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Comparative XANES study of serotransferrin and ovotransferrin at Cu K-edge: evidence of interactions among the metal sites

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Abstract

The Cu site structure of human serotransferrin and hen ovotransferrin using XANES spectroscopy has been investigated. Although the transferrin family proteins have been extensively studied, the results reported herein are the first concerning the structure of the metal site in C-terminal and N-terminal in the whole protein. Our structural data show that these proteins differ with regard to the independence of the two binding sites and the geometry of copper coordination, ranging from a poorly to a significantly distorted octahedron.

Introduction

In this paper, Cu K-edge X-ray Absorption Near Edge Structure (XANES) comparative study of dicupric, monoC and monoN complete form of human serotransferrin and hen ovotransferrin is reported.

The aim of this paper is to show that a structural study (using XANES spectroscopy), gives information on the interaction of two lobes of the transferrin similar to that obtained in a variety of thermodynamic, kinetic and spectroscopic studies. The absence of other structural data regarding this aspect is due to the fact that structural studies on N-terminal and C- terminal are always performed on the half-molecule fragments. The transferrins are a family of metal-carrying glycoproteins. They include serotransferrin, a blood component found in all vertebrates and ovotransferrin, a component of egg white in all species of birds. The main function of human serotransferrin is the transport of iron in the plasma while ovotransferrin has also an antibacterial function via its iron-sequestering capability (Baker & Lindley 1992). The overall structure is approximately the same for all the components of the family: a single polypeptide chain of molecular weight 80 kDa folds into a bilobial structure of about 330 residues, with two specific high affinity metal-binding sites (Aisen & Harris 1989). Metal ions of varying sizes, with oxidation states ranging from $+2$ to $+5$, including Cu^{2+}, Ni^{2+}, Zn^{2+} and Al^{3+} (Smith *et al.* 1991; Congiu Castellano *et al.* 1997) can be accommodated.

The Cu ion occupies the normal Fe-binding site of the protein and utilizes identical ligands. As is known (Legrand *et al.* 1988) three ligands, 2 Tyr and 1 Asp, match the $+3$ charge on the iron ion; the fourth ligand is a neutral His; moreover the iron is coordinated to a carbonate linked to an Arg residue. The geometry of the metal ion and its ligands is found to be a distorted octahedron.

An Electron Paramagnetic Resonance study on copper-ovotransferrin has demonstrated the pH dependence and the anion dependence of the Cu^{2+} binding (Zweier 1980). XAFS studies of chicken dicupric ovotransferrin by Garrat et al. (1991) show that, like Fe^{3+}, the specific binding of Cu^{2+} to transferrin over the pH

range 7–9 has a stoichiometry of 2:1 and requires the presence of a synergistic anion. These data are consistent with a monodentate binding of the synergistic anion in one lobe (presumably at the more labile N-terminal site), and a bidentate binding in the other lobe. The proposed differences in the Cu-anion coordination at the two sites may help to explain why the visible spectra show a considerable difference in λmax of about 20 nm (maxima are observed at 450 nm and at 430–435 nm in the N and C-terminal binding sites, respectively) whereas the two iron-loaded sites are effectively indistinguishable (Williams 1975; Yamamura *et al.* 1984). The differences in EXAFS spectra for solution and freeze-dried samples suggested a reduction in the coordination of the copper with loss of water like that revealed for iron-loaded protein (Hasnain *et al.* 1987).

Studies on the chemical and physical properties of transferrin fragments (N and C-terminal) binding copper or other metal ions like iron, aluminum and zinc have indicated that the two sites are similar but not identical (Aisen *et al.* 1978; Bertini *et al.* 1986): the C site has a greater affinity for metal ions than the N site for most of the transferrins.

However, a recent work (Hirose *et al.* 1996) on copper binding selectivity of N- and C-sites in serotransferrin (human) and ovotransferrin, shows that while the copper binding constants in serotransferrin are different, they are identical for ovotransferrin. These results suggest that the two sites of dicupric serum-Tf and ovo-Tf are kinetically very different.

Synchrotron sources provide very intense continuum radiation that has allowed the development of x-ray absorption spectroscopy of metallo-proteins. In particular the X-ray Absorption Near Edge Structure (XANES) spectrum is determined by multiple scattering (MS) processes of excited inner shell photoelectrons with the neighboring atoms (Durham 1988). As a consequence the spectrum contains electronic and structural information, like the valence state of the photoabsorber or the overall symmetry around it. We also underline that detailed understanding is feasible using XANES only if the nature and position of ligands are well-defined using other methods, such as crystallography; it is also important to compare XANES profiles within a related family of compounds. In this paper we report the XANES results for powder samples considering that detailed multiple scattering calculations have been carried out to investigate the K-edge X-ray absorption near edge structure of a number of copper (II) imidazole powder compounds (Strange *et al.* 1990). The use of powder samples allows the protein damage related to radiolysis of the solvent under X-rays exposition to be limited.

Copper atoms have been found in hesa, penta and tetra coordinated compounds (with tetrahedral or square plane geometry). The structure of hesa-coordinated species ranges from a quite regular to an appreciably distorted octahedral structure, the latter being due to the Jahn-Teller effect clearly characterized in several compounds including copper hydrates in dilute solution. The resulting strong dichroic effect can easily be detected by XANES (Bianconi *et al.* 1988).

Here we present the first comparative XANES study on both dinuclear and mononuclear (monoC and monoN) Cu^{2+} derivatives of serotransferrin and ovotransferrin; our experimental data indicate a significant variation in the geometry of copper coordination.

Materials and methods

Human serum transferrin and ovotransferrin of hen (Sigma, St. Louis, MO, USA) of stated 97% purity was used without further purification.

Mono-and di-cupric forms of the proteins in solution were prepared following the procedure described elsewhere (Harris 1983, 1986). The samples in solution were freeze-dried prior to use.

Dicupric transferrin

Dicupric transferrin was prepared by adding 3 mM $CuCl_2$ to 16 mM apotransferrin (buffer 10 mM HEPES, 5 mM $NaHCO_3$ pH 7.4) until the protein reached about 95% saturation.

Monocupric transferrin, both C and N terminal, was obtained from the complete protein being one of two terminals saturated with iron ions. Optical spectra on monoC-CuTf and monoN-CuTf show a difference in λmax of about 20 nm (maxima are observed at 450 nm and at 430–435 nm in the N and C-terminal binding sites, respectively).

C-terminal (monoC-Cu Tf)

On adding iron in the form of ferrous ammonium sulfate to apotransferrin (buffer 10 mM HEPES, 5 mM $NaHCO_3$ pH 7.4), preferential oxidation and binding to the N site of transferrin occurred (Aisen *et al.* 1978). Carboxy terminal monocupric transferrin was

prepared by adding an appropriate amount of 3 mM $CuCl_2$ to monoFerric-Ntransferrin.

N-terminal (monoN-Cu Tf)

On adding iron in the form of ferric-NTA complex to apotransferrin (buffer 10 mM HEPES, 5 mM $NaHCO_3$ pH 7.4) preferential loading of the C site (Zapolski & Princiotto 1980) occurred. N-terminal monocupric transferrin was prepared by adding an appropriate amount of 3 mM $CuCl_2$ to monoFerric-Ctransferrin.

Differential UV spectroscopy was used to analyze the binding of Cu to the protein. Absorption at 240 nm and 285 nm due to Tyrosine, which participates in metal ion binding in both sites, was monitored by the change in absorption of the more intense band ($\lambda = 240$ nm) as a function of the salt added.

Cu K-edge X-ray fluorescence spectra on powder samples were collected at the LURE Synchrotron facility. A Si (311) channel-cut single crystal was used as monochromator; the energy resolution at the Cu K-edge was about 2 eV and an energy shift of resolved absorption peaks of 0.5 eV was detected. Each spectrum represents a total If/I0 (If = fluorescence count and I0 = photon incident flux measured by a proportional counter) signal averaging of 10 s/point collected at room temperature using a 7-element Energy-resolving Germanium detector from Canberra Industries (Cramer 1992). Calibration of the energy scale was achieved by measuring, at the same time as the fluorescence signal, the intensity transmitted by a metal copper foil placed near the samples. In all the figures, the zero on the energy scale was fixed at the first inflection point of the absorption threshold of metal copper (at 8980.3 eV)

We checked for protein damage due to X-ray absorption by measuring the optical spectra of the samples in solution before and after X-ray exposure.

Results and discussion

In Figure 1a the XANES spectra of lyophilized dicopper serotransferrin (2Cu-Tf), monoC serotransferrin (monoC-Cu Tf) and monoN serotransferrin (monoN-Cu Tf) are reported; the derivatives of the same spectra are presented in Figure 1b.

The very close similarity of these three spectra clearly emerges with no shift in the edge or in the position or intensity of the three main structures named A, B, C (and α, β, χ in the derivative spectra). Using the derivative spectra in Figure 1b, these structures may be located, respectively, at about 8, 18 and 32 eV (the zero of energy scale is at the edge of the copper foil = 8980.3 eV). Also a weak pre-edge structure is present. The physical origin of these peaks in tetragonally distorted Cu^{2+} complex octahedra have been discussed in the past and have been identified as follows: the pre-edge structure has been attributed to the forbidden localized dipole transition 1s→3d (Kau *et al.* 1987); the main peak B arises from the multiple scattering (MS) contribution on the minor plane around the copper, whereas peak A is due to the MS of the two axial ligands and is sensitive to their distance from the metal (Onori *et al.* 1988). The interpretation becomes more and more complicated as the symmetry around the copper center decreases. Peak A is very strong in square planar complexes but can be identified, although much more weakly, also in distorted

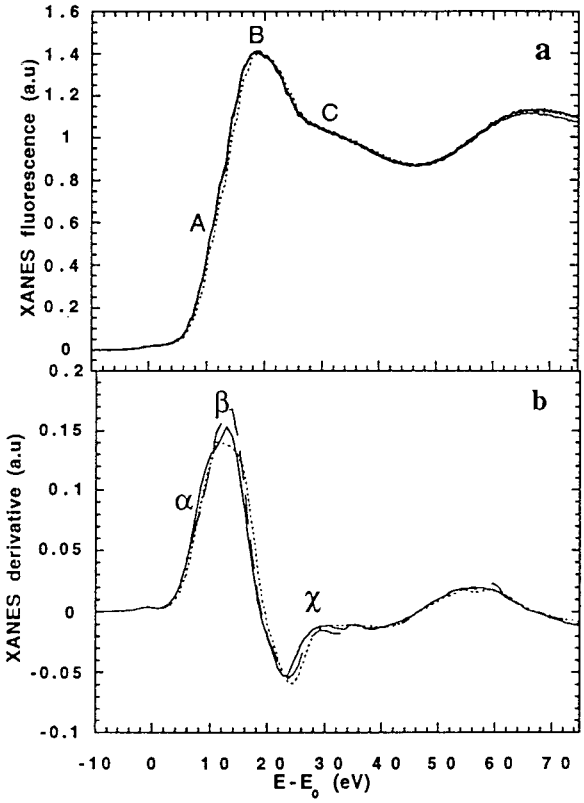

Figure 1. XANES spectra (panel a) and derivative spectra (panel b) of lyophilized: Cu-seroTf (solid line), monoC Cu-seroTf (dotted line) and monoN Cu-seroTf (dashed line). The XANES spectra were normalised by subtracting a polynomial background and then fixing to one the linearly fitted signal beyond the edge. The zero of the energy scale was fixed at the K-edge of a copper metal foil.

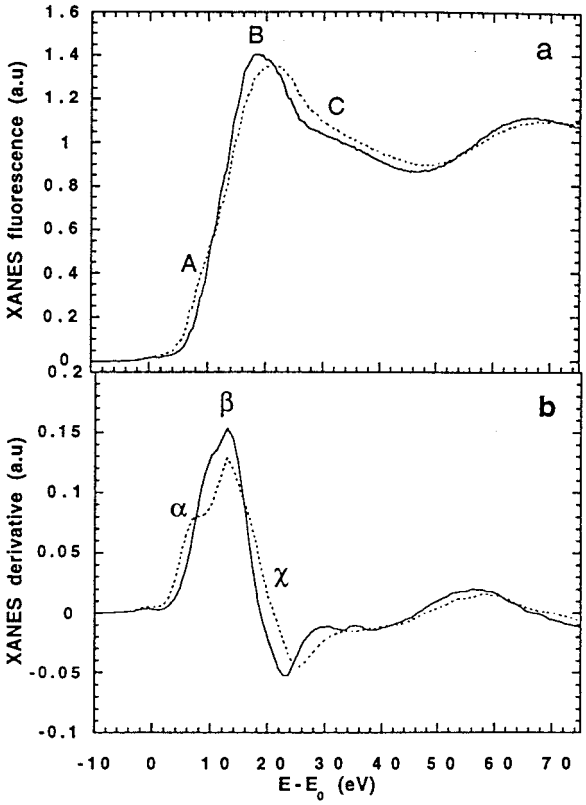

Figure 2. XANES spectra (panel a) and derivative spectra (panel b) of lyophilized dinuclear Cu sero-Tf (solid line), and dinuclear Cu ovo-Tf (dotted line).

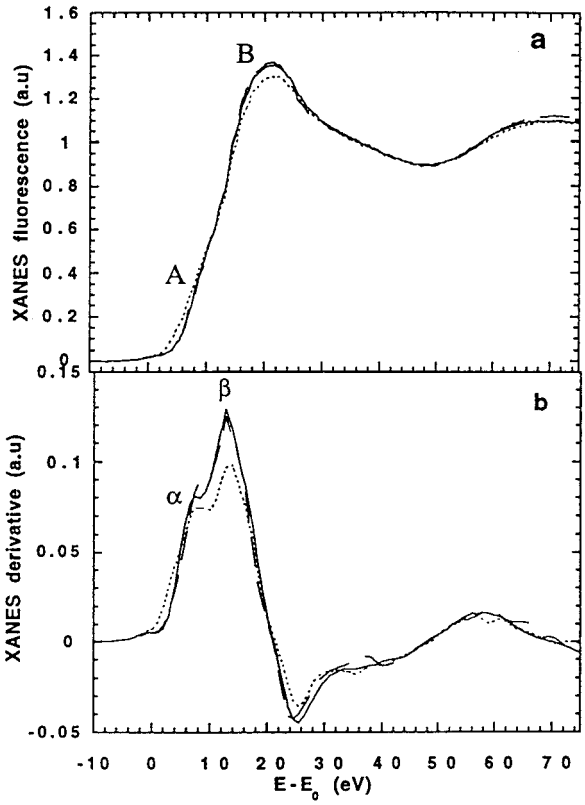

Figure 3. XANES spectra (panel a) and derivative spectra (panel b) of: diCu-ovoTf (solid line), monoC Cu-ovoTf (dotted line) and monoN Cu-ovoTf (dashed line).

octahedrally coordinated species where it is a marker of the Jahn Teller effect (Smith *et al.* 1985; Palladino *et al.* 1993). The interpretation of Peak C is less clear as it falls in the range of full MS. However (Alagna *et al.* 1986) it seems to be influenced by the orientation of the axial ligands as well as by the Jahn Teller distortion (Garcia *et al.* 1986) around copper.

A comparison between the XANES spectra of the three serotransferrins with that reported in literature for model compounds and the theoretical calculation of copper derivatives (Onori *et al.* 1988; Garcia *et al.* 1986; Strange *et al.* 1990) allows us to describe the active sites of the serotransferrins as arranged in a octahedral geometry with little distortion.

From a functional point of view the great similarity between the dinuclear and the mononuclear (monoN and monoC) derivatives suggests that the two Cu sites are also independent, in agreement with the data of Hirose *et al.* (1996), indicating that the N and C copper sites are independent because of the difference in their binding constant.

In Figure 2a XANES spectra of lyophilized dinuclear serotransferrin and ovotransferrin are reported; the corresponding derivatives are shown in Figure 2b. Clear differences emerge between the site structure of the two proteins. The main spectral variations are related to the position of peaks A and B (identified respectively at 6 and 22 eV in the derivative spectra) as well as to the overall shape of the spectra above the main peak B. The geometrical arrangement of active sites in 2 Cu-Ovotransferrin can be still discussed within the framework of a tetragonal distortion of the octahedral cluster. In fact the reported red shift in the position of peak A suggests a greater distortion with respect to 2 Cu-Serotransferrin.

It has also been shown (Garcia *et al.* 1986) that an increase in the intensity of peak C, as actually occurs in 2 Cu-Ovotransferrin, can take place in the presence of a similar Jahn Teller distortion. Moreover, the blue shift and the greater broadening of the main peak B, compared with serotransferrin, suggest greater disorder around the copper involving the

equatorial ligands. Although other rearrangements involving different degrees of freedom in the active site of 2 Cu-Ovotransferrin cannot be excluded, the metal coordination in this a sample is probably apically distorted from an octahedral symmetry.

The XANES and corresponding derivative spectra of 2 Cu-Ovotransferrin, monoC ovotransferrin (monoC-Cu Ovo) and monoN ovotransferrin (monoN-Cu Ovo) are reported in Figures 3a and 3b.

While between 2 Cu-Ovo and monoN-Cu Ovo spectra there are no significant differences, the monoC-Cu Ovo spectrum shows further changes in the intensity of the main peak B and in the shape of A. These changes are similar to those reported between 2 Cu-Serotransferrin and 2 Cu-Ovotransferrin (Figure 2) . A more detailed discussion is possible on the basis of the derivative spectra reported in Figure 3b. Here there is seen to be no shift of the α and β peaks, whereas a further structure appears at about 3 eV in the monoC-Cu Ovo spectrum. With current resolution this new feature cannot be distinguished by a simple larger shape of peak A and is, however, the sign of a larger conformational dynamics associated with the active site of monoC-Cu Ovo.

These results lead to the same conclusions concerning the functional role of ovotransferrin. Because the spectra of dinuclear derivatives are always very close to the monoN, but different from the monoC, a rearrangement of the C site toward the N conformation should occur when the second copper binds to the N lobe. Therefore the two non equivalent copper sites communicate to some extent. This result is supported by the weak positive cooperativity for copper deduced by Hirose *et al.* (1996) who found the same binding constant for this ion.

In conclusion the XANES study of the active site of serotransferrin and ovotransferrin, both in the dinuclear and mononuclear Cu^{2+} derivatives, provides evidence that these two proteins, although belonging to the same family, are different as regards the degree of independence of the two binding sites and in the geometry of copper coordination, which ranges from a poorly to a significantly distorted octahedron.

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