# Synchrotron techniques for metalloproteins and human disease in post genome era

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Metalloproteins make up some 30% of proteins in known genomes. Metalloproteins are a special class of proteins that utilise the unique properties of metal atoms in conjunction with the macromolecular assembly to perform life-sustaining processes. A number of metalloproteins are known to be involved in many disease states including ageing processes. The incorporation of the metal ion is a very tightly regulated process that, in vivo, very often requires specific chaperons to deliver and help incorporate the metal atom in the macromolecule. The lack of or inappropriate incorporation of metals along with genetic factors can lead to the mis-function of these proteins leading to disease. The mis-functions due to genetic alterations that lead to diseases like ALS (amyotrophic lateral sclerosis or motor neuron disease) and Creutzfeld Jacob disease (CJD) are now well recognised. Synchrotron Radiation Sources provide a unique set of structural tools, which in combination can prove extremely powerful in providing a comprehensive picture of these complex biological systems. In particular for metalloproteins, the combined use of X-ray Crystallography, X-ray Solution Scattering and X-ray Spectroscopy (XAFS) is extremely useful. We are currently engaged in a structural study where our aim is to characterize structurally and functionally metalloproteins and then transfer this knowledge to afford the problem of the mis-function of metalloproteins that lead to these terminal illnesses, either due to a gain of function/property or a loss of function/property. In this context, the benefits of adopting the 'philosophy' being developed for the structural genomics effort are highlighted.

## 1. Introduction

It is estimated that about 30% of proteins coded by genomes are metalloproteins which utilise the redox and ligand chemistry of metals to their advantage to perform varied biological functions with specificity and control. Quite often only very small structural changes around the metal atom occur during complex biochemical reactions. A classic example is that of haemoglobin, where a subtle change at the iron centre leads to major changes elsewhere in the protein structure and is responsible for the elegant allosteric mechanism. It perhaps is fair to claim that nowhere in the determination of molecular structure is precision more at a premium than in the case of metalloproteins. In this context, the combined use of high resolution X-ray crystallography and XAFS has proved extremely useful (Hasnain and Hodgson, 1999).

During that last 20-30 years SR techniques have played a major role in structure-function studies of a number of metalloproteins including membrane proteins. In particular, the success of SR X-ray Crystallography is noteworthy where increasingly higher and higher resolution structures are being determined (Yoshikawa et. al. , 2000; Schindelin et.al., 1997);(Messerschmidt et. al., 2001)). Several structures are now known at atomic resolution. Here we take the definition of atomic resolution as a resolution at which individual atoms become clearly resolved, typically 1.2Å (Sheldrick and Schneider 1997). With the increasing number of atomic resolution structures, the realization of the power of a combined techniques approach (XAFS and PX) is becoming more widely appreciated (Hasnain and Strange, 2003).

SR, in addition offers another very valuable technique, namely X-ray scattering (Stuhrmann, 1978;Svergun and Koch, 2002; Stuhrmann, 1981; Grossmann and Hasnain, 1997). For metalloproteins, X-ray scattering has been used to look at the structures of protein-protein complexes (Grossmann et. al. 1999), for elucidating low resolution structures in solution (Grossmann et. al., 1993) (and sometimes also comparing with crystal structure to validate observations in crystal structure, e.g. ligand induced conformational change found in transferrins (Grossmann et. al., 1998)) and for looking at the nature of folding/unfolding of proteins.

In this short article, an overview of the 'combined use of SR X-ray techniques for metalloproteins' will be given, illustrated by a number of examples from work conducted at the author's laboratory. It is beyond the scope of the current paper to review the field exhaustively but the interested reader is encouraged to examine the references given above.

## 2. Metalloproteins and atomic resolution structures

During the last quarter of the previous century X-ray crystallography and high field NMR have transformed biology by providing three dimensional structures of many important biological molecules and their complexes. Structure-Function studies have provided unique insights into the working of complex biological systems. Protein function in the cellular context depends ultimately on the atomic structure and dynamics of individual proteins and their complexes. Enzyme specificity and mechanism, catalysis and regulation are just a few examples where atomic structures are required to give insight into the detailed mechanisms by which cellular processes work and are regulated.

Even though an increasing number of structures are determined at atomic resolution (Dauter et. al., 1997), it still remains an exception. Thus, for example, there are some 250 structures of Cu proteins in the Protein Data Bank (PDB) but only four of these are at atomic resolution, figure 1 and these are all for fairly 'small' proteins. Similar situations exist for metalloproteins containing other metals. The recent atomic resolution structure for MoFe nitrogenase at 1.16Å is to our knowledge the largest metalloprotein (~200kD molecular weight) for which an atomic resolution structure has been determined (Einsle, et. al. 2002). This has not only provided the increased accuracy of the structure but has revealed new features of fundamental biological importance. Thus, a central atom, N, has been identified in the middle of six Fe atoms of the catalytically essential FeMo-cofactor. In previous structures this central atom was not observed as it was masked by the Fourier ripples of the surrounding Fe and S atoms. The electron density of this single N atom does not emerge until the resolution is extended beyond 1.55Å. The same 'Fourier ripples problem' from heavy metal atoms in metalloproteins undermines the accurate definition of metal-ligand bond distances to a level commensurate with the changes in bond length upon oxidation/reduction of these metal atoms.

The small number of atomic resolution structures is a reflection of difficulties in achieving this 'holy grail' for a variety of reasons. The technical advances in sources, monochromators and detectors as well as their stabilities will improve the situation in the coming years. However, our experience shows that crystal annealing is not fully exploited for this particular objective. In the case of four copper proteins on which we have worked recently, the resolution is significantly improved after annealing compared to the first image taken at room temperature. For copper nitrite reductase, where we have structures of several mutants, the crystals typically diffract to

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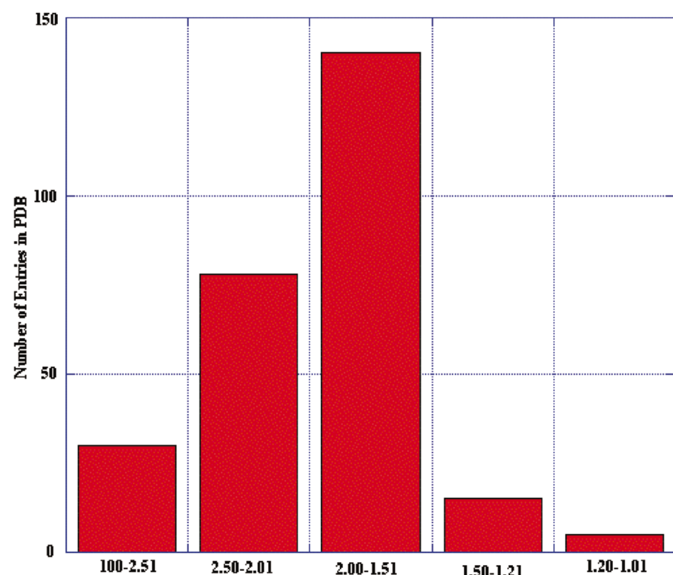


**Figure 1**

Distribution of copper proteins structures in the PDB. The atomic resolution shell includes our recent atomic resolution structures on Cu nitrite reductase and some of its mutants. It does not include our atomic resolution structure of bovine CuZnSOD as it is not yet in the PDB. Prior to this, the highest resolution structures were those of Amicyanin (PDB reference 1BXA, 2RAC both at 1.30Å resolution) and plastocyanin (PDB reference 1PLC at 1.33Å resolution).

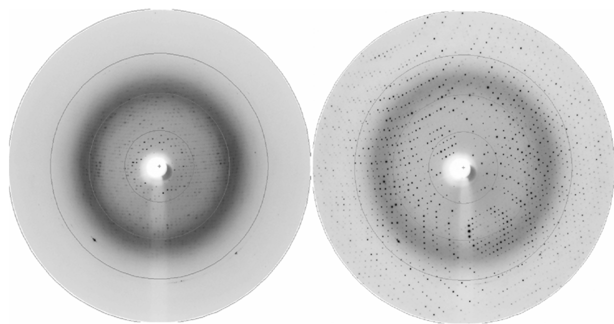

**Figure 2**

Diffraction pictures from the same crystal of Cu nitrite reductase, before and after *in-situ* 'cryo-annealing' of the flash frozen crystal. (pictures taken from Ellis et al. 2002).

1.9Å at room temperatures while annealing has yielded four structures at atomic resolution including that of the native enzyme which has been determined to 1.04Å resolution (Ellis et. al., 2003). Figure 2 shows the diffraction images of the same crystal demonstrating before and after annealing, the positive effect of the annealing method (Ellis, 2002).

## 3. Increased accuracy of metal-ligand distances at atomic resolution and use of XAFS metrical data

It is clear that significant effort is required in order to obtain 'atomic resolution' structures. Thus, in the case of metalloproteins, if one could combine the highly accurate information about the metal's neighbours from XAFS in the crystallographic refinement at more typical resolutions, then an improved picture of the metal sites can be provided. The recent development of 3D XAFS refinement (Binsted and Hasnain, 1996; Cheung et. al., 2000) has paved the way

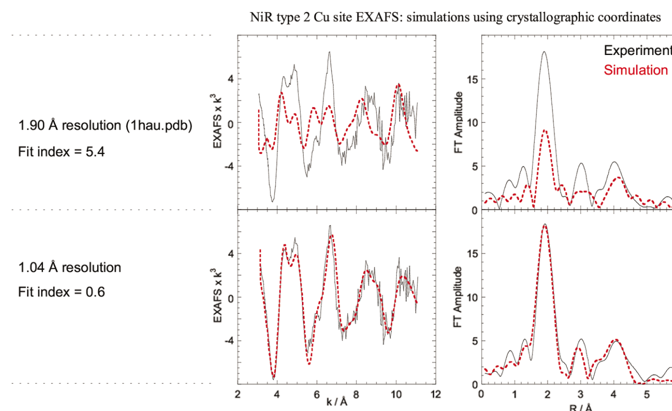


**Figure 3**

Simulation of XAFS data with the 3D XAFS refinement approach (Cheung, 2000) for the type 2 Cu centre of Cu nitrite reductase using the metal-coordinates information from the 1.9Å and 1.04Å crystallographic structures of the enzyme. The 'perfect' simulation of the XAFS data with 1.04Å structure is clearly evident (figure taken from Hasnian and Strange, 2003).

to accomplish this objective. Figure 3 demonstrates the accuracy of the metrical information at atomic resolution in the case of copper nitrite reductase. Here the 1.04Å structure coordinates have been used without any further refinement. The quality of XAFS simulation using crystallographic coordinates at this resolution is excellent and requires only minor adjustment to the parameters for the final simulation.

We thus recently decided to use the XAFS information for FeMo-cofactor in the crystallographic refinement of FeMo nitrogenase structure from kp[1] for which the structure has been determined to 1.65Å (Mayer et. al., 1999) This provided a substantial convergence between the XAFS and PX and is shown in table 1 (Strange et. al., 2003). It is noteworthy that the main difference takes place in the His N position and the homocitrate's O position. The His N moves by some 0.2Å while the O moves by 0.15Å. Rees and co-workers have recently determined the structure of Av Nitogenase at 1.16Å (Einsle et. al., 2002). A comparison with their crystallographic coordinates shows little difference between the XAFS-Restrained 1.65Å Mo structure and the 1.16Å atomic resolution structure thus demonstrating the validity of approach for using XAFS 3D metrical data with 'limited' resolution crystallographic data during their refinement. It clearly can provide an accuracy similar to that obtained at atomic resolutions for the immediate metal environment. Another aspect which is revealed by the atomic resolution structure is that a N atom is located at the centre of six Fe atoms thus providing direct evidence that N2 fixation does not actually take place at the Mo centre. We note that XAFS along with other studies had cast doubts on the direct involvement of Mo in binding as ligand/inhibitors bound enzyme had shown little or no difference. Rees et al have shown that the N atom in the atomic resolution structure is only discernable in the electron density when the resolution is improved beyond 1.55Å. This masking of N at lower resolutions results from the Fourier series ripples of the heavier atoms (Fe and S). We note that this is also the reason for metrical inaccuracies for light atoms. The heavier ligands such as S are already well defined at ~1.6Å.

The above examples are of significant and general interest. Inclusion of XAFS data should be considered in structure refinement

[1] Kp refers to *Klebsiella pneumoniae,* Av refers to *Azotobacter vinelandii.*

**Table 1**

(taken from J. Synchrotron Radiation 10, 197, 2003): Constrained refinements of the Mo K-edge EXAFS using 3D refinement approach. In the first refinement distances are kept at crystallographic values and Debye-Waller ($\sigma^2$) values are refined; in the second refinement the distances are also allowed to vary. A higher value than 0.03 $Å^2$ signifies that the atom is incorrectly placed and that little contribution to EXAFS signal is made. $\Delta R$ is the difference in Mo-ligand distance from the crystallographic value averaged over the two independent $\alpha\beta$ units. Last column includes information from 1.16Å structure (Einsle et. al. Science, 297, 1696-1700 (2002)).

| | First refinement | | Second refinement | | | |
|---|---|---|---|---|---|---|
| Ligand | R / Å | $\sigma^2$ / $Å^2$ | R / Å | $\sigma^2$ / $Å^2$ | ΔR / Å | 1.16 Å structure (Å) |
| | | | | | | |
| Mo-N (His) | 2.48 | 0.11 | 2.29 | 0.003 | -0.17 | 2.29 |
| Mo-O5 (homocitrate) | 2.29 | 0.11 | 2.14 | 0.001 | -0.16 | 2.18 |
| Mo-O7 (homocitrate) | 2.35 | 0.11 | 2.31 | 0.001 | -0.04 | 2.20 |
| | | | | | | |
| Mo-S1B | 2.30 | 0.001 | 2.30 | 0.001 | 0.0 | 2.33 |
| Mo-S3B | 2.38 | 0.001 | 2.37 | 0.001 | -0.01 | 2.37 |
| Mo-S4B | 2.35 | 0.001 | 2.35 | 0.001 | 0.00 | 2.33 |
| | | | | | | |
| Mo-Fe7 | 2.67 | 0.006 | 2.67 | 0.006 | 0.00 | 2.67 |
| Mo-Fe6 | 2.68 | 0.006 | 2.67 | 0.006 | -0.01 | 2.67 |
| Mo-Fe5 | 2.71 | 0.006 | 2.71 | 0.006 | 0.00 | 2.73 |
| | | | | | | |
| Mo-Fe2 | 5.04 | 0.01 | 5.04 | 0.01 | 0.00 | 5.04 |
| Mo-Fe3 | 5.06 | 0.01 | 5.06 | 0.01 | 0.00 | 5.05 |
| Mo-Fe4 | 5.09 | 0.01 | 5.08 | 0.01 | -0.01 | 5.10 |
| | | | | | | |
| Fit index | 12.4 | | 9.5 | | | |
| R-factor | 46 % | | 37 % | | | |

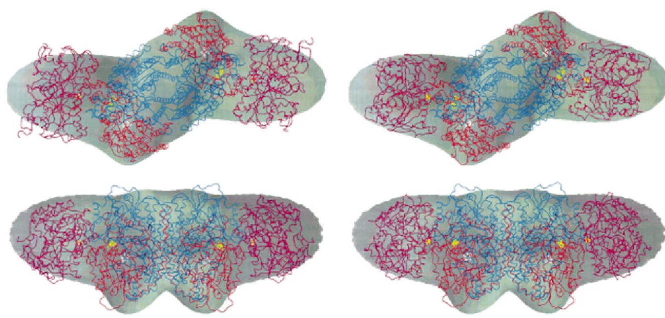

**Figure 4**

Low resolution model obtained from X-ray scattering data for Kp1•(ADP•$AlF_4^-$•Kp2)$_2$ complex, superimposed with the docking model using the crystallographic structures of the component proteins (top) and 3Å crystal structure of Av1•(ADP•$AlF_4^-$•Av2)$_2$ (figure taken from Grossmann et. al. 1999).

of metalloproteins where crystallographic data are not available to atomic resolution.

## 4. Protein-protein complexes and use of X-ray scattering

Proteins do not carry out a biological function in isolation but work in concert with other proteins and ligands to sustain life. It is increasingly recognized that structural studies of individual proteins are not sufficient if we are to solve important biological problems. From recent breakthroughs in high throughput technologies such as bioarrays, it is realized that a single protein may interact with as many as twenty other proteins. Increasing effort is underway to determine structures of protein-protein complexes with significant success. In this respect protein crystallography is on its own as larger and larger structures can only be determined if a stable crystallisable complex can be formed. However, here we would highlight the scope of the X-ray scattering technique for studying complexes at low resolution by means of an example taken from our own work.

Nitrogenase, the enzyme responsible for biological nitrogen fixation, catalyses the ATP-dependent reduction of dinitrogen to ammonia. The formation of a transient electron transfer complex between the MoFe protein (Kp1 or Av1) and the Fe protein (Kp2 or Av2) is an essential feature of the mechanism of nitrogenase. A low resolution (~15Å) structure of Kp1•(ADP•$AlF_4^-$•Kp2)$_2$ was determined (Grossmann et. al., 1997) using solution X-ray scattering data. On the basis of a docking model based on the structures of the individual protein it was clear from this low resolution model that the Fe-protein, Kp2, underwent a substantial conformational rearrangement in the complex with Kp1 remaining essentially unaltered. Subsequent to this study, the crystal structure of Av1•(ADP•$AlF_4^-$•Av2)$_2$ was determined at 3Å resolution (Schindelin et. al., 1997). A comparison of the 15Å solution scattering model and the 3Å crystal structure showed an excellent agreement, figure 4 (Grossmann et. al., 1999). While the crystal structure provided atomic details of interactions between the two partner proteins, the close agreement of the changes in the conformation of Fe protein argues for X-ray scattering as a powerful addition to the structural tool box, particularly in cases where crystallization is not achieved.

## 5. 'Structural genomics philosophy' applied to a copper protein involved in a neurodegenerative disease

There is an increasing body of evidence linking copper with several of the most debilitating major neurodegenerative diseases including motor neuron disease (MND) and Creutzfeldt-Jakob disease (CJD). It has therefore become urgent to understand the role of copper, copper proteins and their metabolism in these diseases. The tight regulation of copper metabolism by the nervous system implies that understanding this link is important for the regulation and control of the pathogenesis of these diseases. For several proteins it has been found that interaction with copper ions and other molecules is linked to a change in fold and that this mis-folding is perhaps the origin of the pathogenesis in some cases. In this section, we highlight our experience with some recent research on SOD1, the gene encoding copper, zinc superoxide dismutase (SOD), where rapid progress has been made by adopting the 'structural genomics' philosophy.

Motor neuron disease or amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterised by the progressive, selective loss of motor neurons in the spinal cord, brain stem and motor cortex (Deng et al., 1993). Some 10% of ALS cases are familial (FALS), and of these a subset of patients exhibit dominant autosomal mutations in SOD1 (Deng et. al. 1993). The sporadic (SALS) and FALS forms of the disease have very similar clinical and pathological features and it is likely that an understanding of the molecular basis of FALS is going to improve our understanding of SALS.

SOD is a critical component of the cellular defences against reactive oxygen species and catalyses the dismutation reaction of superoxide to hydrogen peroxide and oxygen via the cyclic reduction and reoxidation of copper (Fridovich, 1975). Much of what has been known in terms of this enzyme's mechanism has been based on the

**Table 2**

Details of some of the crystallographic structures of human SOD1 and FALS mutants. All of these data have been collected using the MPW 14, station 14.2 with 0.97Å X-rays except for H46R for which data were collected on 14.1 with 1.48Å X-rays.

| | P21 | P21 | C2 | C2 | C2221 | C2221 | P41212 | C2221 |
|---|---|---|---|---|---|---|---|---|
| **Name of the sample** | **Wild type** | **A4V** | **Wild type apo** | **H43R** | **L38V** | **Wild type 2** | **H46R** | **I113T** |
| **Resolution range (Å)** | **50.0-1.8 (1.86-1.8)** | **27 –1.9 (1.97-1.9)** | **50-1.82 (1.89-1.82)** | **50 -1.75 (1.81-1.75)** | **50-1.75 (1.81-1.75)** | **50.0 - 1.75 (1.81 -1.75)** | **50.0-2.15 (2.0-2.15)** | **50-1.6 (1.66-1.6)** |
| **Completeness (%)** | **98.6 (96.3)** | **96.6 (83.0)** | **96.1 (83.6)** | **99.1 (94.6)** | **98.4 (97.0)** | **96.6 (91.2)** | **99.3 (98.6)** | **99.6 (99.7)** |
| **R merge** | **5.2** | **6.3** | **6.1** | **7.1** | **6.5** | **7.8 (48.8)** | **11.2** | **5.1 (42)** |
| **<I>/σI last shell** | **2.7** | **2.3** | **2.8** | **3.0** | **2.0** | **2.0** | **2.0** | **3.0** |
| **Redundancy** | **3.4 (3.0)** | **9.0 (3.0)** | **3.5 (3.0)** | **3.7 (3.2)** | **8.0 (3.0)** | **11.0 (4.0)** | **6.0 (4.0)** | **10.0 (5.0)** |
| **Overall reflections** | **904897** | **2100427** | **176234** | **1128005** | **2031796** | **2844266** | **652196** | **3181893** |
| **Unique reflections** | **265996** | **237766** | **50360** | **306139** | **245194** | **244708** | **35679** | **317774** |
| **Wilson B-factor ($Å^2$)** | **26** | **24** | **26** | **19** | **23** | **23** | **35** | **20.5** |
| **Unit cell parameters** | | | | | | | | |
| **a** | **76.8** | **112.52** | **156.4** | **193.6** | **166.55** | **166.23** | **190.0** | **166.0** |
| **b** | **172.3** | **145.69** | **34.9** | **112.1** | **203.257** | **203.20** | **190.0** | **203.5** |
| **c** | **112.5** | **112.47** | **114.8** | **143.2** | **145.226** | **144.11** | **34.5** | **144.0** |
| | **90** | **90** | **90** | **90.0** | **90.0** | **90** | **90** | **90.0** |
| | **93.5** | **119.96** | **112.26** | **96.0** | **90.0** | **90** | **90** | **90.0** |
| | **90** | **90** | **90** | **90.0** | **90.0** | **90** | **90** | **90.0** |
| **Solvent (%)** | **55.8** | **70.0** | **45.4** | **59.0** | **68** | **68** | **68** | **68** |
| **Number of dimers** | **9** | **6** | **2** | **8** | **6** | **5** | **5** | **5** |
| **Final R cryst (%)** | **18.2** | **22.7** | **23.1** | **16.5** | **16.5** | **17.7** | **20.3** | **17.4** |
| **R-free cryst(%)** | **22.2** | **24.9** | **28.3** | **19.3** | **19.0** | **20.3** | **23.5** | **19.6** |
| **Average B factor ($Å^2$)** | **20.2** | **16.6** | **27.8** | **19.7** | **13.2** | **18.3** | **17.8** | **17.5** |
| **Number of atoms** | **21663** | **13368** | **4321** | **20130** | **12847** | **12527** | **3907** | **12472** |
| **Number of waters** | **1099** | **1230** | **417** | **2522** | **1298** | **1509** | **277** | **1464** |

structure/function studies of the bovine enzyme (Hough and Hasnain, 2003; Blackburn et. al., 1984; Hough and Hasnain, 1999; Hough et. al., 2000; Huttermann et. al., 1988; Tainer et. al., 1983; Murphy et. al., 1997) which has a sequence identitiy with the human counterpart of only 82%. Given this and the fact that many of the single point mutations (some 90) in the human enzyme cause FALS, it became an important goal to determine structure of the human enzyme to high resolution.

SOD1 knockout mice exhibit decreased fertility and recovery from axonal injury but do not develop motor neuron disease (Reaume et. al. 1996) Transgenic mice that over-express WT human SOD1 (in addition to WT mouse SOD1) are normal but those, which over-express FALS mutant SOD1 develop, motor neuron disease (Gurney et. al. 1994). This in conjunction with the finding that many mutant SODs have comparable SOD activity to that of the native indicates that loss of function is not the mechanism of toxicity, but the latter may arise from the gain of a new function or property by mutant SOD1.

Even though it is not clear how so many of the mutants cause FALS, it is clear that these mutations perturb a finely tuned system with terrible consequences in a manner similar to a poor ‘note’ in a well balanced ‘symphony’. It is our belief that the lack of structural results is a major handicap in understanding the well characterised familial form of this disease and requires a high throughput approach of structure determination which is linked to investigations of the cell biology and disease pathology. This concept has prompted the formation of ICOSA[2] (International Consortium On Superoxide Dismutase and Amyotrophic lateral sclerosis) bringing together two structural biology groups, one SOD enzymology group and a MND/SOD clinical group. Several other groups have joined as associates in this effort (see http://www.srs.dl.ac.uk/mbp). Progress on every aspect of the work is shared and information exchanged in an open atmosphere in a way very similar to structural genomics efforts.

Table 2 gives details of some of the structures which have been determined since the formation of ICOSA in early 2002. The progress is due to several reasons, many of these are similar to what is required in a structural genomics effort. To name a few:

Processing and characterisation of large number of mutants,

Availability of (1) to multiple laboratories and wide screening of these for crystallisation in multiple laboratories,

Information exchange of (1) and (2) to all so that an ‘open book’ culture can flourish, successful crystallisation conditions can be tried on other mutants and hence encourage further successes in crystallisation,

Regular meetings of all those involved in the work, 3rd ICOSA meeting is to take place on 4-5 July 2003 in Cheshire.

Large number of structures can be attempted in a high throughput manner so that general trends can be picked allowing, one hopes, the consortium to reach some general conclusions.

[2] The formation of ICOSA resulted from a discussion between the author and Joan Valentine at the first Gordon Conference on Oxidative damage in January 2001. The first ICOSA meeting took place at UCLA on 24-25 March 2002 and most of the results have been obtained since then.

Our results have so far have revealed the importance of understanding how these mutants behave in the absence of the metals, particularly zinc, and how this behaviour is different from that of the metal deficient human wild type enzyme. We have already seen evidence that the metal-deficient mutants can form linear and zig-zag filaments which have some ‘similarity’ to the common structure associated with amyloid-like fibrils. In the case of a Zn-loaded (but copper-deficient) mutant the protein molecules were arranged in a helical pattern, forming a hollow ‘nano-tube’. These crystallographic structures are combined with X-ray solution scattering and XAFS as well as many other biochemical and stability data and it is only through this rigour that ICOSA is likely to provide the breakthrough required for understanding the mechanism by which these mutants acquire their pathogenisity.

### 6. Future directions - structural proteomics of metalloproteins

It is clear that translating a genome sequence to large numbers of metalloproteins structures via a high throughput structure determination (Structural Genomics) approach is of urgent and strategic importance for healthcare as well as fundamental biology of this important class of proteins, which are estimated to make up about 30% of the genome. The above example, however, suggests that it is not sufficient to determine the structure of the native protein as the disease may result from a gain or loss of property/function which may cause it to mis-function. These altered properties in themselves cause the disease or their altered interactions with other proteins may be the cause of the disease. Thus, it is essential that a structure/function approach is adopted in a high throughput manner, i.e., the so-called an ‘integrated structural proteomics’ approach. This philosophy departs from the traditional concept of structural genomics in two important ways: one is that structures of well chosen medicinally important target proteins are carried out (even if proteins of similar folds may exist in the data base) and second that the structure is used to investigate the function and mis-function through a concerted effort of high throughput molecular biology (point mutations) ant high throughput structure determination of these derivative proteins and their complexes. This approach builds on the successes of the Structure-Function approach which has changed the face of biology over the last 30 years, but calls for the utilisation of the high throughput genomics approaches to understand the causes of ‘human diseases’ at the molecular level.

It is now well documented that incorporation of the metal ion is a very tightly regulated process that, in vivo, needs specific chaperons in many cases to deliver and help incorporate the metal atom in the macromolecule. This peculiarity of metalloproteins, however, has not been taken into account by many of the current world-wide structural genomics programmes in setting up high throughput expression system and as such these efforts may miss many of the important metalloproteins as well as isolate them without the native functional metal.

I would like to thank members of the molecular biophysics group, particularly Drs. Svetlana Antonyuk, Mark Ellis, Gunter Grossmann, Mike Hough and Richard Strange, as well as colleagues at the John Innes centre, Robert Eady, Barry Smith and Gary Sawers for long-standing collaboration. It is also my pleasure to acknowledge all of the members of the ICOSA formed in early 2002 for their contribution and intellectual engagement in the high throughput approach for understanding the structural basis of MND.

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