X.a.f.s. studies of chicken dicupric ovotransferrin

Richard C. GARRATT,*†‡§ Robert W. EVANS,† S. Samar HASNAIN,*|| Peter F. LINDLEY‡ and Robert SARRA†

*Molecular Biophysics Group, Daresbury Laboratory, Daresbury, Warrington WA4 4AD, Cheshire, †Division of Biochemistry, United Medical and Dental Schools, Guy's Hospital, London SE1 9RT, and ‡Department of Crystallography, Birkbeck College, Malet St., London WC1E 7HX, U.K.

A comparison of Cu K-edge x.a.f.s. spectra with that of the equivalent Fe K-edge for chicken ovotransferrin (COT) indicates that the metal ions occupy essentially the same binding sites in the protein. However, in the case of the Cu²⁺ complex the metal appears to have reduced co-ordination. Changes are observed in the x.a.f.s. of 90 % -saturated COT (Cu_{1.8}COT) on freeze-drying. The three-dimensional X-ray structures of rabbit serum transferrin and human lactoferrin have shown that the ferric cations are co-ordinated by four protein ligands and a bidentate carbonate anion in a distorted octahedral arrangement [Anderson, Baker, Dodson, Norris, Rumball, Waters & Baker (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1768–1774; Anderson, Baker, Norris, Rice and Baker (1989) *J. Mol. Biol.* **209**, 711–734; Bailey, Evans, Garratt, Gorinsky, Hasnain, Horsburgh, Jhoti, Lindley, Mydin, Sarra & Watson (1988) *Biochemistry* **27**, 5804–5812]. This structural information, together with the differences in e.x.a.f.s. spectra for solution and freeze-dried samples of diferric COT [Hasnain, Evans, Garratt & Lindley (1987) *Biochem. J.* **247**, 369–375] suggests that the synergistic carbonate anion may be capable of behaving as a unidentate linkage to the Cu²⁺ in the dicupric complex. Data for Cu_{1.8}COT are consistent with only three protein ligands bound to Cu²⁺, monodentate binding of the synergistic anion in one lobe and its bidentate binding in the other lobe. Such flexibility in the anion co-ordination may be a requirement for the uptake and release of metals by the transferrins.

INTRODUCTION

The Cu²⁺ complexes of transferrins have been studied extensively. In an analogous manner to Fe³⁺, the specific binding of Cu²⁺ to transferrin over the pH range 7–9 has a stoichiometry of 2:1 and requires the presence of a synergistic anion (Warner & Weber, 1953; Tan & Woodworth, 1969; Zweier & Aisen, 1977). Fe will displace Cu²⁺ from the dicupric complex, but not vice versa (Tan & Woodworth, 1969). These observations have led to the conclusion that Cu²⁺ occupies the normal Fe-binding sites of the protein and that it utilizes identical ligands.

On this basis many spectroscopic investigations of the Cu²⁺-substituted protein have been undertaken in order to probe the nature of the metal binding sites. E.s.r. spectroscopy correctly identified the presence of only one nitrogen ligand over the pH range 7–9, but predicts that, above pH 9.2, only one Cu²⁺ will bind and to a site involving either three or four nitrogen ligands (Zweier & Aisen, 1977; Zweier, 1978, 1980; Froncisz & Aisen, 1982). Subsequent electron-spin-echo studies (Zweier *et al.*, 1979, 1982) identified the nitrogen ligand in the pH 7–9 complex as histidine. Below pH 6, or in the absence of a synergistic anion, only a non-specific complex is formed.

The visible absorption spectra of the Cu²⁺ complex show a greater dissimilarity in λ_{max} for the two lobes of the molecule than is observed for the iron complexes (Williams, 1974, 1975; Yamamura *et al.*, 1984). Maxima are observed at 450 nm and at 430–435 nm in the N- and C-terminal binding sites respectively. The yellow colour of the complex is attributable to the ligation of two tyrosine residues (Pecoraro *et al.*, 1981). Spin-echo studies of the Cu²⁺-oxalate-transferrin complex implicate the direct ligation of the anion to the metal (Zweier *et al.*, 1979, 1982), but this could not be confirmed for the complex involving carbonate

as the anion using either the spin-echo or electron-nuclear-double-resonance (ENDOR) techniques (Zweier *et al.*, 1979, 1982; Roberts *et al.*, 1983).

The recent X-ray crystal-structure analyses of human lactoferrin (Anderson *et al.*, 1987, 1989), rabbit serum transferrin (Bailey *et al.*, 1988) and the N-terminal lobe half-molecule of rabbit serum transferrin (Sarra *et al.*, 1990), have conclusively shown that Fe³⁺ ions in both lobes are approximately octahedrally co-ordinated by two tyrosine residues, a histidine residue and an aspartic acid residue, and a bidentate carbonate anion. This structural information provides a firm template for the interpretation of spectral data. In view of the frequent application of metal substitution in the study of metalloproteins, it is of considerable interest to compare the spectra of the different metal complexes. In the present paper we report an e.x.a.f.s. study of Cu²⁺-loaded COT for both solution and freeze-dried protein samples and their probable structural interpretation.

MATERIALS AND METHODS

COT was isolated from hen's egg white by a modification of the method of Williams (1968) and made Fe³⁺-free by dialysing against two changes of 0.1 M-citric acid, pH 4.7. Ferric citrate so produced was removed by ion-exchange using Amberlite monobed MB-1 resin, and the protein was dialysed exhaustively against deionized water.

Protein was dissolved in 0.1 M-Hepes/0.05 M-NaHCO₃/0.1 M-KCl, pH 7.5, at approx. 10 mg/ml. A sample of the protein was titrated with 0.01 M-CuSO₄, and the increase in A₄₄₀ monitored to determine the volume required to 90 % saturate the remainder of the sample. The undersaturation was to ensure that adventitious Cu²⁺ did not bind at positions other than the specific

Abbreviations used: ENDOR, electron nuclear double resonance; COT, chicken ovotransferrin; Cu_{1.8}COT, 90 % -saturated chicken ovotransferrin; x.a.n.e.s., X-ray absorption near-edge structure.

§ Present address: Instituto de Física e Química de São Carlos, Universidade de São Paulo, CP-369, 13560 São Carlos-SP, Brazil.

|| To whom correspondence should be addressed.

Fig. 1. X.a.n.e.s. (b) and e.p.r. (a) spectra of the solution and freeze-dried $\text{Cu}_{1.8}\text{COT}$

metal-binding sites (Jones & Perkins, 1965; Zweier & Aisen, 1977). After Cu^{2+} loading the sample was left for 3 h at room temperature before desalting into 0.05 M- $(\text{NH}_4)_2\text{SO}_4$ and freeze-drying.

The sample for e.x.a.f.s. measurements was reconstituted in the original buffer at a concentration of approx. 400 mg/ml, and its e.s.r. spectrum was recorded before X-ray exposure. The e.s.r. spectrum was checked after X-ray exposure to ensure sample viability. E.s.r. spectra were obtained as described previously (Hasnain *et al.*, 1987).

X-ray-absorption measurements were made on Station 9.2 at the Synchrotron Radiation Source, Daresbury; the beam energy was 2 GeV, and the average circulating current was 150 mA; the three-pole wiggler magnet was operational at a 5 T magnetic field. An Si(220) double-crystal monochromator was employed at 50% crystal detuning, and fluorescence detection was by three scintillation counters with Ni foil filters. The data used in the analysis were the average of six scans. Data reduction was performed using the EXCALIB and EXBACK routines in the e.x.a.f.s. program suite at Daresbury.

Data analysis utilized rapid-curve-wave single- and multiple-scattering theory (Gurman *et al.*, 1984, 1986) in the program

Fig. 2. E.x.a.f.s. (a) and Fourier transform (b) of solution $\text{Cu}_{1.8}\text{COT}$

The experimental spectrum (—) is shown together with the best theoretical fit (---) using the parameters in Table 1. Note $1 \text{ Å} \equiv 0.1 \text{ nm}$ (SI unit).

EXCURV90 on the raw (i.e. unfiltered) k^3 weighted e.x.a.f.s. data. Phase shifts were those used for the Cu K-edge studies of Cu complexes and Cu_2Zn_2 superoxide dismutase (Blackburn *et al.*, 1987; Strange *et al.*, 1987). An amplitude reduction factor of 0.7, due to multi-electron excitation, was used in the same way as in the previous Cu K-edge x.a.f.s. studies. The imaginary part of the photoelectron self-energy which defines the elastic electron mean free path was set to -1 eV . The photoelectron energy zero, E_0 , was treated as a single overall parameter for the multi-shell fit, as this has been found sufficient for cases where the nature of back-scattering atoms is similar. Fourier transforms were calculated within the plane-wave approximation where they were corrected for the phase shift of the first shell of back-scattering atoms. This gives peaks in the Fourier transform approximately at the correct radial distances (Gurman & Pendry, 1976). The Fourier transform was used for judging the quality of a fit (Perutz *et al.*, 1982). The quality of the structural models is further assessed by the fit index (Binsted *et al.*, 1987) and the agreement factor, R (N. Binsted, R. W. Strange & S. S. Hasnain, unpublished work) according to the equation:

$$R = \sum_i 1/\sigma_i [|\chi_i^{\text{exp}}(k) - \chi_i^{\text{th}}(k)|] \times 100(\%)$$

where χ^{exp} and χ^{th} refer to the experimental and theoretical e.x.a.f.s. spectra and $1/\sigma = k_i^n / \sigma_j^n k^n |\chi_j^{\text{exp}}(k)|$, where n is the weighting.

RESULTS AND DISCUSSION

X-ray absorption near-edge structure (x.a.n.e.s.) and e.s.r. spectra of the freeze-dried and solution 90%-saturated COT ($\text{Cu}_{1.8}\text{COT}$) are shown in Fig. 1, demonstrating significant changes in the stereochemistry of the Cu^{2+} ion on freeze-drying. In order to identify the primary structural difference, e.x.a.f.s. data for both the freeze-dried and solution proteins have been analysed separately using the recently developed constrained-refinement approach of e.x.a.f.s. data analysis (Hasnain & Strange, 1990).

The e.x.a.f.s. spectrum of $\text{Cu}_{1.8}\text{COT}$ is shown in Fig. 2. The lower frequency of the principal wave in the experimental spectrum compared with that observed for Fe_2COT suggests that the first shell distance is shorter in $\text{Cu}_{1.8}\text{COT}$. The mean first shell bond distance of 0.19 nm (1.90 Å) is shorter than that observed for the iron, 0.2 nm (2.0 Å), and is consistent with the change in frequency of the principal wave. The co-ordination number of the first shell refines to 4.5 ± 0.3 if oxygen atoms are assumed to be the co-ordinating atoms.

The full analysis of the spectrum initially assumed ligands identical with those for iron, i.e. two tyrosine residues, one histidine residue and three oxygen atoms originating from an aspartic acid residue and the synergistic anion. For the tyrosine and histidine residues, atoms up to ~ 0.4 nm (~ 4 Å) were included in the simulation. These groups were treated as rigid groups, and thus constrained refinement was carried out incorporating multiple-scattering effects (R. C. Garratt, S. S. Hasnain, P. F. Lindley & R. W. Evans, unpublished work; Hasnain & Strange, 1990).

Because of high correlations on the refinement of the first-shell parameters, it is not possible to assign particular distances to Cu–N or Cu–O unambiguously and, indeed, the only real

Table 1. Final structural parameters for the simulation of $\text{Cu}_{1.8}\text{COT}$

R is the interatomic distance in Å (note 1 Å $\equiv$ 0.1 nm), and A the Debye–Waller factor quoted as $2\sigma^2$ (in Å²). The O/N shell at ~ 0.25 nm (~ 2.5 Å) makes marginal contribution (see the text for details).

(a) Tyrosine

Atom	Solution $\text{Cu}_{1.8}\text{COT}$			Freeze-dried CuCOT		
	R	A	Angle (°)	R	A	Angle (°)
$2 \times \text{O}_\text{H}$	1.90	0.006	—	1.90	0.006	—
$2 \times \text{C}_\text{Z}$	2.77	0.006	117	2.77	0.006	117
$2 \times \text{C}_\text{E1}$	2.89	0.008	87	2.89	0.008	87
$2 \times \text{C}_\text{E2}$	4.09	0.008	146	4.09	0.008	146

(b) Anion and aspartic acid oxygen atoms

Atom/no.	R	A	Atom/no.	R	A
$1.15 \times \text{O}$	1.81	0.003	$1.46 \times \text{O}$	1.85	0.003
$1.46 \times \text{O}$	1.96	0.003	$1.12 \times \text{O}$	2.06	0.003

(c) O/N shell

Atom/no.	R	A	Atom/no.	R	A
$0.3 \times \text{O}$	2.59	0.003	0.5	2.50	0.008
ΔE_0 (eV)...	22.5			23.9	
R -factor (%)...	26.9			26.6

Fig. 3. E.x.a.f.s. (above) and Fourier transform (below) of freeze-dried powder of $\text{Cu}_{1.8}\text{COT}$

The experimental data (—) are shown together with the constrained refinement fit (---) using the parameters in Table 1. Note 1 Å $\equiv$ 0.1 nm.

justification for having separate shells is to enable the calculation of multiple-scattering effects. The multiple-scattering contribution from the outer-shell atoms of the two tyrosine residues and a histidine residue exceeds what is observed in the $\text{Cu}_{1.8}\text{COT}$ samples, suggesting that either one of the tyrosine or the histidine residues is moved significantly from the metal co-ordination compared with the Fe_2COT . A multiple-scattering refinement of the model, where the co-ordination numbers for histidine and tyrosine residues were refined, resulted in the removal of histidine. We note that if histidine was moved to a distance of 0.25 nm (2.5 Å) or more, then its contribution would not be easily identifiable.

Further refinement was carried out with a model based on two tyrosine residues, an aspartic acid oxygen atom and up to two additional oxygen atoms from the synergistic anion. In the case of tyrosine residues, atoms up to ~ 0.4 nm (~ 4 Å) were included and refined as a single unit using the constrained-refinement method. For aspartic acid and other oxygen atoms, distance and co-ordination numbers were refined. Debye–Waller factors for the two oxygen shells were kept the same, as they are at almost the same distance away from the metal and as such should form ‘equally’ strong bonds and thus disorder for the two should be very similar. The best simulation to the data is shown in Fig. 2, and the final structural parameters are given in Table 1.

The data for freeze-dried protein were modelled with the above parameters, which gave an R factor of 50%. As the outer-shell

Fig. 4. The hydrogen-bonding network at the metal-binding site of the *N*-terminal lobe half-molecule of rabbit serum transferrin

Residues Asp-63, Tyr-188, His-249 and Arg-124 were located from Fourier syntheses at 0.23 nm (2.3 Å) resolution; Tyr-95 is not shown. Disruption to this water network may lead to conformational changes in the metal-anion interaction. Water molecules in the vicinity of Arg-124 are shown as small spheres and form part of an extended network of water molecules in the cleft between the two domains which constitute the half-molecule. The metal anion is shown as the larger dotted sphere in the top left hand side of the Figure and the synergistic carbonate anion is immediately to the left of it.

Fig. 5. Probable structure of the Cu^{2+} -binding sites in the *N*- and *C*-lobes of $\text{Cu}_{1.8}\text{COT}$

contribution appeared reasonable in the Fourier transform, the tyrosine contribution was not refined. Refinement of the oxygen shells, presumed to originate from aspartic acid and the bi-carbonate, results in lengthening of both shells of atoms; also, the co-ordination number for these two shells changes somewhat. Final parameters for the best simulation [Fig. 3 ($R = 26\%$)] are given in Table 1.

In both cases, an additional shell of oxygen/nitrogen can be accommodated, which refines to 0.25 nm (2.5 Å) [from a starting position of 0.23 nm (2.3 Å)], with only a marginal improvement, 1%, in the R factor.

The e.x.a.f.s. alone does not allow an unambiguous interpretation of the above observations, but, in conjunction with the known X-ray structures of iron-loaded transferrins and a number of Cu(II) chemical compounds (Prout *et al.*, 1971; McFadden *et al.*, 1975; Wong *et al.*, 1976; Antolini *et al.*, 1982), it is possible to rationalize the results as follows. Studies on freeze-dried

Fe_2COT indicated a reduction in the co-ordination of the iron with loss of water (Hasnain *et al.*, 1987). Since there is no crystallographic evidence for direct ligation of water to Fe^{3+} , this can only be explained as an indirect consequence of the loss of water. Examination of the crystal structure suggests that a likely explanation is a disruption of the water structure in the vicinity of Arg-124 (human serum transferrin numbering), followed by an alteration of the hydrogen-bonding of this residue to the synergistic carbonate anion. Fig. 4 shows the hydrogen-bonding network at the metal-binding site for the *N*-terminal lobe half-molecule of rabbit serum transferrin, together with water molecules close to Arg-124. The disruption of the hydrogen-bonding network could be accompanied by a movement of the arginine residue away from the metal site into the cleft between the two domains, formerly occupied by water molecules; model-binding studies indicate that, in the case of bifunctional synergistic anions such as oxalate, some movement of this residue is required in any case for spatial reasons. A concomitant movement of the carbonate anion and, in particular, a rotation in its own plane, could cause this moiety to become monodentate, thereby leading to a five-co-ordinate ferric complex; subsequent rehydration leads to the reformation of the six-co-ordinate environment with its typical e.x.a.f.s. and e.s.r. spectra (Hasnain *et al.*, 1987). In the case of the dicupric transferrin complex the presence of a monodentate carbonate anion, presumably at the more labile *N*-terminal site, would be consistent with the e.x.a.f.s. simulations. It is difficult to show from the e.x.a.f.s. data alone which of the protein ligands has moved away substantially, but the multiple-scattering calculations suggest that it is one of the ring residues, either tyrosine or histidine, most probably histidine. The requirement for aspartic acid for Cu^{2+} binding is known from the human variant transferrin that lacks the aspartic acid and does not bind Cu^{2+} (R. W. Evans, unpublished work). The proposed differences in the Cu -anion co-ordination at the two sites (Fig. 5) may aid in explaining why the visible spectra show a considerable difference in λ_{max} (~ 20 nm), whereas the spectra for the two iron-loaded sites are effectively indistinguishable (Williams, 1974, 1975; Yamamura *et al.*, 1984). Furthermore, differences in the mode of anion binding for the two metals has been indirectly implied by the observation that oxalate can serve as a synergistic anion at only one of the two sites of Cu^{2+} -loaded human serum transferrin, but binds effectively to both in the case of iron-loaded protein (Zweier *et al.*, 1979).

Apparent differences can thus be observed in the e.x.a.f.s. spectra of copper- and iron-loaded ovotransferrin. These differences highlight the advisability of using the native metal whenever possible in such studies, since even if the metal binds to the same site, the details of the site may be perturbed and adjusted to suit the chemistry of the metal atom. Presumably the ultimate geometry is a compromise between that demanded by the metal and that provided by the protein. The proposed flexibility in the co-ordination of the synergistic anion may well play an important role in the mechanisms of metal uptake and release.

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REFERENCES

- Anderson, B. F., Baker, H. M., Dodson, E. J., Norris, G. E., Rumball, S. V., Waters, J. M. & Baker, E. N. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1768–1774
- Anderson, B. F., Baker, H. M., Norris, G. E., Rice, D. W. & Baker, E. N. (1989) J. Mol. Biol. **209**, 711–734

- Antolini, L., Marcotrigiano, G., Menabue, L., Pellacani, G. C. & Saladini, M. (1982) *Inorg. Chem.* **21**, 2263–2267
- Bailey, S., Evans, R. W., Garratt, R. C., Gorinsky, B., Hasnain, S. S., Horsburgh, C., Jhoti, H., Lindley, P. F., Mydin, A., Sarra, R. & Watson, J. L. (1988) *Biochemistry* **27**, 5804–5812
- Binsted, N., Cook, S. L., Evans, J., Greaves, G. N. & Price, R. J. (1987) *J. Am. Chem. Soc.* **109**, 3669–3678
- Blackburn, N. J., Strange, R. W., McFadden, L. M. & Hasnain, S. S. (1987) *J. Am. Chem. Soc.* **109**, 7162–7168
- Freyberg, D. P., Mockler, G. M. & Sinn, E. (1979) *Aust. J. Chem.* **32**, 321–329
- Francisz, W. & Aisen, P. (1982) *Biochem. Biophys. Acta* **700**, 55–58
- Gurman, S. J. & Pendry, J. B. (1976) *Solid State Commun.* **28**, 287–290
- Gurman, S. J., Binsted, N. & Ross, I. (1984) *J. Phys. Chem.* **17**, 143–151
- Gurman, S. J., Binsted, N. & Ross, I. (1986) *J. Phys. Chem.* **19**, 1845–1861
- Hasnain, S. S. & Strange, R. W. (1990) in *Synchrotron Radiation and Biophysics* (Ed. Hasnain, S. S.), pp. 104–121, Ellis Horwood Ltd., Chichester
- Hasnain, S. S., Evans, R. W., Garratt, R. C. & Lindley, P. F. (1987) *Biochem. J.* **247**, 369–375
- Jones, H. D. C. & Perkins, D. J. (1965) *Biochem. Biophys. Acta* **100**, 122–127
- McFadden, D. L., McPhail, A. T., Garner, C. D. & Mabbs, F. E. (1975) *J. Chem. Soc. Dalton Trans.* 263–268
- Pecoraro, V., Harris, W., Carrano, C. & Raymond, K. (1981) *Biochemistry* **20**, 7033–7039
- Perutz, M. F., Hasnain, S. S., Duke, P. J., Sessler, J. L. & Hahn, J. E. (1982) *Nature (London)* **295**, 535–538
- Prout, C. K., Allison, G. B. & Rossotti, F. J. C. (1971) *J. Chem. Soc. A*, 3331–3335
- Roberts, J. E., Brown, T. G., Hoffman, B. M. & Aisen, P. (1983) *Biochem. Biophys. Acta* **747**, 49–54
- Sarra, R., Garratt, R., Gorinsky, B., Jhoti, H. & Lindley, P. (1990) *Acta Crystallogr.* **B46**, 763–771
- Strange, R. W., Blackburn, N. J., Knowles, P. F. & Hasnain, S. S. (1987) *J. Am. Chem. Soc.* **109**, 7157–7162
- Tan, A. T. & Woodworth, R. C. (1969) *Biochemistry* **8**, 3711–3716
- Warner, R. C. & Weber, I. (1953) *J. Am. Chem. Soc.* **75**, 5094–5101
- Williams, J. (1968) *Biochem. J.* **108**, 57–67
- Williams, J. (1974) *Biochem. J.* **141**, 745–752
- Williams, J. (1975) *Biochem. J.* **149**, 237–244
- Wong, R. Y., Palmer, K. J. & Tomimatsu, Y. (1976) *Acta Crystallogr.* **B32**, 567–571
- Yamamura, T., Hagiwara, S., Nakazato, K. & Satake, K. (1984) *Biochem. Biophys. Res. Commun.* **119**, 298–304
- Zweier, J. L. (1978) *J. Biol. Chem.* **253**, 7616–7621
- Zweier, J. L. (1980) *J. Biol. Chem.* **255**, 2782–2789
- Zweier, J. L. & Aisen, P. (1977) *J. Biol. Chem.* **252**, 6090–6096
- Zweier, J. L., Aisen, P., Peisach, J. & Mims, W. B. (1979) *J. Biol. Chem.* **254**, 3512–3515
- Zweier, J. L., Peisach, J. & Mims, W. B. (1982) *J. Biol. Chem.* **257**, 10314–10316

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