

## Crystallographic studies of copper proteins at room temperature using the Weissenberg geometry.

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### Summary

Rapid and Accurate data collection offered by Weissenberg geometry and an off-line large image plate provides particular advantages for high resolution data collection of redox proteins at room temperatures. Here, some recent results, where this data collection strategy has proven important, are presented and discussed.

### 1. Introduction

Synchrotron radiation has revolutionised the X-ray crystallographic technique over the last twenty years (Johnson & Blundell 1999). Rapid progress in the SR sources from the first generation to the third generation sources have resulted in highly collimated X-ray beam of extreme brilliance making studies of large unit cells tractable on the one hand (Rossmann 1999 & Diprose et al. 1999) and on the other pushing the resolution limits of small to medium sized proteins to 'atomic' resolution. During the mid 80's to mid 90's, Weissenberg geometry with an off-line image plate system adopted at the Sakabe-Tara beamlines have offered particular advantage for rapid data collection at room temperature. In our case, the use of this unique facility has been very helpful as good quality data collection could be accomplished from only one crystal at room temperature. Room temperature data collection is necessary in many cases including when no suitable cryoprotectant is found. In addition, the mosaic spread, in general, is lower at temperatures where crystal is grown as often rapid freezing can lead to an increase in mosaic spread limiting the overall resolution as well as increasing the uncertainty in intensity estimation. However, with the increased brilliance of third generation SR sources, photoreduction is an increasingly serious problem and current speeds of Weissenberg systems with off-line image plate system is not sufficient. Future developments envisaged at the Sakabe-Tara project with a rapid on-line facility would be helpful in this regard (Sakabe et. al. 1997).

Metalloproteins form a large fraction (between a quarter and a third) of all known proteins. These contain metal ions either as a single atom or as part of a cluster and play a variety of life

sustaining roles in the bacterial, plant and animal kingdoms (Harrison, 1985). Some of the fundamental biological processes in which metalloproteins participate include electron storage and transfer, dioxygen binding, storage and activation, and substrate transport, catalysis and activation. Metalloproteins utilise the chemistry of metals to their advantage to perform varied biological functions with specification and control. The redox and ligand chemistry of metals is used to perform a wide variety of chemical reactions in the biosphere. Quite often, these chemical reactions are accompanied by only a small structural change around the metal atom. Thus, high quality data and resolution are essential for providing a structure-based mechanism. Despite much effort, the number of structures which are known at atomic resolution remain limited (see Hasnain & Hodgson 1999). For example, for copper proteins, there are a total of 165 structures known but only amicyanin (MW ~11.5kD) and plastocyanin (10.5kD), both cupredoxins have been determined to a resolution approaching atomic resolution, namely 1.3 and 1.33Å, respectively.

Here a brief review of our studies using the Photon Factory facilities is given. In all of these cases, crystals have either not survived the flash freezing treatment or have shown much increased mosaic spread upon freezing.

## **2. Nitrite Reductase**

Dissimilatory nitrite reductase (NiR) is a key enzyme in the anaerobic respiratory pathway of denitrifying bacteria where nitrate is sequentially reduced to the gaseous products NO, N<sub>2</sub>O or N<sub>2</sub>, leading to a significant loss of fixed nitrogen from the terrestrial environment (Payne, 1985). The NiR from *Alcaligenes xylosoxidans* (NCIMB 11015) (AxNiR) (Abraham *et al.*, 1993) belongs to the group of NiRs which utilise copper at the redox active centres. All copper NiRs isolated so far have a strong band near 600nm arising from a (Cys)S → Cu(II) charge transfer which is characteristic of a type 1 copper centre. The ratio of the intensity of this band to a second charge transfer absorption band at ~460nm determines whether a copper NiR is blue or green in colour. AxNiR belongs to the subset of copper NiRs which are blue in colour and are thought to have azurin as the electron transfer partner.

The trimeric structure of several of these NiRs have now been established by x-ray crystallography (Godden *et al.*, 1991), (Murphy *et al.*, 1995) and solution scattering (Grossmann *et al.*, 1993), (Dodd *et al.*, 1993). The green NiR AfNiR has been shown to be a trimers in solution by x-ray scattering (J.G. Grossmann, personal communication). Two blue NiRs, AxNiR (Grossmann *et al.*, 1993) and

*PaNiR* (J.G. Grossmann, personal communication) have also been shown to possess the trimeric structure by solution x-ray scattering.

### 2.1 The Structure:

Crystallography has confirmed the overall structure of *AxNiR* to be trimeric. The *AxNiR* monomer contains 336 residue and 2 copper atoms, one of which forms a type 1 copper site and the other a type 2 copper site. The type 1 copper is situated in domain I of the monomer, while the type 2 copper resides in the inter-monomer cleft. The two copper atoms are situated 12.6Å apart and are directly connected by residues His129 and Cys130. A second connection between the copper sites is by the polypeptide chain from His89 to His94.

#### 2.1.1. The Type 1 Copper Site:

The type 1 copper atom is ligated by the usual two histidine nitrogens (His89N<sup>?</sup> 1 and His139N<sup>?</sup> 1), one cysteine sulphur (Cys130S<sup>?</sup>) and one methionine sulphur (Met144S<sup>?</sup>) in a distorted trigonal planar geometry. The two histidines and cysteine form the strong planar ligands at distances of 1.9Å, 2.0Å and 2.0Å respectively, while the methionine sulphur forms a weaker axial ligand at 2.6Å. The second axial ligand found in some type 1 copper sites (Dodd *et al.*, 1993), (Baker, 1988), a carbonyl oxygen, is not present in this structure. A carbonyl (Pro88) is found in a position similar to that of a copper ligating carbonyl from a glycine in azurin (Gly45), but at 3.7Å is too distant to form a bond to the copper.

The copper ligand His139 is orientated such that the N<sup>?</sup> 2 nitrogen is exposed to the solvent at the bottom of a small depression in the protein surface. The copper lies ~4Å from the His139 N<sup>?</sup> 2 atom. Surrounding the depression containing the His139 residue is a flat area of protein surface, with the copper atom being ~6Å beneath.

#### 2.1.2. The Type 2 Copper Site:

The type 2 copper atom is ligated by three histidine N<sup>?</sup> 2 atoms and by a putative water molecule. The residues involved are His94 and His129 from one monomer and His300 from the adjacent monomer. The respective ligand distances are 2.1Å, 2.1Å and 2.0Å, with the water molecule 1.7Å from the copper atom. The copper atom is displaced by 0.8Å from the histidine plane in the direction of the water molecule. Two other residues of potential importance situated in the type 2 copper site are Asp92 and His249. Asp92 may form a hydrogen bond to the copper ligating water at a distance of 3.0Å. His249 will not interact with this water as the closest possible hydrogen donor (N<sup>?</sup> 2) is 4.4Å away.

The type 2 copper atom lies at the bottom of a cleft between the monomers, at a distance of  $\sim 12\text{\AA}$  from the protein exterior. The outer edge of the cleft is marked by residues Gln133 and Ala3193. The area between the copper site and the exterior forms a hydrophobic pocket.

### 2.2. Comparison with the Green NiRs:

The structure of  $A_x\text{NiR}$  is very similar to  $A_f\text{NiR}$  and  $A_c\text{NiR}$ . The root mean square deviation in the C $\alpha$  positions for residues 11 to 330 between native  $A_x\text{NiR}$  and  $A_f\text{NiR}$  and  $A_c\text{NiR}$  monomers is  $0.59\text{\AA}$  and  $0.57\text{\AA}$ , respectively; with the RMS deviation between the monomers of  $A_f\text{NiR}$  and  $A_c\text{NiR}$  being  $0.44\text{\AA}$ .

#### 2.2.1. Type 1 Copper Site:

A comparison of the type 1 copper site of  $A_x\text{NiR}$  with those of  $A_f\text{NiR}$  and  $A_c\text{NiR}$  show the copper atom to be closer to the strong ligand plane in the blue NiR,  $A_x\text{NiR}$ . In the case of native  $A_x\text{NiR}$  the copper is  $0.19\text{\AA}$  below the plane (i.e. towards the methionine ligand) and  $0.45\text{\AA}$  from the plane in the case of the nitrite soaked  $A_x\text{NiR}$ . This compares with distances of  $0.47\text{\AA}$  and  $0.56\text{\AA}$  for  $A_f\text{NiR}$  and  $A_c\text{NiR}$ , respectively.

The optical spectroscopic properties of type 1 copper sites have attracted much attention for structural studies. Proteins containing type 1 copper centres can exhibit either a blue or a green colour, depending on the ratio of their absorbance at  $\sim 450\text{nm}$  to that at  $\sim 600\text{nm}$ . It has been suggested that the difference between the "blue" copper proteins, with weak  $\sim 450\text{nm}$  absorbance, and the "green" copper proteins, with strong  $\sim 450\text{nm}$  absorbance, is likely to be owing to the strength of the fourth copper ligand. Adman *et al.* (Adman *et al.*, 1995) have recently speculated that the difference between the green and blue NiRs may be caused by differences in the copper to Met144 S $\gamma$  bond. Such a difference is not observed in either the native or nitrite soaked  $A_x\text{NiR}$  with respect to the  $A_f\text{NiR}$  and  $A_c\text{NiR}$ .

Comparison of the position of the copper atom with respect to the strong ligand plane shows distances of  $0.19\text{\AA}$  in the  $A_x\text{NiR}$  model. The displacement of the copper for  $A_f\text{NiR}$  and  $A_c\text{NiR}$  is  $0.47\text{\AA}$  and  $0.56\text{\AA}$ , respectively. [FRASER - Is this true with 2.1 $\text{\AA}$  structure?] The green colour of the Met121Glu mutant of azurin from *Pseudomonas aeruginosa* (Karlsson *et al.*, 1991); (Karlsson *et al.*, 1993); (Strange *et al.*, 1996) may also arise from a similar movement of the copper from the ligand plane. In fact, the crystal structure of Met121Glu mutant shows a displacement of copper of  $\sim 0.4\text{\AA}$  (Karlsson *et al.*, 1993). Furthermore, a Met121Glu mutant of azurin from *Alcaligenes*

*denitrificans* (Romero *et al.*, 1993), where the 450nm is observed with significant intensity ( $\epsilon \sim 1200 \text{ M}^{-1} \text{ cm}^{-1}$ ), also shows an out-of-plane displacement of copper by  $\sim 0.3 \text{ \AA}$ .

### **2.2.2. Type 2 Copper Site:**

Superpositions of the type 2 copper sites of *AfNiR* and *AcNiR* with the native *AxNiR* give RMS deviations of  $0.18 \text{ \AA}$  and  $0.17 \text{ \AA}$ , with the deviation between *AfNiR* and *AcNiR* being  $0.24 \text{ \AA}$ . The displacement of the copper atom above the plane of the three ligating histidine Ne2 atoms is  $0.78 \text{ \AA}$  for *AxNiR*. This compared with displacements of  $0.72 \text{ \AA}$  and  $0.75 \text{ \AA}$  for *AfNiR* and *AcNiR*, respectively. Therefore all three NIR type 2 copper sites are essentially the same.

We have undertaken studies of the reduced form, substrate-bound enzyme as well some mutants. These are being used to elucidate the structure-based mechanism of this important enzyme.

## **3. Azurin II:**

It has been suggested (Cotton & Wilkinson, 1988), (Williams, 1985), that the copper coordination environment in the blue copper electron transfer proteins is a compromise between the two preferred geometries of the redox states of the copper atom, namely Cu(I) and Cu(II). These distortions of the Cu coordination sphere is thought to result in an energised or entatic state of the Cu (Vallee & Williams, 1968), (Williams, 1995). Such an environment should result in a lowering of the activation energy between the two redox states allowing rapid electron transfer to take place. A study of the crystallographic structures of a fully oxidised and reduced form of AzII has been performed in order to compare the structures of the copper sites at the same high resolution of  $1.75 \text{ \AA}$ . The rapid data collection and the length of the crystal have allowed data collection to be undertaken from one crystal with each spot on the crystal exposed for a single image thus minimising photoreduction.

### **3.1. Comparison of Oxidised and Reduced Azurin II:**

The structures of oxidised and reduced AzII are very similar indeed. A superposition of all the protein atoms and copper atom gives an RMS fit of  $0.08 \text{ \AA}$ . Superposition of the water molecules in each model gives an RMS fit of  $0.17 \text{ \AA}$ . When superimposed on the  $1.9 \text{ \AA}$  structure of AzII, both oxidised and reduced models have an RMS fit of  $0.10 \text{ \AA}$ .

#### **3.1.1. The Copper Sites:**

Comparison of the copper sites of the oxidised and reduced models of AzII show there to be very little change in structure. The copper geometry consists of three strong planar ligands (namely His46N<sup>?</sup> 1, His117N<sup>?</sup> 1 and Cys112S<sup>?</sup> ) which are arranged in a distorted trigonal plane about the

copper atom. Two other atoms form bonds to the copper atom. These ligands are Gly45O and Met121S<sup>?</sup> which are in axial positions with respect to the trigonal plane. The transition from oxidised to reduced forms results in only small changes in the positions of the ligands. The ligand distances change very little being essentially the same in the two structures. The ligand distance changes are well within experimental errors. The His46N<sup>?</sup> 1 copper bond is shortened by 0.01Å in the reduced structure, with His117N<sup>?</sup> 1 and Cys112S<sup>?</sup> bond lengths increasing by 0.03Å and 0.02Å respectively. The bond length to Gly45O increases by 0.03Å, while the Met121S<sup>?</sup> distance remains unchanged. In both refinements the position of the copper atom is essentially in the plane, the deviation being 0.01Å. The only notable difference between the oxidised and reduced copper sites is the change in B-factor of the copper atom from 15.5Å<sup>2</sup> and 9.5Å<sup>2</sup>. The reduction in B-factor may partially be due to a slightly larger copper atom in the reduced state, copper (I), as compared to the oxidised state, copper (II).

These minimal changes are consistent with the requirements for fast electron transfer (Marcus & Sutin, 1985). Similar small changes have been observed between the oxidised and reduced forms of AzAD (Shepard *et al.*, 1990) which have been studied at 1.8Å and 1.9Å, respectively. These structures support the view that the protein structure surrounding the copper atom provides an environment which is optimised for the function of biological electron transfer. The rigidity of the AzII structure, with the copper site being constrained by hydrogen bonding and van der Waal interactions, allows little movement upon change in redox state of the copper atom. The copper atom is held in a geometry which is an intermediate between the trigonal planar favoured by copper (I) and the bipyramidal trigonal favoured by copper (II). Such a state is said to be entatic in which the copper atom is bound in an energised conformation (Vallee & Williams, 1968), (Williams, 1995). However, calculations of the energy states of other blue copper sites have revealed there to be little strain casting doubt upon the idea of the copper binding being entatic (Ryde *et al.*, 1996).

#### **4. Superoxide Dismutase**

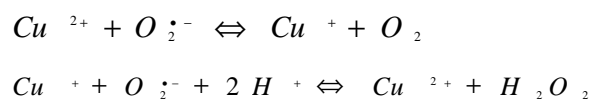
CuZn superoxide dismutases (CuZnSODs) are metalloenzymes which protect cells from oxygen toxicity by catalysing the dismutation of superoxide (O<sub>2</sub><sup>·-</sup>) into molecular oxygen and hydrogen peroxide (Fridovich, 1975). The enzymes are of clinical importance as therapeutic agents in pathological conditions related to oxidative stress such as ionizing radiation damage and lung and tissue damage (White *et al.*, 1991). Single site mutants of human CuZnSOD have been linked to the development of familial amyotrophic lateral sclerosis (Deng *et al.*, 1993). Thus there is considerable interest in understanding the structural basis of the catalytic mechanism of this enzyme.

#### **4.1. The structure**

The molecule is a homodimer of molecular weight 32kDa. The two subunits are related by an approximate two-fold axis along the dimeric interface. Each subunit folds into an eight stranded antiparallel beta barrel connected by three external loops. Each subunit contains one copper and one zinc ion separated by approximately 6Å, forming a dimetallic catalytic centre. The active site is located at the base of a shallow channel, some 10Å beneath the protein surface. A number of charged residues along the active site rim provide electrostatic guidance to the substrate.

#### **4.2. Catalytic mechanism**

The generally accepted mechanism for the dismutation of superoxide by CuZnSOD was proposed by Tainer (1982) and involved cyclic reduction and reoxidation of copper by successive molecules of superoxide (Tainer *et al.*, 1983).



The key step of this proposed mechanism is the formation of three coordinate copper in the reduced enzyme. EXAFS studies of reduced CuZnSOD in solution confirmed this (Blackburn *et al.*, 1985, Murphy *et al.*, 1997), but a crystallographic study on the same system suggested that copper was five coordinate in the reduced CuZnSOD (Rypniewski *et al.*, 1995).

#### **4.3. Crystal structures of bovine CuZnSOD**

A crystallographic study of bovine CuZnSOD at 1.65Å resolution (Hough & Hasnain, 1999) yielded a structure in which one subunit contained a three-coordinate Cu(I) site whilst the other contained a five coordinate, Cu(II) site. This was the first capture of both the oxidised and reduced stages of the catalytic mechanism in a single crystal structure. In order to further probe the observed asymmetry between the subunits a number of additional structures have been determined using data collected at beamline BL6A2 from similar crystals to the original study, but collected over a range of X-ray exposure times. We observed time dependent photoreduction of Cu(II) to Cu(I) selectively in one of the two subunits, indicated by a transition between five coordinate and three coordinate copper ligation. The fully Cu(II), intermediate mixed Cu(II)/Cu(I) and fully Cu(I) conformations were observed over increasing X-ray exposure times. Copper in the second subunit (subunit B) remained in a five coordinate Cu(II) conformation in all structures, regardless of X-ray exposure.

#### **4.4. The importance of mobility of the Glu119-Leu142 loop region for catalysis**

A clear asymmetry was found to be present between the temperature factors for the two subunits in the loop region between residues Glu119 and Leu142. This loop forms one side of the active site channel and contains residues involved in the electrostatic guidance of the substrate to the catalytic centre. In subunit A, which was observed to undergo photoreduction, this loop region is highly mobile and involved in few intermolecular contacts. In subunit B, which does not undergo photoreduction, this loop region has very low temperature factors and is heavily involved in crystal contacts between the subunits. We conclude that the mobility of the Glu119-Leu142 loop region may be essential for correct catalytic function of CuZn superoxide dismutases, and that this freedom is often not present in the crystalline form.



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