex structure but the bond in that complex formed between the iron and the cyanide carbon. This structure is also the first that describes nitrite binding to a peroxidase.

Overall this study is another step in understanding the big picture of substrate and inhibitor binding for one of the most important of the heme peroxidases, human myeloperoxidase. Since this enzyme is implicated in so many processes in physiology and pathophysiology further studies including crystallographic ones are needed to understand its mechanisms of catalysis and inhibition.
REFERENCES


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Appendix: Structural Studies of Herpes Simplex Virus Type 1 UL8 Protein

Background information and significance

One of the most important processes in biological systems is duplex DNA replication. The molecular machinery responsible for it is the replisome, an assembly of macromolecules that can perform DNA replication with the rate and exactitude suitable for every biological system.

There are many similarities between the events that take place during DNA replication in different systems. These similarities are partly the result of the functions performed by various factors involved in the process. The process was investigated the most in prokaryotes (E. coli, phage T4 and phage T7) (Benkovic et al., 2001), but there is also a lot of information about replication in eukaryotes (C. cerevisiae mainly) as well (Lehman and Boehmer, 1999).

Some of these functions can be performed by a single subunit or by a complex of multiple subunits. Among the similar proteins distributed in various organisms, the following distinguish (reviewed by Benkovic et al., 2001):

1. The origin binding protein (DnaA in E. coli, ORC in C. cerevisiae) binds to the replication origin(s) and locally melts the DNA strands.

2. The replicative helicase (DnaB in E. coli, gp41 in phage T4, gp4 in phage T7) functions as a motor protein that translocates along single stranded DNA (ssDNA) while unwinding the double stranded DNA (dsDNA) in a process coupled to NTP hydrolysis.
3. The primase (DnaG in *E. coli*, gp61 in phage T4, gp5 in phage T7) catalyzes formation of RNA primers for DNA synthesis (because DNA polymerases can only elongate existing primers). Together with the replicative helicase it can form the primosome.

4. DNA polymerase (DNA pol III in *E. coli*, gp43 in phage T4, gp5 in phage T7) catalyzes addition of nucleotides at the growing fork.

5. DNA polymerase clamp (β subunit of DNA pol III in *E. coli*, gp45 in phage T4, *E. coli* thioredoxin in phage T7, PCNA in *C. cerevisiae*) tethers the core polymerase to DNA and therefore increases its processivity.

6. The helicase loading protein (DnaC in *E. coli*, gp59 in phage T4) has the function to recruit the helicase to the fork.

7. The single-stranded DNA-binding protein (SSB in *E. coli*, gp32 in phage T4, gp2.5 in phage T7, RP-A in *C. cerevisiae*) binds to unpaired single DNA strands preventing them from reannealing and involvement in unwanted secondary structures.

Other functions/proteins are also present in these systems including: a proofreading exonuclease, a DNA polymerase clamp loader, and a 5′-3′-exonuclease (Lehman and Boehmer, 1999).

An interesting model for studying replication that offers great insights into the way the process occurs in eukaryotes is the one provided by herpes simplex virus type 1 (HSV-1) replication.
HSV-1 is a member of the *Herspesviridae* family of viruses which also includes Herpes simplex virus type 2 (a very important human pathogen), cytomegalovirus, varicella zoster virus, Epstein-Barr virus, human herpes viruses 6, 7 and 8 (the last being associated with Kaposi’s sarcoma).

The HSV-1 is the most extensively studied virus among the ones in its family. It is a double stranded DNA virus with a genome of 152 kb, 75 open reading frames and three origins of replication. Three repeated regions a, b and c separate its genome into two unique regions $U_L$ (unique long) comprising 82 % and $U_S$ (unique short) comprising 18 % (Boehmer and Lehman, 1997).

HSV-1 genome encodes seven gene products essential for DNA replication: UL30 and UL42 form the highly processive heterodimeric DNA polymerase; UL5, UL8 and UL52 constitute a heterotrimeric helicase-primase; UL29 gene encodes for a single-stranded DNA-binding protein (SSB, also named ICP8); and UL9 is the origin binding protein (Boehmer and Lehman, 1997).

A lot of information regarding the protein-protein and protein-DNA interactions that are involved in the formation of the replication machinery is available and much is known about the functions of most of the components.

Replication starts with the recognition of the origins of replication by the UL9 protein. The N-terminal domain of UL9 binds to ssDNA and has helicase and ATPase activities, while the C-terminal domain recognizes and binds to the origin of replication (Boehmer and Lehman, 1997). Based on its ATPase and 3′-5′ helicase activities, it locally melts the duplex DNA at these sites (Boehmer *et al.*, 1993). It was found that upon interaction with ICP8 the UL9 mediated origin
unwinding is enhanced (Lee and Lehman, 1999). Once the origin is open the other replication proteins are able to assemble the replication fork.

The three subunit helicase-primase complex unwinds the DNA in a 5'-3' polarity and synthesizes the RNA primers that will be extended by the polymerase complex (Crute and Lehman, 1991).

Most of the information about the HSV-1 replication proteins comes from kinetic and biochemical studies. Structure-function studies are an alternative approach that would answer very important questions about the protein and DNA topology at the replication fork and its implication at the functional level.

My goal was to better understand the interactions between the proteins that participate in the HSV-1 replication at the atomic level and how these interactions are reflected in the roles played by these proteins. In order to do that I wanted to employ X-ray crystallographic studies. My main focus was the UL8 protein.

The helicase and the primase activities of the heterotrimeric helicase-primase complex are associated with the UL5 and UL52 subunits, respectively. The two subunits are codependent, but they do not require the presence of UL8 to perform their catalytic functions (Calder and Stow, 1990; Dodson and Lehman, 1991). UL8 protein, comprised of 750 amino acids and having a molecular mass of 79,921 Da (McGeoch et al., 1988), is of uncertain function. Being part of the helicase-primase complex, it was shown that it does not have any known catalytic or DNA binding activity (Dodson and Lehman, 1991; Parry et al., 1993).
UL8 has been reported to stimulate primer synthesis by the UL5/52 core enzyme (Tenney et al., 1994) and it is thought to increase their utilization by stabilizing the association between nascent oligoribonucleotides and template DNA (Sherman et al., 1992). It has been proposed that UL8 acts as a loading protein for the helicase-primase on ICP8-coated DNA templates (Tanguy Le Gac et al., 1996). It is also involved in interactions with UL30 (Marsden et al., 1997) and UL9. UL8 has also a very interesting role in the localization of UL52 at the nuclear level in cells infected with HSV (Marsden et al., 1996).

Due to its various interactions and observed and putative functions, UL8 seemed a very interesting protein to be studied by X-ray crystallography. I believed that by solving the atomic structure of UL8 and investigating which residues or domains are important for its function not only I would have obtained a lot of data that can confirm the information that was already present in literature, but also this would have pushed these studies farther to shed new light on how the replisome functions in HSV-1 or in other eukaryotic systems.

**Research proposal and experimental method**

In this research project, I wanted to use X-ray crystallography to determine the atomic structure of the UL8 protein. For that I needed highly purified protein solutions. In order to obtain such purified proteins I followed the following steps:

*Spodoptera frugiperda* Sf9 cells were infected with *Autographa californica* nuclear polyhedrosis virus recombinant for the HSV-1 UL8, gene. 48 hrs post-infection, cells were harvested, washed in phosphate-buffered saline, and frozen at -80°C.
Sf9 UL8 frozen cells were thawed on ice, resuspended and allowed to swell on ice. Protease inhibitors were added in order to prevent protein degradation. Then, cells were lysed with pestle B in a Dounce homogenizer. After extraction the debris was pelleted by ultracentrifugation. The next step involved solid ammonium sulfate fractionation. After resuspension the sample was applied to a hydroxyapatite column and the protein eluted with a linear gradient of ammonium sulfate. For further purification the protein was applied to an ion exchange column (Resource Q).

The purified protein was concentrated to 20-30 mg/ml using Centricon concentrators (Milipore Inc.) and then stored in small aliquots at -80°C.

**Preparation for crystallography**

Pure fractions were concentrated to different concentrations with change/no change of storage buffer (to 20mM HEPES, pH 7.5, 40 mM NaCl) using a Milipore Centricon concentrator or Centricon centrifugal filter devices (50,000 MWCO), according to manufacturer's manual. Crystal trials were set up using the vapor diffusion hanging drop method with 1ml solution in the reservoir and a drop was assembled usually with 2 μl of protein solution (at different concentrations) mixed with 2 μl of solution from the reservoir at 4°C or room temperature. Attempts were made to optimize the conditions that looked favorable for obtaining good crystals by variation of different parameters: precipitant concentration, protein concentration, pH, temperature, etc.


**Preliminary results**

**Protein expression and purification**

I have been successful in expressing and purifying the UL8 protein. 2 liters of Sf9 cells with a cell density of 2 million/ml were infected at a m.o.i. (multiplicity of infection) of 5 with *Autographa californica* nuclear polyhedrosis virus recombinant for the HSV-1 UL8. After 48 hrs the infected cells were centrifuged for 10 min., washed in phosphate-buffered saline, and frozen at -80\(^\circ\) C.

After lysis and protein extraction, addition of ammonium sulfate to a saturation of 25% precipitated UL8 protein (Fig. A.1).

![SDS-PAGE gel](image)

**Fig. A1**  SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining of ammonium sulfate cuts (1 – High molecular weight standard, 2 – Sf9 whole cell lysate, 3 – Sf9 whole cell lysate expressing UL8, 4 – 0% supernatant, 5- 25% pellet, 6 – 25% supernatant, 7 -35% pellet, 8 – 35% supernatant, 9 - 45% pellet, 10 – 45% supernatant).
The 25% ammonium sulfate pellet was resuspended and the sample was desalted. The desalted sample was loaded onto a 5-ml Econo-Pac HTP cartridge (Bio-Rad). Proteins were eluted with a 50-ml linear gradient of ammonium sulfate (0-390 M) in 50 mM imidazole HCl, pH 7.0, 1 mM dithiothreitol, 10% glycerol, and 50 mM NaCl and 1-ml fractions were collected (Fig. A.2 left). The desalted eluate was applied on a Resource Q column and proteins were eluted with a 10-ml linear gradient of NaCl (0.05-0.4 M) in 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10% glycerol, 0.1 mM EDTA, and 50 mM NaCl) and 0.3-ml fractions were collected (Fig. A.2 right).

Fig. A.2
Left: Elution profile on the hydroxyapatite column. 1-ml fractions were collected.
Right: Elution profile on Resource Q column. 0.3-ml fractions were collected

UV absorbance at 280 nm (blue)
Conductivity (red)
Crystal trials

The drops were set up usually combining 2 μl protein solution at various concentrations and 2 μl precipitant. The reservoirs contained 1 ml of precipitant solution.

The first trials were based on the conditions used for Crystal screen I (Hampton Research, CA. Most of the conditions showed precipitation. Some of them yielded crystals, but on a strong background of precipitation (Fig. A.3).

Further trials involved scanning conditions in the vicinity of the ones that yielded crystals, and attempts to decrease precipitation in the drops and to reduce the number of centers of nucleation. Seeding attempts were also carried out. So far, none of these trials yielded better crystals for X-ray diffraction experiments. However, crystals formed repetitively. It has been observed that there is a tendency for the UL8 protein to yield crystals at a pH of 6.5 to 7.6 and at a concentration of ammonium sulfate between 0.04 and 0.16 M.

Fig A.3 Pictures showing rod-like shaped protein crystals stained with Izit Crystal Dye (Hampton Research) and a large level of protein precipitation
The crystals were too small to examine by X-ray diffraction and, since repeated attempts to obtain larger crystals were unsuccessful, the project had to be abandoned.

REFERENCES


