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Aluminum site structure in serum transferrin and lactoferrin revealed by synchrotron radiation X-ray spectroscopy

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The Al site structure of serum transferrin and lactoferrin is investigated using X-ray absorption near edge structure (XANES) spectroscopy. Al K-edge spectra in the mono- and dialuminum forms of the proteins have been recorded for the first time. Our results show that the aluminium ion is hexa-coordinated in an octahedral-like symmetry and that the monoaluminum form, where only the C-terminal binding site is saturated, has an increased structural distortion around the metal site.

Keywords: synchrotron radiation, transferrins, X-ray spectroscopy

Introduction

The transferrin protein family includes: (i) serum transferrin, responsible for iron transport in the plasma of vertebrates; and (ii) lactoferrin, found in milk and other secretions. Lactoferrin protects the cells from damage by free radicals and inhibits bacterial growth (Aisen & Harris 1989; Baker & Lindley 1992). Both serum transferrin and lactoferrin are metal-binding glycoproteins able to bind more than 20 metal ions including iron, zinc, copper, aluminum and gallium, as well as a large number of lanthanides and actinides. *In vivo*, they are saturated only partially (about 30%) with ferric iron. The single polypeptide chain of molecular weight 80 kDa folds in a bilobial structure of about 330 residues, joined by a short connecting peptide with two specific high affinity metal-binding sites which, although similar, are not identical (Baldwin *et al.* 1982)

Two conformational states, referred to as 'closed' and 'open', related to the mechanism of iron uptake and release are detected from X-ray diffraction (Anderson *et al.* 1987) and X-ray uptake and release are detected from X-ray diffraction (Anderson *et al.* 1987) and X-ray small angle scattering (Grossmann *et al.* 1992). X-ray crystallographic studies on iron-serotransferrin (Bayley *et al.* 1988) and iron-lactoferrin (Anderson *et al.* 1990) have been reported. The iron in the diferric molecule is in the hexacoordinated high-spin form Fe^{3+}. The geometry of the metal ion and its six ligands (five oxygen atoms and one nitrogen atom) is found to be a distorted octahedral (Bayley *et al.* 1988, Anderson *et al.* 1990, Day *et al.* 1993). Three anionic ligands, 2 Tyr and 1 Asp, match the +3 charge on the metal ion; the fourth ligand is a neutral His (Legrand *et al.* 1988); moreover the iron is coordinated to a carbonate linked to an Arg residue and probably, as suggested elsewhere, to a water molecule (Bayley *et al.* 1988). EXAFS (extended X-ray absorption fine structure) investigation on ovotrasferrin (Garrat *et al.* 1986) and serum transferrin containing an iron-binding

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site (Bertini *et al*. 1986) has confirmed the hexacoordination of the metal site.

The role of transferrins in the transport of many metal ions has biomedical relevance; of particular interest is the transport of aluminum. In fact, it has been proposed that transferrin carries aluminum to the brain by using a specific serotransferrin receptor which is independent of the metal being transported. Although the hexacoordinated ionic radius of $A1^{3+}$ is smaller with respect to the ionic radius of Fe^{3+} (0.54 versus 0.65 Å), there is considerable evidence that the former has a high affinity for the iron-binding sites of transferrins. This is correlated to the higher concentration of $A1^{3+}$ in the brain of subjects with degenerative disorders such as senile dementia of the Alzheimer's type (Roskams & Connor 1990).

The lack of both unpaired electrons and *d* electrons in $A1^{3+}$ makes this metal ion undetectable by ESR and other direct optical techniques. Many indirect spectroscopic techniques have been used (Battistuzzi *et al*. 1995) to study the binding of aluminum to transferrins. These include UV visible spectroscopy, X-ray scattering, and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopies. In a recent review (Aramini *et al*. 1996) the results obtained by these spectroscopic techniques have been described. We have shown (Congiu-Castellano *et al*. 1994), by using X-ray small angle scattering (SAXS) in solution, that the pH value of the micro-environment plays a critical role in the conformational changes of the transferrin-containing iron or aluminum ions.

The results of X-ray absorption spectroscopy on aluminum K-edge in proteins (George 1993) are here reported for the first time. The local structure of the Al binding site is revealed, using XANES (X-ray absorption near edge structure) spectroscopy with a synchrotron radiation source, providing information on the higher order correlation functions of atomic distribution near the selected atom (Bianconi *et al*. 1985). The experimental equipment, described in the following section, has allowed us to overcome, in this energy range, the problem of signal detection of low Z elements in disordered and diluted samples such as biological systems. On the other hand, very low concentration of the metal in the samples restricts the possibility to extend the data to the high energy region.

Materials and methods

Solutions of mono- and dialuminum forms of the proteins were prepared as described elsewhere (Thompson *et al*. 1986). Human serum transferrin and lactoferrin (Sigma, St. Louis, MO, USA) of stated 97% purity were used without further purification. For the preparation of the dialuminum transferrin, Al was added to apotransferrin, 0.2 mM l^{-1} in 0.100 M l^{-1} HEPES, 0.025 M l^{-1} $NaHCO_3$, pH 7.5 using 2 mM $AlNH_4(SO_4)_2$ in 2 mM l^{-1} HCl until the protein reached about 95% saturation. The carboxy terminal monoaluminum transferrin was prepared by adding the appropriate amount of Al to the protein in 0.100 M l^{-1} MES, 2 mM l^{-1} $NaHCO_3$, pH 5.5.

Difference UV spectrophotometry was used to analyse the binding of Al^{3+} to the proteins. Absorption maxima at 240 nm and 288 nm, due to tyrosines which participate in metal ion binding in both sites, were monitored and the change in the absorption of the more intense band ($\lambda = 240$ nm) as a function of the titrant added, was related to the binding process.

Al K-edge XANES spectra were collected at the SA32 beam line of the Super-ACO storage ring at LURE (Orsay, France). The beam line uses a toroidal mirror and a two-crystal type monochromator. In the present experiment, we used two quartz ($10\bar{1}0$) crystals which allow a resolution of 0.4 eV at 1600 eV. The incident beam intensity was monitored by a thin berillium film TEY monitor. The absorption was measured by the fluorescence yield using a windowless Ge seven-element array, with an energy resolution of about 150 eV at 5.9 KeV, mounted at 90° to the incident light direction in the plane of incidence. Despite the characteristics of the experimental equipment, the signal-to-noise ratio was very low because of the high dilution of the samples. The spectra shown are the sums of about 80 different scans; for this reason, we have carried out the experiment only in the near edge energy range. The samples were prepared as thin film on a Ta stripe. Partially dehydrated protein samples were deposited on the sample holder and allowed to dry at room temperature. The stripe was then mounted in a UHV chamber by means of a transfer assembly. Given the dilution values, no correction for the self-absorption effect has been necessary.

Results and discussion

Figure 1a shows the XANES spectra of dialuminum transferrin (2Al-TF) and dialuminum lactoferrin (2Al-LAC); Figure 1b shows the derivative spectra. The overall shapes of the spectra, and the positions and the intensity ratio of the main features suggest a strong similarity between the active sites of the two proteins. In fact, both spectra show the presence of four structures A,B,C,D, (α,β,χ,δ in the derivative spectra) that occur at the same energy. The energy separation (3.8 ± 0.2 eV) between the two main peaks B and C of the edge, the position of feature D at 29.0 ± 0.4 eV (the higher error value arises from the lower S/N ratio in correspondence of D) and the position of a very weak feature A at

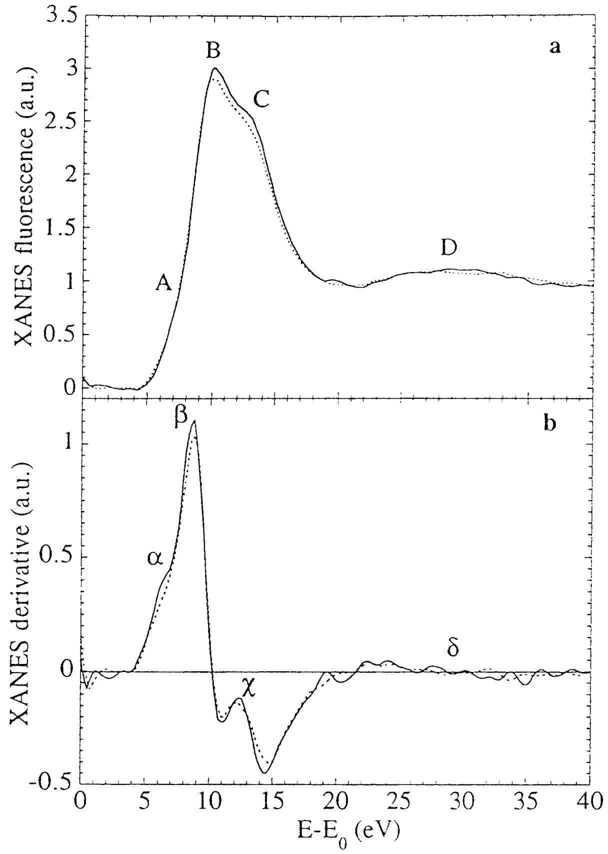

Figure 1. XANES spectra (panel a) and derivative spectra (panel b) of: 2Al-LAC (solid line) and 2Al-TF (dotted line). The XANES spectra were normalised by subtracting a polynomial background and then fixing to one the linearly fitted signal beyond the edge. The zero of the energy scale was fixed at the K-edge of a metal aluminum foil.

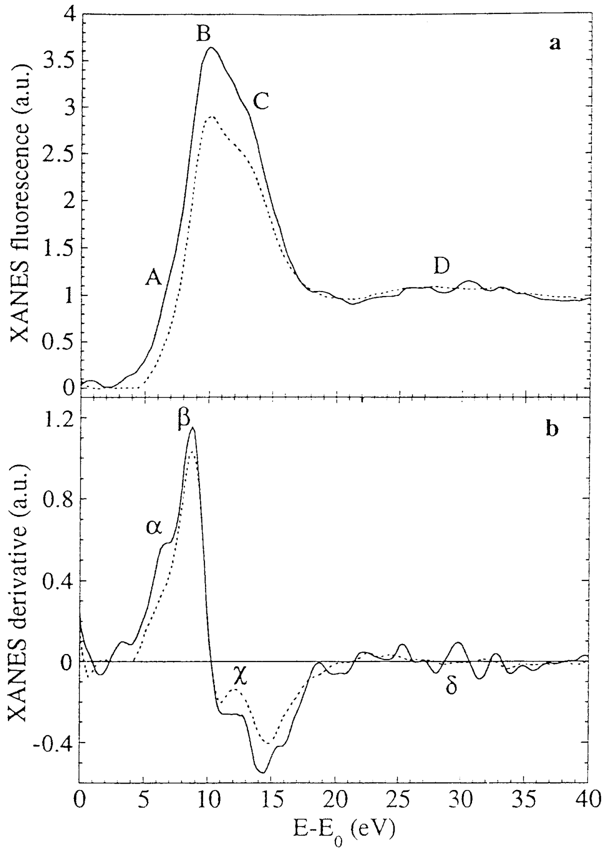

Figure 2. XANES spectra (panel a) and derivative spectra (panel b) of 1Al-TF (solid line) and 2Al-TF (dotted line).

about 6.2 ± 0.2 eV above the edge, are measured from the derivative spectra.

Figure 2 shows the experimental XANES spectra of 2Al-TF and 1Al-TF and their derivative spectra. Despite the overall shape of the spectra being somewhat similar, meaningful differences between these samples are observed. Even if peaks B, C and D occur at the same energy (and we will later point out the importance of these observations concerning the coordination geometry of Al), the 1Al-TF spectrum exhibits a stronger intensity of the B and C features and a 0.7 eV red shift (measured at half maximum) of the edge with respect to the 2Al-TF spectrum. We note that the red shift in the 1Al-TF spectrum (Figure 2a) is due to the enhancement of feature A (feature α at about 6.4 eV in Figure 2b) that is nearly absent in the 2Al-TF spectrum. A more quantitative analysis is in progress to assign such a feature to structural parameters involved in the rearrangement of the metal ion site.

The coordination geometry of Al in transferrin and lactoferrin is deduced by comparing the XANES spectra of the proteins with the spectrum of an aluminum salt of known structure. We have used as a model compound [$NH_4Al(SO_4)_2 \cdot 12H_2O$], in which the metal atom is bound to oxygens from six water molecules (Lipson 1935) in an octahedral geometry. Moreover, the aluminum coordination has been extensively studied in mineral science by XANES (McKeown 1989, Froba *et al.* 1995, Ildefonse *et al.* 1995, Wu *et al.* 1996). This technique allows us to distinguish between six (octahedral) and four (tetrahedral) aluminum coordination. It has been pointed out that the main peak B (attributed to transition between the 1*s* core level and the unoccupied 3*p* state) in tetrahedral compounds is red-shifted about 2.5–3 eV relative to octahedral compounds (Wong *et al.* 1994), going from about 1569 eV (octahedral compounds) to 1566 eV (tetrahedral compounds). This well known red shift is induced from the more attractive tetrahedral potential with respect to the octahedral case (Cabarett *et al.* 1996).

Figure 3 shows the 2Al-TF and 1Al-TF XANES spectra, as well as the spectrum of the hexacoordinated aluminum salt. The comparison shows that the main peak B of both transferrins and the salt occurs at the same energy. It suggests that in both transferrin and lactoferrin the aluminum atom is hexacoordinated in an octahedral-like geometry in agreement with some recent results of ^{27}Al NMR spectroscopy (Aramini *et al.* 1996), which report characteristic chemical shifts of such a coordination. The broadness of the peaks B and C in the proteins is due to the higher degree of structural disorder. In the salt the peaks B and C are sharper and with a different energy separation. The smaller energy separation in the protein could be related (Natoli 1984) to higher average distances around aluminum. Moreover, the rising edge of the salt spectrum is better fitted by the 2Al-TF spectrum, suggesting that aluminum in 1Al-TF has a higher degree of distortion from octahedral symmetry.

SAXS measurement carried out on aluminum transferrin samples prepared in the same chemical conditions (Congiu-Castellano *et al.* 1994) have demonstrated a marked pH-dependent conformational change between 2Al-TF ('closed' conformation) and 1Al-TF ('open' conformation) inducing a 3Å increase of gyration radius that can drive, according to XANES results, a structural rearrangement at the active site level.

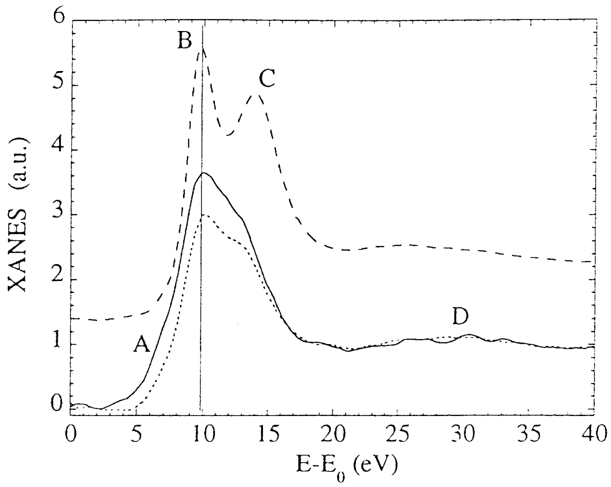

Figure 3. XANES spectra of [$NH_4Al(SO_4) \cdot 12H_2O$] (dashed line); 1Al-TF (solid line) and 2Al-TF (dotted line). The spectra of [$NH_4Al(SO_4) \cdot 12H_2O$] is shifted up by 1.4 eV for clarity. Peak B (used as a six-coordination marker) occurs at the same energy in all the spectra.

In conclusion we have shown that (i) Al site structure is very similar in the dialuminum forms of transferrin and lactoferrin; (ii) Al in both transferrin and lactoferrin in accomodated in a hexacoordinated site with octahedral-like symmetry; and (iii) the pH-dependent conformational rearrangement, observed by comparing the mono- and dialuminum forms, is associated with a structural distortion around the Al ion site.

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