X-ray structural studies of some metalloproteins

S. Samar Hasnain

Molecular Biophysics Group, SERC Daresbury Laboratory, Warrington, Cheshire, WA4 4AD, U.K.

Abstract

Metalloproteins are an important class of proteins and perform a variety of biological processes. They are ideally suited for Synchrotron Radiation studies. In particular, X-ray region of the spectrum has been very effectively exploited not only for crystallographic structure determinations but X-ray absorption fine structure (XAFS) and more recently X-ray solution scattering techniques have also been used in providing unique information which have helped in improving our understanding of the structure/function relationships for several metalloproteins.

INTRODUCTION

The complementarity (ref. 1) of protein crystallographic (PX) and XAFS techniques in the study of the environments of metals in metalloproteins has been obvious since the first application of XAFS to the electron transport protein, rubredoxin (ref. 2). The PX technique provides a unique insight into the overall assembly of the protein which, in turn, may indicate possible functional mechanisms for such an assembly. Relatively few protein structures have been elucidated to high resolution (<1.7Å). Thus, details such as the local environments of metal atoms in metalloproteins are not as precisely defined as would be routinely expected for a "small molecule" crystallographic structure. However, such detail is essential for understanding the chemistry of these metal (functional) centres because it is the chemistry of these metals which are used by nature in tuning their effectiveness for a biological function. In contrast, with XAFS only the local environment of the metal site can be investigated due to the short mean free path of the X-ray generated photoelectron whose scattering gives rise to the structure of the XAFS data. The localised nature of this phenomenon means that structure of the metal site can be probed with an accuracy often approaching that routinely achieved in "small molecule" crystallography. An additional advantage is that XAFS data can be collected on both aqueous and crystalline proteins with same resolution. Thus, in addition to accurate definition of the metal site in the native protein, it is possible to study changes in the local chemistry upon a biochemical reaction. These changes are often quite subtle and well within the error limits of a crystallographic determination.

X-ray solution scattering has been extensively used for investigating assembly of macromolecules for some years (ref. 3). It has only been recently used for defining molecular conformation of metalloproteins in solution and has provided functional insight into the role of domain/lobe movements in transferrins (ref. 4-6). These studies have benefited from crystallographic structures of native or related proteins, demonstrating the complementarity of solution scattering and crystallography.

NITROGENASES

Biological nitrogen fixation, the reduction of N2 to NH3, is catalysed by the enzyme nitrogenase. Molybdenum has long been known to have an involvement in this process and Mo-containing nitrogenases have been isolated from a wide range of nitrogen-fixing organisms. In addition, two Mo-independent
nitrogenases have been recently identified. In the *Azotobacter vinelandii* (ref.7) system, there are strong similarities between the three nitrogenase systems. Each comprises a specific Fe-protein and a larger protein which contains Mo and Fe (MoFe-protein of Mo-nitrogenase); V and Fe (VFe-protein of V-nitrogenase) or only Fe in the case of the third nitrogenase. For the Mo- and V-nitrogenases, it has been shown that the role of the Fe-protein is that of a specific, ATP-dependent, electron donor to its partner protein. The ability of components of the three nitrogenases to form functional hybrid nitrogenases when recombined shows them to be closely related (ref. 8). The derived amino acid sequences also indicate a high degree of homology, particularly in residues which have been identified as potential ligands of the redox centres of the MoFe-proteins (i.e. FeMoco, an iron- and molybdenum-containing cofactor, which is the likely substrate binding site of Mo-nitrogenase and other, spectroscopically unusual FeS clusters, the 'P-centres') (ref. 9,10).

The involvement of Mo in a polynuclear cluster containing S and Fe was definitively demonstrated by the first EXAFS application to nitrogenase (ref. 11) as long ago as in 1978, which inspired several groups worldwide to synthesise chemical model compounds for FeMoco (For a review, see ref. 12). Mo K-edge XAFS studies also established the distorted octahedral geometry around the Mo centre, suggesting that Mo can not participate in substrate binding without a change in the coordination number or a change in the liganding groups. Furthermore, using the XAFS data at the Fe K-edge (ref. 13) the trigonal coordination of Fe was established in the FeMo-cofactor. This unusual character for Fe is central to both of the crystallographic models (ref. 14,15) where six core Fe atoms are modelled as three coordinate Fe. XAFS studies at the Fe K-edge also showed for the first time, the existence of an extended framework (Fe-Fe distance of 3.7Å) for FeMoco involving Mo-S(n)-Fe(m) and Fe-S(n)-Fe(m) units, a model recently observed by Rees (ref. 15) and Bolin (ref. 14) in their independent crystallographic structures of the MoFe-nitrogenase. The quality of agreement between the EXAFS model and the crystallographic models for the Mo-Fe-S cluster is excellent, e.g. our Fe K-edge EXAFS showed that Fe atom, on average, was surrounded by 3.0±0.3 S @ 2.20±0.02Å, 0.8±0.2 Mo @ 2.70±0.08Å and 2.2±0.4 and 1.3±0.2 Fe atoms @ 2.64±0.08Å and 3.68±0.37Å, respectively, while in the crystallographic model six Fe atoms have an average coordination of 2.9S, 0.4Mo and 0.3N (or O) with 3.4 and 1.7 Fe atoms located at ~2.7 and ~3.8Å. For the inner shell, crystallographic values of the interatomic distances are not not quoted by Rees or Bolin at this stage.

For the VFe-protein, we have shown (ref. 16), using XAFS, that V is in a very similar environment (~30 @ 2.15Å, ~3S @ 2.31Å and 3Fe @ 2.75Å) to that of Mo in Mo-nitrogenase (ref. 12) and also that the VFe-protein contains a vanadium-iron cofactor (FeVaco) analogous to FeMoco of the MoFe protein (ref. 17). The requirement of nifV and nifB (genes implicated in FeMoco biosynthesis) for the function of all three nitrogenases strongly suggests that the third nitrogenase contains a cofactor analogous to FeMoco and FeVaco but is thought to contain only Fe (ref. 8,18).

Preliminary X-ray crystallographic structure analysis at 5Å of the MoFe protein from *Clostridium pasteurianum* showed that the FeMoco centres are separated by 70Å from each other and are separated from the "P" centres by 17Å (ref. 14). This structure is now determined to 2.2Å resolution (Bolin et al. personal communication). The X-ray structure of the MoFe-protein of *A. vinelandii* has been reported at 2.7Å resolution (ref. 15) enabling the polypeptide ligands to FeMoco and the "P" centres to be identified and also providing structural models for these redox centres. In addition, the X-ray structure of the Fe-protein from *A. vinelandii* has been determined to a resolution of 2.9Å (ref. 19).

Our preliminary X-ray solution scattering investigation of the MoFe- and VFe-nitrogenases has shown substantial differences, not only in the overall size of the molecule but also in the subunit organisation. The crossover points observed in the scattering patterns, not only show the differences due to the extra subunit in the case of the VFe-protein (Ac1) but also the differences in the organisation of the protomers in the a2b2 structure. The crystal structure of the MoFe-protein is likely to help in achieving a quantitative interpretation of the X-ray scattering data and thus provide insight into the molecular organisation of these proteins in solution.
X-ray structural studies of some metalloproteins

The mechanism of biological electron transfer has long been a subject of intense interest (ref. 20,21). Many of the proteins involved in electron transfer reactions associated with soluble components of both aerobic and anaerobic energy-generating systems contain copper (ref. 22). The copper centres of these proteins play an essential role both in substrate activation and also as the redox centre of the electron donors or acceptors of these enzymes. Denitrifying bacteria utilize the high mid-point potential of the NO$_3^-$ / NO$_2^-$ couple to exploit nitrate as an electron acceptor under anaerobiosis for electron transfer reactions which generate a proton electro-chemical gradient across the cytoplasmic membrane. Nitrite, the product of nitrate reduction, is then further reduced by haem or copper containing nitrite reductases (depending upon the species), both types of enzyme utilize small blue copper proteins, e.g. azurins, as electron donors (ref. 23). The X-ray structure of several of these donors have been determined (ref. 22). The copper containing nitrite reductases have both type I and type II Cu centres, only one structure has been determined (ref. 24).

The three dimensional structure of several of the small blue copper proteins have shown that the coordination sphere around copper consists of two nitrogens (His-117 and His-46, numbering is that of azurin from Pseudomonas aeruginosa, AzP), two sulphur donors (Cys-112 and Met-121) and in the case of azurins, an oxygen (residue Gly-45), arranged in a distorted trigonal bipyramid. There is considerable interest in how the same basic framework, found for all structurally characterised blue proteins, is fine tuned to give redox potentials ranging from 184mV(St) to 680mV rusticyanin (Ru) from Thiobacillus Ferro-oxidans. In particular, the role of methionine (or another fourth ligand), has been extensively discussed in terms of fine tuning the redox potential (21,25). To directly address this, we have undertaken an investigation of the structure/function relationship through site mutations of methionine in AzP (ref. 26). We have used a variety of physical techniques which are able to look at the Cu centre directly, including XAFS, UV/Vis spectroscopy and electron paramagnetic resonance (EPR). Redox potential measurements have also been made. For the purpose of present discussion, results on two mutants are of particular interest, one where the methionine-121 amino acid residue has been replaced by an aspartate (Asp-121) and a second in which the amino acid chain has been terminated at residue 120, to give End-121. The XAFS and spectrochemical studies on azurin and these methionine mutants show that both the spectral and redox properties of the Cu(II) centre in azurin can be modified substantially by the single mutation. Even though, the methionine ligand is not essential for producing a blue copper site, the Cu centre responds positively to the ligand which occupies this place. In mutants where the mutated residue is short or when it is absent (as is the case for the end mutant) and is not able to reach Cu, an O, presumably from gly 45 moves closer in and thus stabilises the Cu(II) site. The presence of methionine, thus, may be seen as a constraint for Cu to adopt its ideal Cu (II) coordination and as such facilitate the redox role which Cu plays in these proteins.

Copper containing nitrite reductase (NiRs) have been characterised from a variety of bacteria and reports of their visible and E.P.R. spectra, subunit number and molecular weights (Mr) vary (ref. 27). The purified NiR from Achromobacter xylosoxidans (AxNiR) is blue in colour, and has been reported to contain only the Type 1 copper centre and 1.6 copper atoms in a dimer of Mr 70 kD (ref. 28). However, the native Mr of this enzyme has been reported (ref. 29) as 149 kD (sedimentation velocity centrifugation) compared with the 70 kD determined by gel filtration analysis. The NiR from Achromobacter cycloclastes (AcNiR) is reported as a green protein, of native Mr of 69 kD determined by gel filtration, with two identical subunits (37 kD) and containing 3 copper atoms/dimer (ref. 30). E.p.r spectroscopy has shown that it contains both Type 1 and Type 2 copper centres (ref. 31). Recently the X-ray structure of AcNiR has been solved (ref. 24), and shows surprisingly that crystals of this enzyme contained trimeric molecules. Since the degree of association in the crystalline state might be biased by crystal packing forces and crystallisation conditions (e.g. high sample concentration), we have applied to apply small angle X-ray scattering to AxNiR in order to obtain direct information about the overall shape of this molecule in solution (ref. 32). The X-ray scattering pattern collected on dilute solutions of 5 mg/ml and 10 mg/ml clearly shows the trimeric nature of the AxNiR enzyme at these concentration. In addition, a hydration layer surrounding the trimeric structure has been defined by combining molecular dynamics with X-ray scattering. This example demonstrates that the analysis of solution X-ray scattering experiments in combination with high resolution crystallographic data represents a powerful means for structure determination in solution.
IRON (METAL) TRANSPORT

Transferrins are a homologous family of glycoproteins with molecular weights of approximately 80,000 Dalton. They consist of two homologous lobes. Each lobe is made up of two domains containing a single high affinity metal binding site in the interdomain cleft. The N- and C-terminal binding sites are similar, but not identical. Both sites bind ferric iron as an octahedral high spin complex through the phenolic groups of two tyrosines, the imidazole group of a histidine, the carboxylic group of an aspartate and two oxygens of a (bi)carbonate anion (ref. 33,34).

Recent crystallographic structure determinations of diferric human lactoferrin (ref. 34) and rabbit serum transferrin (ref. 33) have provided a major insight into the structural organisation of these proteins. XAFS studies on iron and copper loaded transferrins and their lobes have provided detailed stereochemical information for both solution and freeze-dried proteins (ref. 35,36). PX and XAFS techniques have generally been in good agreement and have provided complementary and often supportive information (ref. 1,37). In addition to the metal-loaded proteins, the crystallographic structure of apo-lactoferrin has become available which shows a major conformational change in the N-lobe; domain NII appears to move substantially which allows the inter-domain cleft to open. In contrast, no conformational change appears to take place in the C-lobe of the apo-lactoferrin crystal structure (ref. 38).

We have used solution X-ray scattering technique for studying the structural changes which may take place upon uptake and release of iron from serum and chicken ovo-(COT) transferrins and human lactoferrin in solution. We have also used the isolated N- and C-lobes of the COT to provide information about conformational changes of the individual lobes. These studies reveal that both lobes undergo a change which is consistent with an opening of the inter-domain cleft when the iron is removed from the protein. The opening in the C-lobe is similar to that observed in the N-lobe of apo-lactoferrin crystal structure. The simulation of scattering data, using the analysis programme, DALAI (see article of Pantos & Bordas, this volume), over the medium angle range provides an accurate estimation of the opening of the lobes when iron is removed (ref. 4). These results set forth the importance of studying conformational changes of metalloproteins directly in solution, as the crystal packing forces and crystallisation conditions may artificially stabilize a particular conformation of the molecule, as would appear to be the case for the X-ray structure of apo-lactoferrin.

The mechanism of transferrin binding to the receptor as well as the effect of the receptor on iron release from transferrin is not yet well understood. However, it is known that the affinity of the apo-protein for the receptor is low and that receptor preferentially binds when iron is incorporated in both lobes. Thus, it is likely that the conformational change of the protein increases the specificity of receptor binding and thus facilitates the incorporation of iron into the iron-requiring cells. Further support to this idea is obtained from solution X-ray scattering results on transferrins loaded with a variety (Cu, In, Al & Hf) of non-physiological metals (ref. 5). Some of the metals, e.g., Hf, do not induce conformational changes in a manner similar to iron.

These experiments have led us to suggest that the "correct" co-ordination of metal ion is crucial for inducing the closed conformation and that the closed conformation is likely to be of functional importance for recognition by the transferrin receptor. Fourier of the iron ligands are provided by the protein, only one of these, Asp, originates strictly from domain I. An examination of the crystal structure and hydrogen bonding network suggests that this particular ligand may play an important role in inducing the conformational change upon metal binding. Recently, it has become possible to express mutants of the amino-terminal half-molecule of human serum transferrin (hTf/2N), a fragment of 337 residues incorporating a single iron binding site (ref. 39). We have examined two mutants, Asp63 -> Ser63 (D63S) and Asp63 -> Cys63 (D63C) by X-ray solution scattering in both the apo- and holo- forms (ref. 6). In the case of D63C, the cysteine residue is blocked by a disulphide bond formation and thus is not available for ligation to iron. Consequently, the lobe is expected to remain open. In the case of D63S, the mutation to an uncharged residue would weaken the strength of hydrogen bonds between the two domains and would probably reduce the closure of the interdomain cleft. Indeed, X-ray scattering curves (holo- vs apo-) for the D63C mutant are almost
indistinguishable, thus indicating no structural change upon binding of iron showing that with the non-availability of this ligand for iron binding, the trigger mechanism for domain closure is completely switched off. The scattering curves for the D63S mutant show a small difference only in the low angle scattering range suggesting only a small closure of the molecule upon iron binding compared to the wild type. Although the serine side chain is of only a marginally different length, the substitution obviously results in a major perturbation at the iron binding site, resulting in an "inhibition" of the domain closure. In this case, the negatively charged Asp is replaced by an uncharged polar group. The energy contribution (ref. 40) for a hydrogen bond to a charged residue is three fold more than the contribution due to an uncharged residue. The hydrogen bonds in this mutant may therefore not be sufficiently strong to tighten the metal binding cavity and to bring the two domains closer together when iron is bound.

CONCLUSION

The above examples clearly show the complementarity of the three techniques for studying structure/function relationships of metalloproteins. Now that it is becoming possible to carry out time resolved XAFS studies via Quick EXAFS (ref. 41), the combination of these techniques is likely to receive further impetus in the coming years.

Acknowledgements

I would like to thank a number of colleagues & collaborators, particularly G. Grossmann, L. Murphy, R. Strange, M. Neu, P. Lindley, R. R. Eady, Z. Abraham, B. Smith, E. T. Adman, R. W. Evans, B. Reinhammar, G. Karlsson, H. Appel, N. Binsted, A. Mason and R. Woodworth. I would also like to thank the SERC and the Daresbury Laboratory for provision of resources.

REFERENCES

20. F. A Armstrong; H. A. O. Hill, and N.J.Walton, Quarterly Rev. of Biophys. 18, 261(1986).