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An extended-X-ray-absorption-fine-structure study of freeze-dried and solution ovotransferrin

Evidence for water co-ordination at the metal-binding sites

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Our previous extended-X-ray-absorption-fine-structure (e.x.a.f.s.) study has shown that the probable iron environment in chicken ovotransferrin involves two low-Z ligands (consistent with phenolate linkages) at 0.185(1) nm and four low-Z ligands at 0.204(1) nm [Garratt, Evans, Hasnain & Lindley (1986) Biochem. J. **233**, 479–484]. Herein we provide additional information from the e.x.a.f.s. and near-edge structure suggestive of a decrease in the co-ordination number of ovotransferrin-bound iron upon freeze-drying. These effects are reversible, and exposure of the freeze-dried material to a humid atmosphere results in reversion to the solution spectra. Progressive rehydration was monitored by using e.p.r. spectroscopy and was confirmed by recording the high-resolution X-ray-absorption near-edge structure (x.a.n.e.s.). The results suggest the presence of a labile water molecule at the iron-binding sites of ovotransferrin in solution.

INTRODUCTION

The presence of co-ordinated water to metal ions in metalloproteins is not uncommon and is often thought to play an important role in the biological functioning of such proteins. For example, it is suggested that the enzymic mechanisms of CuZn superoxide dismutase, carbonic anhydrase and the zinc peptidases thermolysin and carboxypeptidase A are critically dependent on the displacement or catalytic involvement of a solvent water molecule (Lipscomb, 1983; Hangauer *et al.*, 1984; Cass, 1985). Even though some evidence for water co-ordination has been provided in several cases by the crystallographic studies, it is not always possible to define accurately the position of a co-ordinated water because of limitations in resolution. In contrast, in the e.x.a.f.s. analysis the presence of a first-shell water molecule may be more readily detected, but it is not easy to distinguish such ligands from other low-Z species such as N(histidine) and O(tyrosine). In the present paper we report a comparative study of the aqueous and freeze-dried forms of chicken ovotransferrin, which show changes compatible with the loss of a ligand (water) upon freeze-drying. A similar observation has been reported for CuZn superoxide dismutase, where the water ligand at 0.224 nm was found to be absent in the freeze-dried material (Blackburn *et al.*, 1984).

The role of ovotransferrin in egg-white is presumed to be as a bacteriostatic agent acting via its iron-sequestering capability. Much research effort may have centred on ovotransferrin because of its evolutionary relationship with serum transferrin, the principal iron-transport protein of human plasma. Ovotransferrin is similarly related to lactoferrin, a basic protein from secretory fluids and melanotransferrin (p97 protein) expressed on the surface of human melanoma cells (for reviews see Chasteen, 1983; Brock, 1985).

The transferrins are single-chain glycoproteins of M_r 80000, capable of binding a maximum of two Fe^{3+} ions concomitant with two synergistic anions (usually HCO_3^- or CO_3^{2-}) at specific metal-binding sites. Although other metals have been reported bound to serum transferrin *in vivo* (e.g. Evans, 1976; Trapp, 1983), and have served as useful spectroscopic probes *in vitro* (Aisen *et al.*, 1969; Cannon & Chasteen, 1975; Zweier & Aisen, 1977), the only well-established functions of the protein are concerned with its interaction with iron.

The detailed structure of the metal-binding sites remains obscure despite considerable effort in the application of many physical and chemical techniques. E.p.r. and Mössbauer spectroscopies have established that the iron lies in a rhombic ligand field but are in disagreement about the extent of the departure from octahedral symmetry (Aasa, 1972; Tsang *et al.*, 1976). More recently, e.x.a.f.s. studies have demonstrated the first co-ordination sphere to consist of a split shell of low-Z ligands (Garratt *et al.*, 1986). Although only two first-shell distances could be identified [two ligands at 0.185(1) nm and four at 0.204(1) nm], the limited extent of the data range means that the possibility of a further splitting in the first shell cannot be precluded.

The asymmetry of the ligand field arises largely from co-ordination by several different types of ligand. Tyrosine and histidine residues are thought to contribute the only protein-derived ligands (see Brock, 1985), though the exact numbers of each remains uncertain. The synergistic anion is also believed to be directly bound to the metal and interlocked to the protein via

Abbreviations used: e.x.a.f.s., extended X-ray-absorption fine structure; x.a.n.e.s., X-ray-absorption near-edge structure; SRS, Synchrotron Radiation Source.

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electrostatic interaction (Schlabach & Bates, 1975; Najarian *et al.*, 1978; Harris & Gelb, 1980; Zweier *et al.*, 1981; Schneider *et al.*, 1984). Over recent years the favoured model for the iron-binding sites that has emerged involves two tyrosine residues, two histidine residues and the (bi)carbonate anion with the sixth possible co-ordination site occupied by a water or hydroxy-group moiety (Koenig & Schillinger, 1969; Gaber *et al.*, 1970; Villafranca *et al.*, 1976). Recent nuclear-magnetic-relaxation dispersion experiments on the VO^{2+}, Cu^{2+} and Gd^{3+} derivatives of human serum transferrin also suggest indirect, second-shell, water co-ordination, presumably via an intervening ligand (Bertini *et al.*, 1985; O'Hara & Koenig, 1986).

Since the transferrins are capable of both sequestering and releasing ferric iron under appropriate physiological conditions, it is evident that the metal-binding sites are accessible to bulk solvent, as illustrated by Koenig & Schillinger (1969). The exchange of iron between transferrin and iron chelators and its uptake from simple ferrous salts indicates that the binding of iron to transferrin is not solely thermodynamically dictated, but depends on the accessibility of the donor species to the metal-binding sites. For example, most Fe(III) chelate donors preferentially donate iron to the *N*-terminal domain of transferrin rather than the thermodynamically more stable *C*-terminal site (Aisen *et al.*, 1978; Evans & Williams, 1978). Furthermore, the mechanism by which iron is removed from serum transferrin in the CURL (compartment of uncoupling of receptor and ligand) of the recipient cell probably involves ligand protonation and ligand exchange mechanisms, and the obvious candidates vulnerable to chelate attack are the anion and solvent molecule. Experiments by Bates and his co-workers (Cowart *et al.*, 1982, 1986) demonstrate the existence of quaternary complexes of the form chelate–Fe^{3+}–transferrin–CO_3^{2-} in which the tyrosine residues appear undisplaced, and it seems plausible that the water ligand may be absent from such a complex.

In the present work we have used X-ray-absorption spectroscopy and e.p.r. to observe changes at the metal-binding sites as a function of dehydration and rehydration. The abilities of these techniques to distinguish small differences in site conformations in metalloproteins have long been exploited (Palmer, 1985; Hasnain, 1987). The opportunity to extract some physically meaningful parameters from the e.x.a.f.s. and x.a.n.e.s. seemed to us particularly attractive and stimulated the current investigation.

EXPERIMENTAL

Ovotransferrin

Ovotransferrin was prepared from chicken egg-white by the method of Williams (1968), and made diferric by addition of excess iron(III) nitrilotriacetate and desalted on Sephadex G-25 before use. Freeze-dried diferric chicken ovotransferrin was prepared by shell-freezing a solution of iron-saturated protein in 0.05 M-NH_4HCO_3 with liquid N_2 and freeze-drying at a temperature of -60 °C and under a vacuum of 6.7 Pa (5×10^{-2} Torr) for approx. 20 h on an Edwards model EF03 freeze-dryer.

E.p.r. spectroscopy

All e.p.r. spectra were recorded at 77 K with a Varian E-9 EPR spectrometer operating at X-band frequencies (approx. 9 GHz). For all spectra a scan rate of 25 mT/min was used and a time constant of 1 s. The microwave power, modulation frequency and modulation amplitude were 10 mW, 100 kHz and 0.5 mT respectively.

Measurements on the freeze-dried protein were made on samples freshly packed into e.p.r. tubes, sealed with Parafilm and frozen in liquid N_2. Samples prepared in this fashion could be stored at 77 K and would give reproducible spectra for upwards of a week. Solution spectra were recorded in 0.05 M-NH_4HCO_3 at a concentration of typically 200 mg/ml.

Rehydration of the freeze-dried powder was performed by suspending the protein in a gauze net over a beaker of water at approx. 30 °C, and covered with a second beaker to maintain a moist atmosphere. Samples were taken at intervals, packed into e.p.r. tubes and spectra recorded. A sample after 3 days' exposure was taken for X-ray-absorption measurements.

X-ray-absorption spectroscopy

X-ray-absorption spectra (x.a.n.e.s. and e.x.a.f.s.) were measured on the recently commissioned high-brightness station 8.1 of the SERC Daresbury Laboratory Synchrotron Radiation Source (SRS). A detailed description of this station is given elsewhere (Van der Hoek *et al.*, 1986), but briefly it is equipped with a slitless monochromator system that provides high photon intensity without loss of energy resolution, and this is ideally suited for e.x.a.f.s. and x.a.n.e.s. studies of metalloproteins. Data were collected in fluorescence mode as fluorescence excitation spectra (Hasnain *et al.*, 1983), with the use of up to four NaI scintillation counters. Monochromatic radiation was produced by using a slitless Si(111) or Si(220) double-crystal X-ray monochromator. The second crystal was offset from the Bragg angle and the incident intensity at the sample was maintained at half maximum in order to minimize harmonic contamination. During the course of the measurements the SRS beam energy was 2 GeV and the average circulating current was approx. 200 mA.

Freeze-dried samples were packed into Teflon sample cells and sealed with Parafilm. Solution spectra were recorded in 0.1 M-Mes buffer, pH 7.2, in identical cells at a concentration of typically 400 mg/ml. Spectra presented here are the averages of 12 scans for the solution sample, two scans for the freeze-dried sample and seven scans for the freeze-dried sample after rehydration.

Data reduction and analysis was performed by using the e.x.a.f.s. program suite at the Daresbury Laboratory. Simulations utilizing '*ab initio*' phase-shifts and the single-scattering spherical-wave formalism of Lee & Pendry (1975) were performed by using the fast curved-wave algorithm of Gurman *et al.* (1984).

RESULTS

X.a.n.e.s.

Fig. 1 shows the Fe *K*-absorption edge for diferric chicken ovotransferrin in solution and as a freeze-dried powder before and after rehydration. Changes in the features indicated (*a* and *b*) demonstrate that geometrical alteration of the metal-binding sites has occurred as a consequence of freeze-drying but that the perturbations are reversible. The increase in intensity of the pre-edge transition *a* indicates a lower co-ordination for the iron in the freeze-dried state.

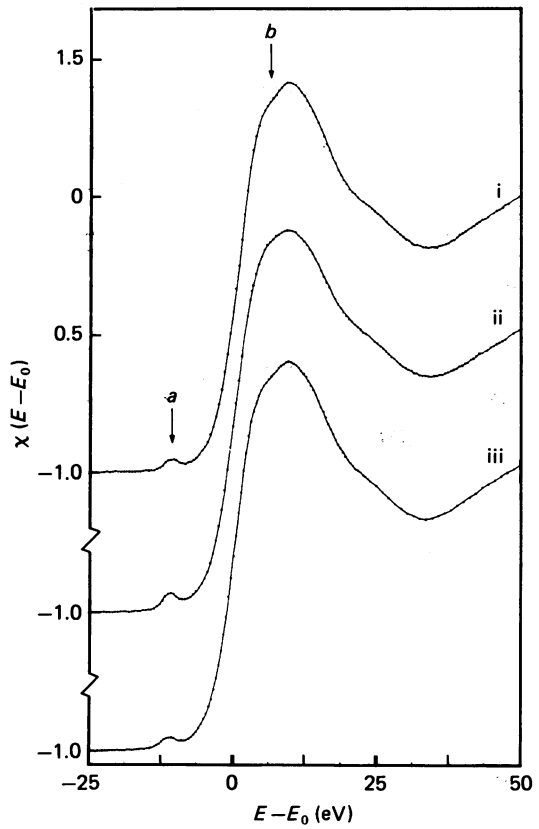

Fig. 1. Fe *K*-edge x.a.n.e.s. spectra of diferric chicken ovotransferrin

Spectrum i, solution spectrum recorded in 0.1 M-Mes buffer, pH 7.2; spectrum ii, freeze-dried powder spectrum; spectrum iii, freeze-dried powder spectrum after 3 days' exposure to a humid atmosphere. Enhancement of the features *a* and *b* in spectrum ii indicates structural perturbation of the iron sites that can be reversed by allowing the protein to rehydrate.

E.x.a.f.s.

Fig. 2 compares the *k*-space e.x.a.f.s. spectra and Fourier transforms (inset) of the two forms of diferric chicken ovotransferrin after normalization, background removal and k^3-weighting of the raw data. Background subtraction involved firstly the definition of an edge step constant (c) due to the atomic fluorescence. A smooth polynomial (μ_A) was then fitted through the e.x.a.f.s to mimic the spectrum in the absence of back-scattering neighbours. The oscillatory component of the fluorescence (the e.x.a.f.s.) is then given by:

$$\frac{\mu-\mu_A}{c}$$

where μ is the experimentally measured fluorescence.

The loss of amplitude of the e.x.a.f.s. oscillations of the freeze-dried sample and the consequent diminution of the first-shell peak in the Fourier transform indicates a decrease in the longer components' contribution within the first shell. Simulation of the freeze-dried protein started from parameters derived previously for the protein in solution (Garratt *et al.*, 1986) (Fig. 3*a*), and involved a first-shell co-ordination of two oxygen or nitrogen (O/N) ligands at 0.185 nm and four O/N ligands at 0.204 nm. Assuming a five-co-ordinate system by removal of one of the shorter ligands (0.185 nm) results in a worsening of the fit index from 7.5 to 13.4 (Fig. 3*b*). The fit index (F.I.) is defined as the following residue between theory and experiment:

$$\text{F.I.} = \sum_k k^3[\chi_{\text{exp.}}(k)-\chi_{\text{theor.}}(k)]^2$$

where k is the wavevector of the photoelectron. However, a second model involving removal of one of the ligands at 0.204 nm results in an improvement of the fit index to 4.4, and the amplitudes of most of the e.x.a.f.s. features are reproduced faithfully (Fig. 3*c*). A non-constrained refinement of either model reduced the fit index to 1.8, but the second is preferred since it generates comparable Debye–Waller factors for the two components of the first shell (0.00005 nm^2 at 0.186 nm and 0.00008 nm^2 at 0.203 nm) compared with the substantial disparity produced by the first model (0.00001 nm^2 at 0.183 nm and 0.00011 nm^2 at 0.200 nm). The implied Fe–OH_2 bond distance of approx. 0.204 nm is in good agreement with those found in small-molecule high-spin Fe(III) complexes (Thundarthil *et al.*, 1976, 1977; Hair & Beattie, 1977). Fig. 3(*d*) shows the theoretical fit to the second model after refinement and incorporates a first shell consisting of two O/N ligands at 0.186 nm and three O/N ligands at 0.203 nm. Furthermore, a single oxygen atom refining to a distance of 0.206 nm can be seen to account successfully for the significant features of the difference spectrum between the two experimental measurements (Fig. 4).

E.p.r.

The perturbations of the e.x.a.f.s and x.a.n.e.s. spectra upon freeze-drying are reflected in the e.p.r. spectra by loss of structure in the $g' = 4.3$ (150 mT) region. In Fig. 5 spectra are plotted as a function of the magnetic field strength in millitesla. The freeze-dried spectrum is then monitored as a function of time of exposure to a humid atmosphere, up to a maximum of 5 days (spectra b–i). The final sample (spectrum j) is of protein, after the rehydration procedure, dissolved in 0.05 M-NH_4HCO_3.

DISCUSSION

The sensitivity of the x.a.n.e.s. region of the X-ray-absorption spectrum to geometrical changes at metal-co-ordination sites makes it an excellent tool for studying metalloproteins under different conditions. The enhanced features *a* and *b* (Fig. 1) in the x.a.n.e.s. spectrum of freeze-dried diferric chicken ovotransferrin over the solution form indicate significant structural alteration as a consequence of dehydration. Roe *et al.* (1984), using over 20 model Fe(III) complexes, have observed a significant negative correlation between the intensities of the pre-edge transition *a* and the co-ordination number. The implication for ovotransferrin is that at least one ligand has been lost from the first co-ordination sphere as a result of freeze-drying.

E.p.r. spectroscopy of the freeze-dried sample confirms that the normal (solution) electronic structure of the iron sites is disrupted. The spectrum is essentially the same as that reported for freeze-dried human serum transferrin (Windle *et al.*, 1963). From the e.p.r. spectra alone it is not possible to assert that the changes due to freeze-drying are solely (or even principally) due to the removal

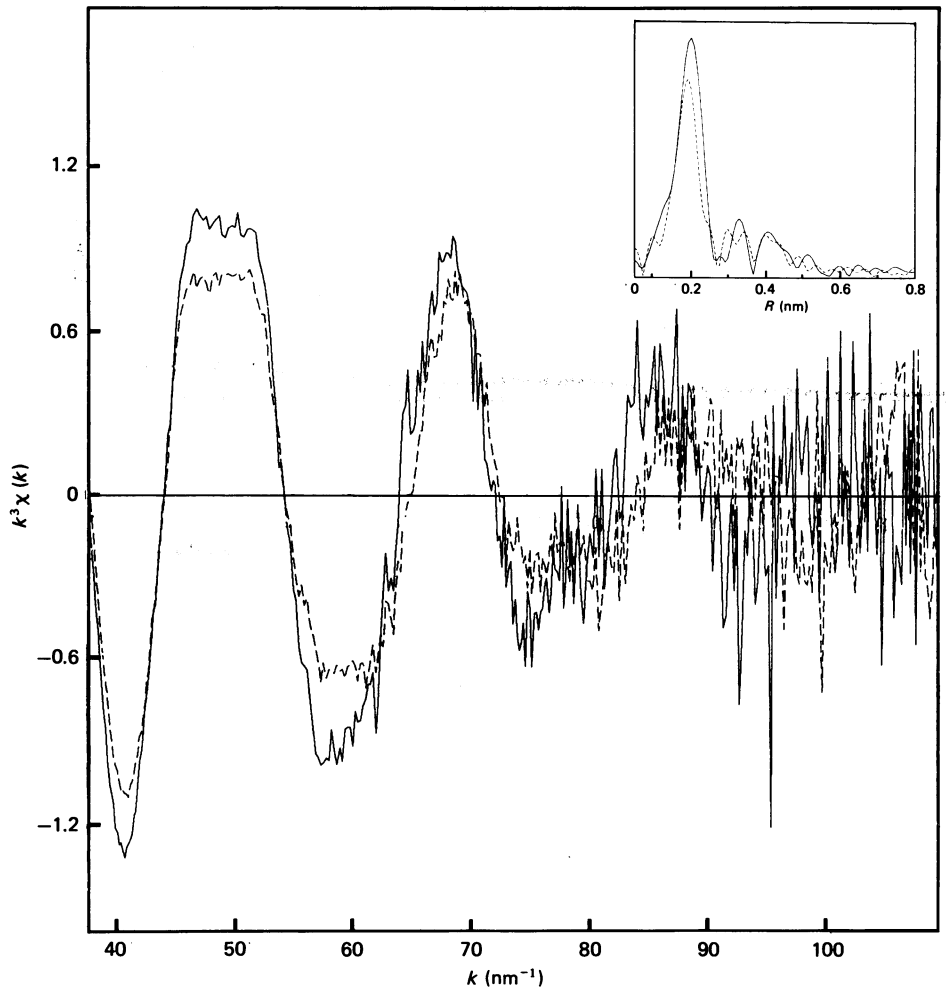

Fig. 2. Fe *K*-edge e.x.a.f.s. spectra and their Fourier transforms (inset) of diferric chicken ovotransferrin

Spectra are plotted as a function of k after normalization, background removal and k^3-weighting. ——, 0.1 mM-Mes buffer, pH 7.2, solution sample; – – – –, freeze-dried sample.

of a ligand from the first co-ordination sphere, since the procedure will inevitably involve the removal of structural water from the exterior of the protein. However, the evidence from x.a.n.e.s. and e.x.a.f.s. (see below) strongly suggests that this is the case. Furthermore, it has been shown previously that substitution of a single ligand (the synergistic anion) can produce dramatic perturbations of the e.p.r. spectrum in the $g' = 4.3$ region (Aisen *et al.*, 1973).

The strongest evidence that a ligand has been removed by freeze-drying comes from the e.x.a.f.s. spectrum and its Fourier transform (Fig. 2). The amplitude of the contributory waves of the e.x.a.f.s. function are dependent on co-ordination number (N) as well as the size of the back-scattering species (Z) and their respective Debye–Waller factors ($2\sigma^2$). The correlation of the N and $2\sigma^2$ parameters and the restricted k-range of the usable data mean that it is often not possible to obtain co-ordination number to an accuracy of better than 10%. However, by monitoring differences in the e.x.a.f.s. spectrum between comparable samples it is possible that changes in co-ordination number can be more readily extracted. We suggest that the decrease in the amplitude of the major shell contribution in the spectrum of the freeze-dried sample arises from a decrease in co-ordination.

The re-arrangement of atoms in the second co-ordination shell, at approx. 0.3 nm, is consistent with small changes in ligand geometry, but provides no convincing evidence for the loss of a bulky group from the binding sites. For example, the Debye–Waller factors for the carbon atoms in the shells at approx. 0.29 nm and approx. 0.31 nm have decreased from 0.00011 nm^2 to 0.00007 nm^2 and from 0.00014 nm^2 to 0.00005 nm^2 respectively. This decrease in the Debye–Waller factors suggests a diminution in the static/dynamic disorder for these atoms in the freeze-dried protein or a need for an increased co-ordination in this shell. Clearly, the evidence suggests that the decrease in the first-shell co-ordination does not come from the loss of a bulky (side-chain) ligand. Rather, the data suggest that a single isolated first-shell atom (i.e. a solvent water molecule) is co-ordinated to ovotransferrin in solution but lost as a consequence of freeze-drying. A similar observation has been previously reported for the copper site of CuZn superoxide dismutase (Blackburn *et al.*, 1984).

We have shown previously (Garratt *et al.*, 1986) that the e.x.a.f.s. spectrum of diferric chicken ovotransferrin

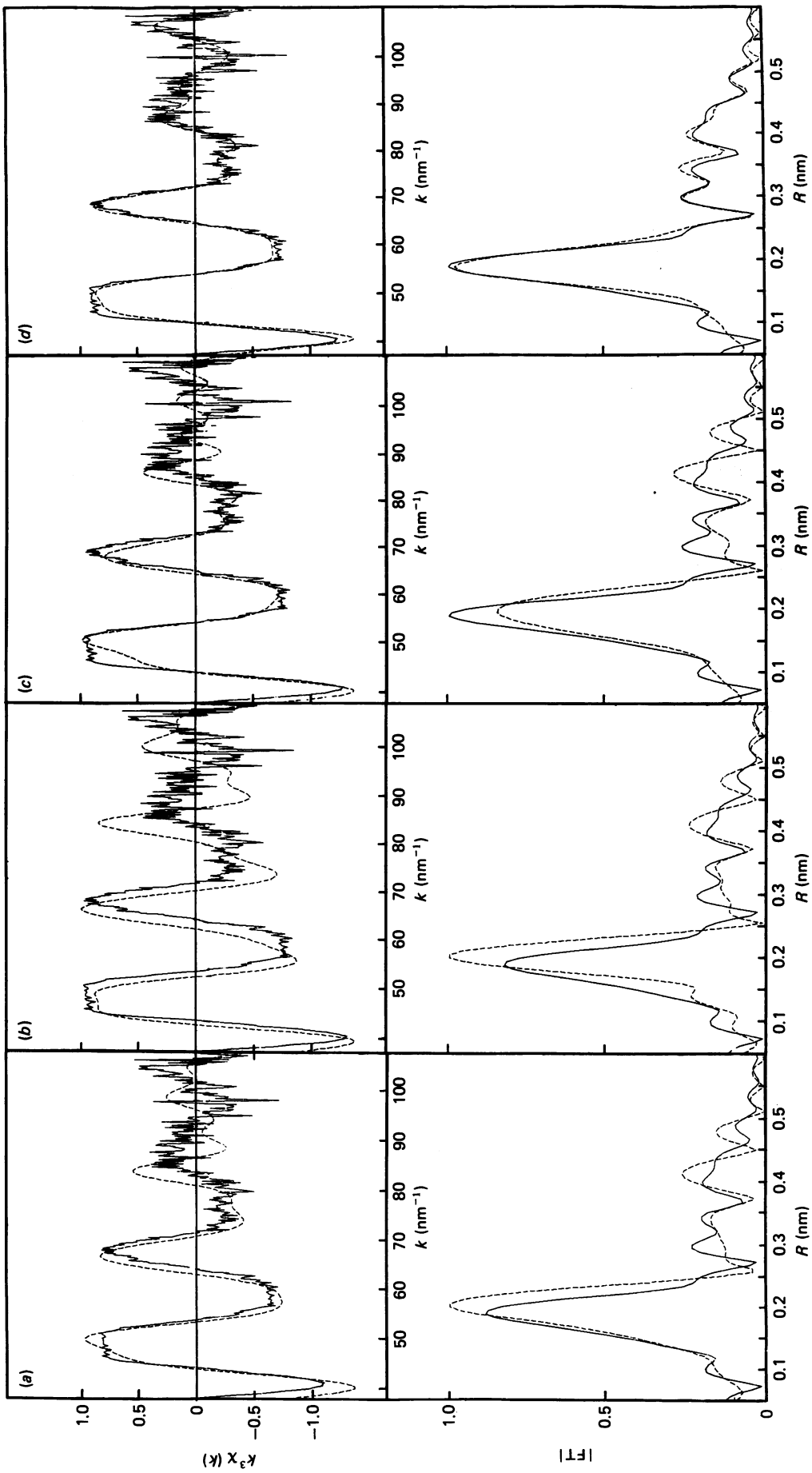

Fig. 3. Simulation of the e.x.a.f.s. spectrum of freeze-dried chicken ovotransferrin

(*a*) Assuming theoretical parameters derived previously for the solution spectrum (see the text), and involving a six-co-ordinate first shell of two O/N ligands at 0.185 nm ($2\sigma^2$ 0.00006 nm^2) and four O/N ligands at 0.204 nm ($2\sigma^2$ 0.00003 nm^2), where $2\sigma^2$ is the Debye–Waller-type disorder parameter. (*b*) Assuming a five-co-ordinate model of one O/N ligand at 0.185 nm and four at 0.204 nm. (*c*) Assuming a five-co-ordinate model of two O/N ligands at 0.185 nm and three at 0.204 nm. (*d*) After refinement of the model in (*c*), giving refined first-shell distances of 0.186(1) nm ($2\sigma^2$ 0.00005 nm^2) and 0.203 nm ($2\sigma^2$ 0.00008 nm^2). In all cases the continuous line is the experimental spectrum, which is constant across the four panels, and the broken line is the theoretical simulation.

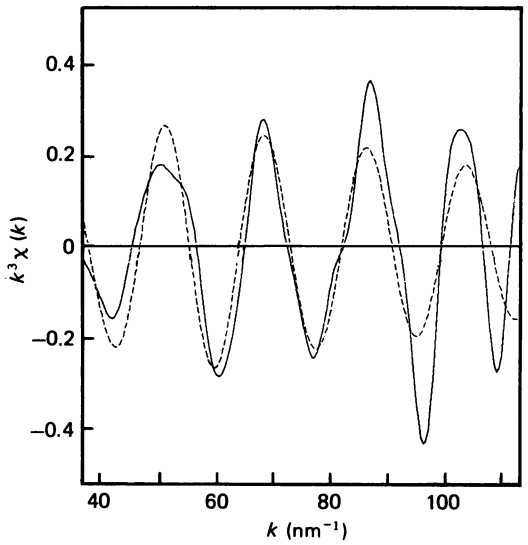

Fig. 4. Simulation of the solution minus freeze-dried ovotransferrin difference spectrum

——, Difference between the two experimental measurements after Fourier filtering the raw data using a square window in R-space from 0.02 nm to 0.5 nm (before phase-shift correction). – – – –, Theoretical simulation assuming a single oxygen atom at a distance of 0.206 nm ($2\sigma^2$ 00003 nm^2).

in solution can only be adequately simulated by using a split first-shell co-ordination involving two O/N ligands at 0.185(1) nm and four O/N ligands at 0.204(1) nm. Simulation of both the freeze-dried ovotransferrin spectrum and the solution minus freeze-dried ovotransferrin difference spectrum are consistent with the absence of one of the longer first-shell ligands (0.204 nm) from the freeze-dried protein. Hair & Beattie (1977) have made a comparative assessment of six-co-ordinate Fe(III)–OH_2 bond distances observed in known crystal structures and find a distribution between 0.198 nm and 0.208 nm with a trend towards the longer distance as the number of non-water ligands increases. The data presented here are entirely consistent with this observation, and it seems likely that the normal Fe–water bond distance in ovotransferrin is approx. 0.204 nm.

Data from both x.a.n.e.s. and e.p.r. measurements indicate that the protein will readily acquire water if exposed to a moist atmosphere. Spectra from freeze-dried samples that have been exposed to a humid environment for more than about 1 day have almost entirely regained the features of a solution spectrum. Clearly, the removal of water at the ovotransferrin binding sites is a reversible event. Indeed, the observation by Aasa *et al.* (1963) that the e.p.r. spectrum of freeze-dried human serum transferrin 'had the same general appearance at 77 K, only slightly less resolved', compared with their solution spectrum, is probably explained by the presence of significant residual water in their preparation.

The importance of a relatively weakly co-ordinated water molecule at the binding sites of the transferrins (presuming this to be generally true) is not completely clear. However, the requirement of serum transferrin to be able to bind and release iron in appropriate circumstances means that exposure to bulk solvent is

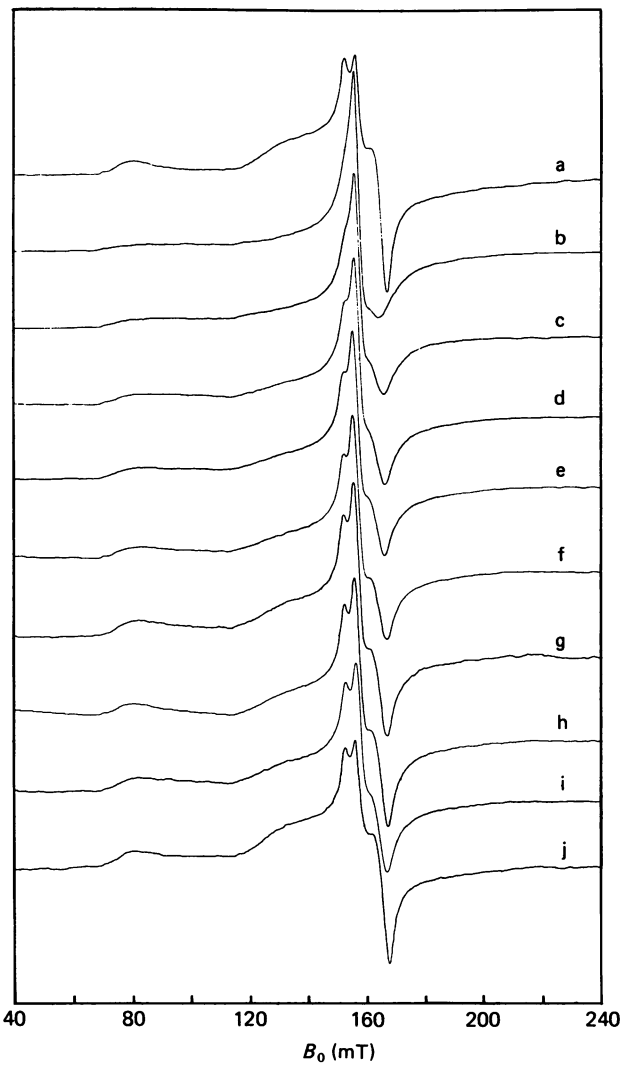

Fig. 5. E.p.r. spectra of diferric ovotransferrin over the time course of rehydration

Spectrum a, in 0.05 M-NH_4HCO_3; spectrum b, immediately after freeze-drying; spectra c–i, after exposure to a humid atmosphere for 5 min, 15 min, 30 min, 60 min, 2 h, 24 h and 5 days respectively; spectrum j, after reconstitution of the freeze-dried powder in 0.05 M-NH_4HCO_3. The features of the solution ovotransferrin spectrum are almost entirely regained by the freeze-dried protein over the time course of the rehydration.

important, as has been pointed out previously (Aisen, 1980). Cowart *et al.* (1982, 1986) have demonstrated the formation of intermediates during the exchange of iron between transferrin and small molecules of potential biological importance. Such intermediates involve the retention of the normal (bi)carbonate and tyrosine linkages in conjunction with the iron chelator. It is tempting to speculate that the co-ordinated water molecule is the ligand that undergoes exchange during the interaction of such molecules with transferrin *in vivo*.

Note added in proof (received 14 August 1987)

Recent medium-resolution X-ray structure determinations of human lactoferrin (Anderson *et al.*, 1987)

and rabbit serum transferrin (Bailey *et al.*, 1987) have indicated that four protein ligands are directly involved in iron binding, two tyrosine residues, one histidine residue and an aspartic acid residue. A fifth ligand, an arginine residue, may also be involved via a bridging bicarbonate anion. Although these X-ray studies are not of sufficiently high a resolution to permit the determination of water sites, they do not preclude a water molecule at the iron-binding site.

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