Iron and copper *K*-edge XAS study of serotransferrin and ovotransferrin

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The active metal site structure of transferrin with iron and copper atoms is investigated using metal *K*-XANES. Theoretical analysis of experimental data has been performed on the basis of full multiple-scattering theory. This approach made it possible to study the origin of XANES fine details and to investigate the local structure around active metal sites. A deep insight into the local structure and electronic subsystem of Fe, Cu transferrins is obtained. For example, in the case of Cu substitution of Fe in the active centre, the best fit of theoretical spectra to experiment has been obtained for distances 3% smaller between the Cu atom and the nearest neighbours.

Keywords: transferrin; metalloproteins; active site structure; XANES.

1. Introduction

Transferrin constitutes a homogeneous group of glycoproteins widely distributed in the extra-cellular fluids of vertebrates. This protein has the following physicochemical and biological properties: it is constituted of a single polypeptide chain of molecular weight ~80 kDa organized in two similar, but not identical, iron-binding sites disposed in the N- and C-terminal halves of the molecule. The transferrin binds two Fe^{3+} ions and the binding depends on the concomitant binding of carbonate (or bicarbonate) synergistic anions that form a bridge between the metal and the protein.

In this work we have studied two members of the transferrin family: the human serotransferrin from blood plasma and the hen ovotransferrin from avian egg white. These proteins also specifically bind a variety of other metal ions present in the blood. However, the transferrin mainly transports iron because of the high abundance of iron in relation to other metal ions.

Previous X-ray crystallographic studies of serotransferrin and ovotransferrin (Macgillivray *et al.*, 1998) have determined the three-dimensional structure of the protein and their spatial conformation of the iron-binding site. On the basis of the results a model has been proposed in which the iron is hexacoordinated to 2 Tyr, 1 His and 1 Asp residue, and (bi)carbonate linked to an Arg residue. An EXAFS study on serotransferrin fragments (Garrat *et al.*, 1986) with iron ions provides the distance between the ion and its ligands with an error of 0.02 Å. A SAXS study (Grossmann *et al.*, 1998) of transferrin with metal ions shows a 'closed–open' change of the structure of the ligands around the metal ion sites, dependent on the nature of the ion. This conformation change could not depend on the nature of the ion but on the pH (Congiu-Castellano *et al.*, 1997). Studies of ovotransferrin with copper (Garrat *et al.*, 1991; Smith *et al.*, 1992; Baker *et al.*, 1991) show a different organization of the ligands around the ion for two sites. Fe *K*-pre-edge analysis (Roe *et al.*, 1984) shows that the coordination number of the active metal site in ovotransferrin is between six and seven.

Synchrotron radiation sources provide very intense continuum radiation that allows the development of X-ray absorption spectroscopy of metalloproteins. In particular, the X-ray absorption near-edge structure (XANES) spectrum is determined by multiple-scattering processes of excited inner-shell photoelectrons with the neighboring atoms (Durham, 1988). As a consequence, the spectrum contains electronic and structural information, such as the valence state of the photoabsorbing ion or the overall symmetry around it. We also underline that detailed understanding is feasible by XANES only if the nature and the position of ligands are well defined by other methods, such as EXAFS, EPR *etc*. It is also important that XANES profiles are compared within a family of related compounds. In the present study, we have concentrated on the XANES region; work on EXAFS analysis is in progress.

Here we have performed a comparison of theoretical and experimental XANES spectra at the *K* edge of iron and copper in the N-terminal of serotransferrin and ovotransferrin.

2. Materials and methods

Human serum transferrin and hen ovotransferrin (Sigma, St Louis, MO, USA) (97% stated purity) were used without further purification. Monocupric and dicupric forms of the proteins in solution were prepared following procedures described elsewhere (Harris, 1986) and freeze-dried prior to use.

It is well known that XAFS spectra of transferrin in freeze-drying, solution and crystal forms differ slightly from one another. The use of powder samples allows the protein damage related to radiolysis under X-ray exposition to be limited. Because there are no data on the local structure of transferrin in solution, we used Protein Data Bank (PDB) data for the crystal form of transferrin.

The Fe, Cu *K*-edge X-ray fluorescence spectra of powder samples were collected at the LURE synchrotron facility. Si(111) and Si(311) channel-cut single crystals were used as the monochromator; the energy resolution at the Cu *K* edge was ~3 eV, and a 0.5 eV energy shift of resolved absorption peaks could be detected. Each spectrum represents a total of I_f/I_0 (I_f = fluorescence count and I_0 = photon incident flux measured by a proportional counter) signal averaging of 10 s per point collected at room temperature by using a seven-element energy-resolving Ge detector from Canberra Industries (Cramer, 1988).

3. Results and discussion

The XANES spectra show three main structures: *a* in the pre-edge, *b* and *c* near the absorption edge. The first weak absorption (*a*) is associated with the $1s \rightarrow 3d$ transition; there are two possible sources for this peak: quadrupole transitions or a $3d$–$4p$ mixing due to vibronic interactions. It was estimated that the quadrupole transitions are three orders of magnitude weaker than those observed. This leaves the even–odd orbital mixing due to vibronic interactions as the most probable origin of peak *a*. The intense absorption above 7120 eV was identified as a dipole $1s \rightarrow 4p$ transition.

The algorithm of the full multiple-scattering method that we used in this study has been described earlier (Della Longa *et al.*, 1995). We have chosen the transferrin structure from the PDB (PDB entry 1a8e; Macgillivray *et al.*, 1998). Atomic charge densities were obtained using the self-consistent Dirac–Slater method. Phase shifts were calculated for molecule muffin-tin (MT) potential with touching MT spheres.

XANES above the metal *K* edge of metalloproteins is generated by multiple scattering of the excited photoelectron within a cluster (*i.e.* a group of atoms around a metal ion of the active centre) consisting of a few shells of atoms. The first step in the multiple-scattering analysis of XAFS data is to determine the size of a representative cluster of neighbour atoms around the absorbing Fe atom which will reproduce fully all of the fine structure of the spectrum. The analysis of the dependence of the main structures in XAFS spectra on cluster size shows that the convergence with cluster size is reached just for a cluster of three shells. Therefore, all the following XAFS calculations were performed using this cluster size.

In order to perform a direct comparison with experimental data one must take into account the broadening of the spectra according to three factors: the core-hole lifetime, the finite mean free path of the photoelectron and the experimental resolution. For the Fe *K* core-hole bandwidth the value of 1.25 eV (Fuggle & Inglesfield, 1992) has been used. The mean free path of the photoelectron function has been taken from Muller *et al.* (1982). For the experimental energy resolution, a value of 3 eV has been used. All of these factors have been treated as contributing to the imaginary part of the self-energy term used in the calculation.

In Fig. 1 a comparison of experimental and theoretical Fe *K*-edge XANES of mono (N) Fe-transferrin is presented. We have analyzed several models of transferrin local structure from the PDB, and the result obtained using the 1a8e (Macgillivray *et al*., 1998) data is the best (Varoli-Piazza *et al.*, 2000). As one can see, the agreement of the theoretical results with experimental data is good enough. Thus, one can suppose the local structure around the Fe active site in mono (N) Fe-Tf obtained by Macgillivray *et al.* (1998; PDB entry 1a8e) to be quite realistic.

In the case of transferrin with substituted metal ions, *e.g.* mono (N) Cu-transferrin, the situation is more complicated. There are no structural data available in the literature or in the PDB, except radial distributions given by EXAFS (Garrat *et al.*, 1986, 1991). As a first approximation we suggested the structure around the Cu ion in transferrin to be close to the structure of Fe-Tf. Then we tried to vary the local structure parameters (coordinates of the first-shell atoms) assuming the same symmetry of the shell. The best results were

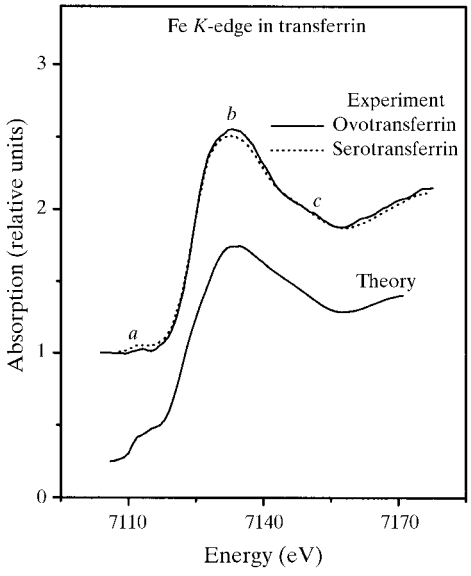

Figure 1
Comparison of experimental and theoretical Fe *K*-edge XAFS of mono (N) transferrin.

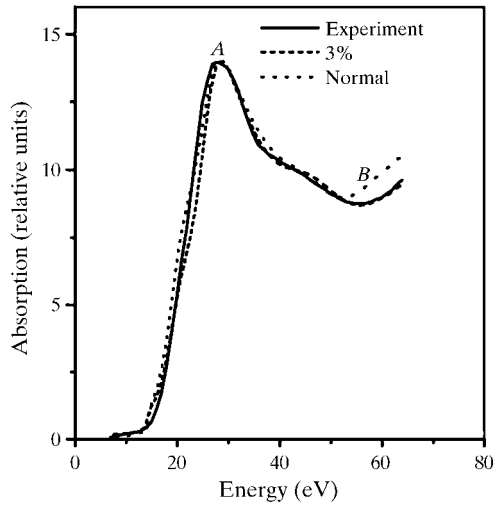

Figure 2
Comparison of experimental and theoretical Cu *K*-edge XAFS of mono (N) transferrin, calculated using the Fe-transferrin model structure (PDB 1a8e) and the 3%-compressed first-shell model.

obtained when the first-shell atoms were compressed by 3% (*i.e.* distances from Cu to the first-shell atoms were decreased by 3%; see Fig. 2). As a parameter we have used the energy distance between main maximum *A* and high-energy minimum *B*. Experimentally this value is equal to 27.8 eV, for 'normal' structure (1a8e) it is equal to 22.5 eV, and for structure with 3% reduced interatomic distance it is equal to 27.1 eV. This result supports the idea that substitution of Fe by Cu ion in mono (N) Tf leads to more compact surroundings of the active metal centre. In the earlier EXAFS study of Cu-transferrin (Garrat *et al.*, 1991), the first shell was found to be compressed by about 5% in comparison with Fe-transferrin (0.19 nm Cu-Tr; 0.20 nm Fe-Tr). One might obtain a similar result by fitting the Fe transferrin EXAFS, *i.e.* the EXAFS bond lengths are compressed by 3% (or more) compared with the crystal structure values. For an example, see the case of azurin (Cheung *et al.*, 2000).

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