THE TWO METAL-BINDING SITES OF HUMAN SEROTRANSFERRIN AND LACTOTRANSFERRIN

Differences in histidine coordination as revealed by EPR of cupric complexes

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1. Introduction

We have demonstrated in a previous paper [1], using the diethyl pyrocarbonate (DEP) as specific reagent, that in both human serotransferrin (STF) and lactotransferrin (LTF) histidine residues are directly involved in the iron-binding sites. It was also demonstrated that one of the binding sites contains 3 reactive histidine residues in STF and only one in LTF whereas the second site in both proteins was not affected by the DEP reaction.

EPR spectra of ferric transferrin complex distinguish the 2 iron-binding sites [2–5] but they do not exhibit the hyperfine structure necessary for the identification of nitrogenous ligands. The nitrogen hyperfine structure is observed in the EPR spectra of copper-substituted transferrin [5,6]. Two different spectra were reported by Aasa et al. and Aisen et al. [4,5] for copper–STF in neutral solutions. They observed a signal corresponding to 3 or 4 nitrogen atoms bound to the cupric ions in a bicarbonate free system but to only 1 nitrogen atom in the presence of bicarbonate. However, the 2 binding sites could not be distinguished in these systems and the possibility of obtaining stable bicarbonate-free complexes has been questioned by Price and Gibson [7] and even eliminated for the ferric complexes.

EPR spectra of cupric complexes have been recorded in the present work in order to differentiate the 2 metal-binding sites of human STF and LTF using their different histidine reactivity toward DEP. We demonstrate that:

(i) ^{63}Cu–STF and ^{63}Cu–LTF may exhibit EPR spectra with 2 components, a 'sharp' one corresponding to the binding of one nitrogen atom and a 'broad' one corresponding to the binding of 3 or 4 nitrogen atoms on the cupric ions. The latter mode of binding shows a greater difference between ^{63}Cu–STF and ^{63}Cu–LTF than the former.

(ii) After carbethoxylation of apo-STF and apo-LTF, only 1 cupric ion can be bound, corresponding to the disappearance of the 'broad' EPR component.

2. Materials and methods

STF was prepared by the method of Roop and Putnam [8] and LTF [9] was isolated from human milk according to Chéron et al. [10]. Their apo-derivatives were obtained by dissolving native proteins in 0.1 M phosphate buffer, pH 4.6, containing 1% EDTA, dialyzing several times against the same buffer to remove free metal ions, then, against distilled

Abbreviations: His-CM, carbethoxylated histidine, HisCM-LTF, apo-lactotransferrin with carbethoxylated histidine; HisCM ^{63}Cu–LTF, carbethoxylated His-LTF after cupric saturation; Fe^{3+}–HisCM-LTF, ferric complex of carbethoxymethylated lactotransferrin

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water to remove the EDTA. After lyophilisation the amount of iron was determined by using ferrozine reagent [11].

The lyophilised apoderivatives were dissolved in 0.1 M Tris–HCl, containing 0.05 M sodium bicarbonate, pH 8.6. $^{63}CuCl_2$ in Tris–HCl sodium bicarbonate buffer was used as cupric source and 5 μg ^{63}Cu/mg protein was added to the solutions. $^{63}CuCl_2$ was prepared by action of gaseous Cl_2 on metallic ^{63}Cu purchased from CEA, France.

Excess of reagents were removed by gel filtration on a Sephadex G-25 column. The amount of copper in the lyophilised metal–protein complexes was determined according to Peterson et al. [12]. Protein concentrations were estimated by the method of Lowry et al. [13] using bovine serum albumin as a standard.

Specific modification of histidine residues was measured by difference spectra using a Beckman DB-6 spectrophotometer as previously described [1].

The EPR spectra were obtained at the temperature of liquid nitrogen in deuterium oxide, 0.1 M KCl adjusted to uncorrected p*D* 7.5 with NaOD. They were recorded on a Varian E-3 spectrometer operated at 9.15 GHz using a non-saturating microwave power and a field modulation adapted to the spectral resolution. The *g*-values were calibrated with a DPPH standard.

3. Results

3.1. *Cupric saturation of apo-STF, apo-LTF and their carbethoxylated derivatives*

The determination of cupric ions in the saturated ^{63}Cu–STF and ^{63}Cu–LTF gave a ratio of 2 metal ions/molecule protein. The optical spectra of these cupric complexes exhibit a maximum of absorption at 432 nm. The absorbance values $E^{1\%}_{1\,cm}$ are given in table 1.

Carbethoxylation was carried out on apo-STF, apo-LTF and on their ^{63}Cu complexes. Six histidine residues were modified by carbethoxylation of apo-LTF and the HisCM-LTF was able to bind only 1 cupric ion. The characteristic visible absorption of this complex was similar to that of unmodified ^{63}Cu–LTF but a 40% decrease of the molar absorption was observed (table 1). When carbethoxylation was carried out on the previously saturated cupric LTF, only 5 histidine residues were modified. This result is in good agreement with the number of modified histidines in the Fe^{3+}–HisCM-LTF [1].

In the case of apo-STF, 14 histidine residues were carbethoxylated and only 1 cupric ion was found to be bound to the protein. A blue shift of 12 nm was observed in the visible spectrum and a decrease of 34% in the molar absorption was noticed.

The number of modified histidines could not be determined when carbethoxylation was carried out on cupric saturated STF, since the cupric ions were slowly removed during the reaction.

3.2. *EPR spectra*

The EPR spectra of the unmodified proteins substituted by ^{63}Cu exhibit 2 independent subspectra which are easily analyzed in terms of tetragonal symmetry (table 2; figs 1a and 2a). The parallel components of these two subspectra can be separated by their 'broad' or 'sharp' profile. The 'broad' spectrum is characterized by a larger $A^{Cu}_{\parallel}$ hyperfine

Table 1
Characteristics of the cupric complexes of STF and LTF reconstituted from unmodified and carbethoxylated (HisCM) apotransferrins

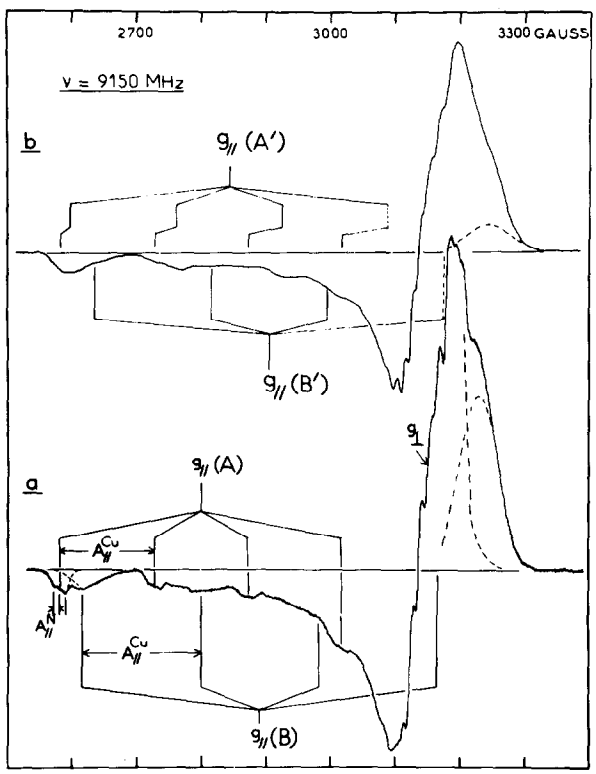
Protein	No. fixed ^{63}Cu ions	No. of His-CM	Absorbance ($E^{1\%}_{1\,cm}$ 432 nm)
^{63}Cu–STF	2	0	0.45
^{63}Cu–HisCM-STF	1	14	0.30[a]
^{63}Cu–LTF	2	0	0.50
^{63}Cu–HisCM-LTF	1	6	0.30
HisCM ^{63}Cu–LTF	2	5	0.50

[a] The value is given at the maximum of absorbance which was shifted to 420 nm

Table 2
EPR characteristics of ^{63}Cu–STF, ^{63}Cu–HisCM-STF, ^{63}Cu–LTF and ^{63}Cu–HisCM-LTF

Protein		$g_{//}$	$g_{\perp}$	$A_{//}^{^{63}Cu}$ (G)	$A_{//}^{N}$ (G)	His involved at the binding site
^{63}Cu–STF	Signal A	2.30 (7)	2.04 (9)[a]	145	9.0	1
	Signal B	2.23 (8)	2.04 (9)[a]	180	n.r.[b]	3 at least
^{63}Cu–HisCM-STF	Signal A'	2.27	2.04	150	9.0	1
^{63}Cu–LTF	Signal A	2.31 (4)	2.04 (7)[a]	140	9.4	1
	Signal B	2.17 (7)	2.04 (7)[a]	200	n.r.[b]	3 at least
^{63}Cu–HisCM-LTF	Signal A	2.31	2.04	140	9.4	1

[a] Signal A and B undistinguishable. The last figure between brackets is uncertain
[b] Not resolved



interaction and by a smaller $g_{//}$-value. It does not exhibit a well hyperfine structure arising from nitrogen interactions, but the profile is compatible with the interaction of the unpaired electron spin with at least 3 nitrogen nuclei assumming a coupling constant of 10–15 G [4]. On the other hand, the 'sharp' spectrum exhibits a hyperfine structure, well revolved for the low field line, corresponding to a single nitrogen. The perpendicular components are not sufficiently resolved to be analyzed.

The relative population of the 2 sites, as estimated from crude integration, tends to a ratio 1 : 1 for the saturated systems. It does not appear to depend significantly upon the degree of saturation of the apotransferrins.

The EPR spectra of the carbethoxylated proteins

Fig.1. EPR spectra at 77°K of ^{63}Cu substituted LTF (a) with untreated apo-LTF, (b) with carbethoxylated apo-LTF, (c) carbethoxylation carried out after saturation. A and B in the untreated complex (a) correspond to two equally populated distinct binding sites. The A site is not modified upon carbethoxylation. The reaction is not complete in (b) resulting in a population of a modified B' site. Both sites cannot be resolved in (c) though the complex includes two cupric ions.

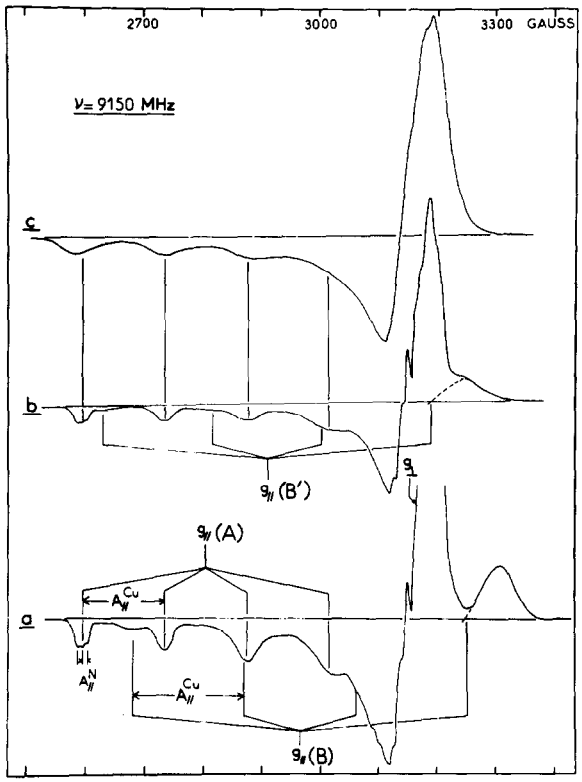


Fig.2. EPR spectra at 77°K of ^{63}Cu substituted STF (a) with untreated apo-STF, (saturated A and B site), (b) with carbethoxylated apo-STF (modified and saturated A' site). The reaction is not complete in the latter resulting in the presence of some unmodified A species and a population of a modified B' site.

are characterized by the absence (LTF) or slight modification (STF) of the 'sharp' spectrum (table 2; fig.1b and 2b) together with a decrease in intensity, down to zero, of the 'broad' spectrum. The disappearance of this signal during the carbethoxylation reaction is parallel with a progressive increase of $A_{//}^{Cu}$ and decrease of the $g_{//}$-value together with some broadening which can be assigned to sample heterogeneity.

4. Discussion

The 2 tetragonal copper signals observed simultaneously in the unmodified proteins correspond to those observed separately, depending on experimental conditions, by Aasa and Aisen [5] when corrected for the copper isotopic effect upon the hyperfine structure. These authors assigned these signals to non-distinguishable sites in the presence and absence of bicarbonate, respectively. The present investigations were carried out under atmosphere conditions and bicarbonate should have been present in any circumstance. Rather we propose that the observed 'broad' and 'sharp' signals arise from cupric ions at the 2 different binding sites. In fact, the stoichiometric ratio of saturation is near unity for the untreated proteins. Non-specific binding of cupric ions to the proteins and the presence of foreign complexes due to the buffer were carefully prevented using purification by gel filtration chromatography rather than less efficient dialysis. Furthermore the broad signal exhibits EPR characteristics quite different from those of unspecifically bound cupric ions [5] and from aqueous or buffer complexes. Unspecific buffer complexes could hardly give different spectra in the 2 protein preparations. The 'bicarbonate free' ferric complex has already been shown to be an artefact due to citrate [7]. The most convincing evidence for the assignment of the 'broad' signal to one of the specific sites of the metal-ion fixation is its behaviour upon carbethoxylation of the histidines:

(i) The population of this site decreases progressively as the reaction proceeds.

(ii) The EPR characterictics of the corresponding signal are progressively modified with an increase of the average of $g_{//}$-value, a decrease of $A_{//}^{Cu}$ together with a line broadening suggesting some heterogeneity of the site. Such an observation is consistent with a decrease of the number of nitrogen atoms coordinated on the cupric ion [14].

The relative pH lability of the 'sharp' and 'broad' copper sites of transferrins allows one to identify these sites with the sites A and B, respectively, described for vanadyl, chromium and mixed ferric STF complexes [15,16]. The absence of simultaneous observation of the 2 sites in the cupric complexes by Aasa and Aisen [5] cannot be clearly interpreted at the moment. However, it may be suggested that the

instability of the B site at acidic pH and, the conversion of the A site characteristics toward a structure similar to that of the B site in the alkaline range [15,16] may be an explanation for this different behaviour, since the preparation procedures may differ to some extent.

Site A exhibits a single coordinated nitrogen, which is not accessible to the DEP treatment even in the apoproteins. This site is quite similar, as revealed by its EPR characteristics in both STF and LTF. However, moderate changes both in the optical absorption (table 1) and EPR spectra (fig.2b) upon DEP treatment occur in the STF, probably due to indirect conformation perturbation of the metal-ion environment. In contrast, site B contains in both proteins at least 3 or more probably 4 coordinated nitrogen atoms. The nature of this site shows a greater degree of difference in STF and LTF. These differences which are observed through the EPR characteristics, (table 2) are also reflected by their relative stability with respect to the DEP treatment of the cupric proteins. There is a rapid loss of 1 cupric ion in the cupric STF with disappearance of the broad signal. In contrast, cupric LTF does not loose a metal ion although some histidine reaction occurs at the broad site. The EPR signal of this site appears superimposed on the sharp signal (fig.1c) when the reaction is completed, suggesting that one coordinated nitrogen at least has not reacted with DEP. These differences in the behavior of the 2 binding sites may be due to the number of histidine residues accessible to carbethoxylation (table 1) as already observed for the ferric complexes of the 2 transferrins [1].

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