An extended-X-ray-absorption-fine-structure investigation of diferric transferrins and their iron-binding fragments

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Iron *K*-edge extended-X-ray-absorption-fine-structure (e.x.a.f.s.) spectra were recorded for diferric human and rabbit serum transferrins and for diferric chicken ovotransferrin in aqueous solution; for ovotransferrin e.x.a.f.s spectra from the *N*-terminal and *C*-terminal domain fragments were also measured. The overall spectral profiles closely resemble one another, indicating similar iron-binding sites. The simulation of the diferric ovotransferrin spectrum suggests a first co-ordination shell consisting of six low-*Z* ligands (nitrogen/oxygen), two ligands at a distance of approx. 0.185 nm (1.85 Å) and four ligands at approx. 0.204 nm (2.04 Å). The two shorter distances may correspond to Fe–O (tyrosine), whereas the longer distance is consistent with Fe–N (histidine) and Fe–O (water). Detailed analysis of the spectra of the *N*-terminal and *C*-terminal fragments indicates a difference in the short ligand distance.

INTRODUCTION

The transferrins are a group of two-sited iron-binding glycoproteins found in blood plasma (serum transferrin), egg-white (ovotransferrin) and body fluids (lactoferrin). All are single-chain polypeptides of M_r approx. 80000 that are each capable of binding two ferric ions at specific binding sites. The physiological role whereby serum transferrin must be capable of sequestering iron at sites of absorption and haem degradation as well as releasing it at sites of storage and utilization has led to a considerable research effort centred on the nature of the metal-binding sites [for reviews see Aisen (1980) and Chasteen (1983)].

Perhaps the most unusual characteristic of Fe(III) binding to transferrin is the obligatory requirement for concomitant binding of a synergistic anion (Schade *et al.*, 1949). Physiologically this is known to derive from carbonic acid (HCO_3^- or CO_3^{2-}), and the most recent magnetic-resonance evidence suggests that the anion is directly ligated to the metal (Campbell & Chasteen, 1977; Najarian *et al.*, 1978; Zweier *et al.*, 1979), a suggestion subsequently supported by e.x.a.f.s. measurements (Roe *et al.*, 1984; Schneider *et al.*, 1984).

The nature of the remaining ligands has been the subject of much debate (see Aisen, 1980). Tyrosine residues have been implicated by chemical and spectroscopic techniques, although the number involved, whether two or three, has been disputed; careful u.v. difference experiments (Pecoraro *et al.*, 1981) suggest only two per Fe(III) ion. One or more nitrogen ligands from histidine imidazole groups are also thought to be involved (Rogers *et al.*, 1977; Zweier & Aisen, 1977), and proton-relaxation studies have indicated a water molecule (or OH^-) to be the final possible ligand (Gaber *et al.*, 1970).

Many studies, including those with single-domain iron-containing fragments and monoferric transferrins, have indicated that the two sites are not identical in several of their physical and chemical properties (see

Aisen, 1980). The significance of these findings, however, and the possibility of structural and functional heterogeneity between the sites have remained unanswered questions.

The detailed structure of each of the iron-binding sites will ultimately only be solved by a high-resolution crystal structure. The size of the protein and the weak diffraction of crystals of rabbit plasma transferrin, currently under investigation (Gorinsky *et al.*, 1979), mean that a more specific technique to probe the iron environment is highly desirable. The possibility that e.x.a.f.s. will ultimately enable us to refine the crystal structure in the region of the Fe^{3+} ions as well as to probe the sites independently, by use of the *N*-terminal and *C*-terminal domain fragments, has stimulated the current investigation.

MATERIALS AND METHODS

Chicken ovotransferrin was isolated from egg-white as described in the literature (Williams, 1968), and made diferric by addition of excess ferric nitrilotriacetate. Unbound chelate was removed by gel filtration.

Evans *et al.* (1985) have described a method for the production and separation of both the *N*-terminal and *C*-terminal domain fragments of chicken ovotransferrin by tryptic digestion followed by anion-exchange chromatography in the Mono-Q column on the Pharmacia fast-protein-liquid-chromatography system. For present purposes this method was scaled up and separation performed on a conventional ion-exchange column with DEAE-Sephacrose 4B.

Tryptic-digestion conditions were as described previously (Evans *et al.*, 1985). Incubations were performed at 37 °C for up to 18 h, then samples were diluted 5-fold and applied to a column (12 cm × 2.5 cm) of DEAE-Sephacrose 4B equilibrated in 20 mM-Tris/HCl buffer, pH 8.0. Fragments were eluted from the column by using a linear 0–0.35M-NaCl gradient in the same buffer (total

volume 600 ml). Polyacrylamide-gel electrophoresis in the presence of SDS and immunodiffusion (Ouchterlony, 1958) indicate that the C-terminal fragment is eluted first from the column, followed by a high- M_r form of the N-terminal fragment (M_r approx. 40000) and finally a lower- M_r form of the N-terminal fragment (M_r approx. 32000).

It is known (Evans *et al.*, 1985) that the digestion procedure generates an 80000- M_r species consisting of non-covalently bound associated N-terminal and C-terminal fragments, which separate during the electrophoretic process. Only those C-terminal fragment fractions assessed from the SDS/polyacrylamide-gel electrophoresis and immunodiffusion plates to be free from such contamination were desalted, freeze-dried and retained for e.x.a.f.s. determination. To remove this 80000- M_r contaminant from the pooled 40000- M_r N-terminal fragment fractions, further purification on a column of Sephacryl S-200 equilibrated in 50 mM- NaHCO_3 was performed. Only the unassociated fragment peak was freeze-dried and retained for e.x.a.f.s. determination. The 32000- M_r N-terminal fragment species was not further used in the current investigation.

The purity of both the C-terminal and 40000- M_r N-terminal fragment preparations was further assessed by their elution profiles from a Mono-Q anion-exchange column in the Pharmacia fast-protein-liquid-chromatography apparatus. In each case the preparations were free from significant contamination by the other domain fragment.

Human serum transferrin was isolated by immuno-affinity chromatography (Evans *et al.*, 1982) from normal human serum. Rabbit serum transferrin was isolated by the method of Baker *et al.* (1968) and contaminating haemopexin removed by the method of Martinez-Medellin & Schulman (1972), with the modification that the elution from the SP- (sulphopropyl)-Sephadex column was achieved with a linear 0–0.4 M-NaCl gradient. Rabbit and human serum transferrins were made diferric by addition of excess ferric nitrilotriacetate, desalted, freeze-dried and retained for e.x.a.f.s. determination.

Protein samples were dissolved in 0.1 M- NaHCO_3 and concentrated in Amicon Minicon concentration cells. Final concentrations in iron were 10.9 mM (diferric chicken ovotransferrin), 5.4 mM (C-terminal fragment of chicken ovotransferrin), 3.6 mM (N-terminal fragment of chicken ovotransferrin), 9.1 mM (diferric human serum transferrin and 6.7 mM (diferric rabbit serum transferrin). Protein solutions were contained at room temperature within polytetrafluoroethane sample holders with Mylar windows for the determination of e.x.a.f.s. Measurements were made on two separate occasions at the Daresbury synchrotron radiation source. Data on diferric human serum transferrin were collected when the synchrotron radiation source was operating at 1.8 GeV at 150 mA by using an Si 111 monochromator. The data on diferric chicken ovotransferrin, on its iron-binding fragments and on diferric rabbit serum transferrin were collected during the session when a 580 mm-long double-focusing mirror was used in conjunction with a channel-cut Si 111 monochromator to increase the intensity and brightness of the X-ray beam on the sample. For diferric chicken ovotransferrin and its iron-binding fragments the synchrotron radiation source was operating at 2 GeV with an average current of 150 mA,

and for diferric rabbit serum transferrin the energy was 1.8 GeV.

Spectra presented here are the averages of two (diferric chicken ovotransferrin), seven (C-terminal fragment of chicken ovotransferrin), nine (N-terminal fragment of chicken ovotransferrin) and six (diferric human serum transferrin) scans. The spectrum for diferric rabbit serum transferrin is from a single scan only.

The single-scattering spherical-wave method for computation of theoretical e.x.a.f.s., by using phase shifts derived from 'ab-initio' calculations as described in the literature (Lee & Pendry, 1975; Perutz *et al.*, 1982; Blackburn *et al.*, 1984), was used, and for this purpose e.x.a.f.s. $[\chi(k)]$ is plotted in k -space, with k^2 weighting to highlight the amplitude at higher k . Both the radial distances and Debye-Waller factors, σ^2 , were allowed to float in the refinement procedure.

RESULTS AND DISCUSSION

Fig. 1 shows the e.x.a.f.s. spectra for diferric chicken ovotransferrin, diferric human serum transferrin and diferric rabbit serum transferrin. The spectra attenuate rapidly and oscillations are not easily visible at photon energies greater than 300 eV above the edge. The overall forms of the spectra are almost identical, indicating great similarity in the iron-binding sites for all three species. Small differences, however, do exist. The spectrum for

Fig. 1. Fe K-edge e.x.a.f.s. of diferric transferrins

(a) Chicken ovotransferrin; (b) human serum transferrin; (c) rabbit serum transferrin. E_0 is defined as the mid-point of the absorption edge $\chi(E)$ is the normalized e.x.a.f.s.

Fig. 2. (a) Fe K-edge e.x.a.f.s. and (b) Fourier transforms of the *N*-terminal iron-binding fragment from chicken ovotransferrin

Continuous lines represent the experimental spectra, broken lines the simulated spectra. Experimental spectrum is shifted by $\Delta E_0 = 26.2$ eV, as was required for theoretical simulation. A value of 0.22 was obtained for the fit-index parameter. Data range was 35–108 nm⁻¹ (3.5–10.8 Å⁻¹). E.x.a.f.s. is presented as $\chi(k) \times k^2$ plotted versus k , where k is in Å⁻¹ (equivalent to 10 nm⁻¹).

diferic human serum transferrin attenuates most rapidly, showing little sign of a third maximum, possibly reflecting greater disorder in the iron environment in the human transferrin, or marginal differences in the ligand distances between the two sites.

Fig. 3. (a) Fe K-edge e.x.a.f.s. and (b) Fourier transform of the *C*-terminal iron-binding fragment from chicken ovotransferrin

Continuous lines represent the experimental spectra, broken lines the simulated spectra. ΔE_0 was 26.2 eV. A value of 0.09 was obtained for the fit-index parameter.

Diferic rabbit serum transferrin shows a somewhat narrower second e.x.a.f.s. peak, whose maximum is shifted towards higher photoelectron energy. No attempt has been made to analyse this spectrum, as data are from only a single scan and the signal-to-noise ratio is limited.

Figs. 2 and 3 show the e.x.a.f.s. spectra and Fourier transforms for the *N*-terminal and *C*-terminal fragments of chicken ovotransferrin respectively. Both have good

Fig. 4. (a) Fe K-edge e.x.a.f.s. and (b) Fourier transforms of the diferric chicken ovotransferrin

Continuous lines represent the experimental spectra, broken lines the simulated spectra. ΔE_0 was 26.9 eV. A value of 0.20 was obtained for the fit-index parameter.

signal-to-noise ratios, and they show similar e.x.a.f.s. profiles, thus suggesting the two sites to be similar. In the transferrins the two metal-binding sites are known to differ in several important respects, including pH-stability (Princiottio & Zapolski, 1975; Lestas, 1976), Cu(II) and Fe(III) e.p.r. spectra (Zweier, 1978; Butterworth *et al.*, 1975) and their binding constants for iron (Aisen *et al.*, 1978). The e.x.a.f.s. spectra for the *N*-terminal and *C*-terminal fragments of chicken ovotransferrin show similar profiles but differ from each other in the second beat region [53–70 nm⁻¹ (5.3–7 Å⁻¹)], where the shoulder

on the rising edge of the beat is better defined in the case of the *N*-terminal fragment compared with the *C*-terminal fragment. A closer examination of the other spectra reveals changes in the same region; for example in the spectrum for diferric human serum transferrin this shoulder is almost non-existent.

The large number of beats in the e.x.a.f.s. profiles suggests that contributions from a number of shells (distances) of atoms are present. This is further confirmed by the Fourier transforms, which show contributions from atoms up to approx. 0.45 nm (4.5 Å) from the iron atom. The broad nature of the first beat in the e.x.a.f.s. pattern $K = 40\text{--}55\text{ nm}^{-1}$ (4–5.5 Å⁻¹) is typical of more than one radius present in the first shell. This is clearly shown in the Fourier transforms, where a shoulder on the main peak is observed for the *C*-terminal fragment and a small peak at approx. 0.17 nm (1.7 Å) for the *N*-terminal fragment.

Detailed analysis of the e.x.a.f.s. spectra shows that the main differences in the *C*-terminal and *N*-terminal fragments arise because of this shell. Theoretical simulations of the spectra shown in Figs. 2–4 are the optimum fits to the data obtained by using parameters given in Table 1.

Theoretical calculations were performed by gradually including shells of atoms to simulate the observed experimental spectra. Thus the atoms listed in Table 1 represent the minimum number of atoms required for the simulation. For the first shell a total of six low-*Z* atoms (nitrogen and oxygen) were required, and for the second and third shells another 33 low-*Z* atoms needed to be included. Two separate distances, corresponding to two atoms at approx. 0.185 nm (1.85 Å) and four atoms at approx. 0.204 nm (2.04 Å) gave a satisfactory simulation for the first shell. The e.x.a.f.s. technique, however, is not sufficiently discriminating to enable the nature of these six first-shell atoms to be assessed unambiguously, particularly when they are located at two distances within 0.02 nm (0.2 Å), so that models involving two oxygen atoms at approx. 0.185 nm (1.85 Å) plus two oxygen and two nitrogen atoms at approx. 0.204 nm (2.04 Å) or alternatively two oxygen atoms at approx. 0.185 nm (1.85 Å) and four nitrogen atoms at approx. 0.204 nm (2.04 Å), for example, give equally good simulations. A comparison with known Fe(III) octahedral complexes (Holt *et al.*, 1979; Heistand *et al.*, 1982; Lauffer *et al.*, 1983) suggests the assignment of the shorter distance to Fe–O (phenolic) whereas the longer distance is consistent with Fe–O (carboxylate) and/or Fe–N. In the Fe(III) complex *NN'*-ethylenebis(*o*-hydroxyphenylglycine) (Bailey *et al.*, 1981) the mean Fe–O (phenolic), Fe–O (carboxylate) and Fe–N distances are 0.191 ± 0.002 nm (1.91 ± 0.02 Å), 0.204 ± 0.004 nm (2.04 ± 0.04 Å) and 0.215 ± 0.002 nm (2.15 ± 0.02 Å) respectively. In the Fe(III)–methanol complex with *N*-{2-[(*o*-hydroxyphenyl)glycino]ethyl}salicylideneimine (Carrano *et al.*, 1985) the mean Fe–O (phenolic) distance, 0.190 ± 0.0001 nm (1.90 ± 0.01 Å), is again shorter than the mean Fe–N, 0.215 ± 0.002 nm (2.15 ± 0.02 Å); the Fe–O (methanol) separation is 0.205 ± 0.001 nm (2.05 ± 0.01 Å). The longer distance could also correspond to Fe–O (water), 0.209 ± 0.002 nm (2.09 ± 0.02 Å) (Thundarathil *et al.*, 1977).

Table 1 shows that for all the spectral simulations the nature and distances of the atoms remain essentially the same, although the shorter Fe–ligand distance changes

Table 1. Parameters used to simulate the e.x.a.f.s. spectra of the C-terminal and N-terminal iron-binding fragments from chicken ovotransferrin and of the diferric protein

R is the distance of atoms from Fe atom in nm (10 Å) and σ^2 is the correlated Debye–Waller term in nm² (100 Å). E_0 is defined as the mid-point of the absorption edge.

Shell	Atom	No.	C-Terminal fragment of ovotransferrin		N-Terminal fragment of ovotransferrin		Diferric ovotransferrin	
			R (nm)	$2\sigma^2$ (nm ²)	R (nm)	$2\sigma^2$ (nm ²)	R (nm)	$2\sigma^2$ (nm ²)
1	O	2	0.187	0.00005	0.183	0.00005	0.185	0.00006
	N	4	0.206	0.00005	0.204	0.00002	0.204	0.00003
2	C	3	0.291	0.00012	0.293	0.00013	0.294	0.00011
	C	5	0.314	0.00010	0.321	0.00011	0.319	0.00014
	C	3	0.341	0.00006	0.347	0.00003	0.345	0.00002
3	C	4	0.399	0.00002	0.405	0.00008	0.404	0.00007
	N	6	0.419	0.00003	0.421	0.00005	0.421	0.00003
	O	6	0.430	0.00015	0.429	0.00007	0.432	0.00011
	C	6	0.456	0.00008	0.456	0.00005	0.457	0.00008
Fit-index parameter (k^2 weighting)			0.085		0.220		0.197	
E_0			26.21 eV		26.21 eV		26.88 eV	

by 0.004 nm (0.04 Å) in the two ovotransferrin fragments and takes an intermediate value in diferric chicken ovotransferrin. This short distance may correspond to Fe–tyrosine. In the second shell a minimum of 11 carbon atoms are required, three at approx. 0.294 ± 0.001 nm (2.94 ± 0.01 Å), five at approx. 0.319 ± 0.001 nm (3.19 ± 0.01 Å) and three at approx. 0.345 ± 0.001 nm (3.45 ± 0.01 Å); some of the shorter values may correspond to histidine $C_{(\beta)}$ carbon atoms. In the CuZn superoxide dismutase, where Cu is known to ligate to histidine residues, $C_{(\beta)}$ atoms were found at 0.292 and 0.305 nm (2.92 and 3.05 Å) (Blackburn *et al.*, 1984); the Cu– $N_{(\alpha)}$ distance is 0.20 nm (2.0 Å). This splitting of the Cu– $C_{(\beta)}$ separations was taken to indicate twisting of the histidine ring relative to the Cu– $N_{(\alpha)}$ bond. However, tyrosine ligands, if the Fe–O–C angle is about 130° [cf. $128.7 \pm 0.5^\circ$ in the NN' -ethylenebis-(*o*-hydroxyphenylglycine) (Bailey *et al.*, 1981)], would also give an Fe– $C_{(\beta)}$ separation in the region of 0.29 nm (2.9 Å). The Fe– $C_{(\gamma)}$ separation will depend on the inclination of the phenyl ring to the plane defined by Fe–O– $C_{(\beta)}$. Thus the carbon atoms observed in the range 0.29–0.34 nm (2.9–3.4 Å) may originate from the histidine and/or tyrosine residues.

At this stage we can only speculate as to the precise nature of the ligands at the iron-binding sites of transferrin. Models involving two or three histidine residues cannot be eliminated. There is evidence from the present spectra of sufficient numbers of atoms to account for two tyrosine residues as well as two histidine residues, and the two Fe–ligand distances of approx. 0.185 nm (1.85 Å) are not inconsistent with Fe–phenolate-type linkage.

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