Electron Paramagnetic Resonance Evidence for a Distinction between the Two Iron-binding Sites in Transferrin and in Conalbumin*  

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SUMMARY

The electron paramagnetic resonance spectra of aqueous solutions of both transferrin and conalbumin have been studied as a function of increasing sodium perchlorate concentration. Characteristic changes are observed which provide direct evidence for a distinction between the two metal-binding sites under certain conditions in the naturally occurring iron derivatives of each protein. This may be of physiological importance in the case of transferrin. Differences in the behavior of the two proteins were also observed.

Conalbumin, from avian egg white and transferrin, obtained from pooled human blood serum, are each capable of binding 2 atoms of iron per molecule to form similar, very stable high spin Fe(II) complexes which are amenable to study by electron paramagnetic resonance. This technique is sensitive to small changes in the electronic environment about the paramagnetic atom and much useful information about the properties of these molecules has been obtained by EPR spectroscopy (1, 2).

There is continued interest in the problem of whether or not the two iron-binding sites in each protein have the same properties. The iron-binding constants for each site are nearly identical (1, 3). However, Aisen et al. (4) have described EPR experiments which demonstrate a heterogeneity of the sites in the chromium derivative of transferrin. Evidence for dissimilar sites in a bicarbonate-free iron complex of transferrin (5) was not substantiated (6). Ryall (7) suggests by kinetic studies that the two sites in normal transferrin differ in their reactivity towards thioglycollic acid. Fletcher and Huehns (8) have shown a difference between the two sites under more physiological conditions in a bicarbonate-free iron complex of transferrin (9) and (8) signals under various conditions, both a distinction between conalbumin and transferrin, and differences between the two iron-binding sites in each protein.

EXPERIMENTAL PROCEDURE

Type I apocatalbumin was from the Sigma Chemical Company; lyophilized apotransferrin was prepared by Behringwerke and purchased from Hoechst Pharmaceuticals, Ltd. Aqueous solutions of the apoproteins were purified by dialysis versus 0.1 M NaClO₄, followed by several changes of distilled water to remove small amounts of metal-chelating agents used in the manufacture of the apoproteins. Other chemicals were of the highest purity available and used without further purification. Distilled water was used throughout. EPR spectra were measured at a frequency near 9.2 GHz (X band) using a Varian E-12 spectrometer, operating at temperatures down to 90° K. pH measurements were made with a Vibron model 30A pH meter. Ultraviolet/visible spectra were recorded on a Cary 15 spectrophotometer.

Fe₂-conalbumin and Fe₂-transferrin were prepared by addition of the calculated amount of Fe(C₃O₄)₃ to a 10% solution of the apoprotein which was usually unbuffered in water. (The molecular weight was taken as 80,000 in each case.) Solid NaHCO₃ was added for the dual purpose of increasing the pH to the desired value (generally 7.5) and also to ensure that sufficient bicarbonate was present for complete iron binding (9); the desired amount of solid NaClO₄ was then added. About 0.3 ml of this solution was transferred to a calibrated EPR tube and frozen; a separate quantity was used to record the electronic spectrum if required.

The usual method of freezing was by slow immersion into liquid nitrogen over a period of about 40 s. Plunging into an acetone-Dry Ice bath was faster but gave the same results. Experiments were also carried out in a rapid freezing apparatus (10-12) in which the solution was squirted into isopentane at -140° where it froze in about 4 ms. Again the same EPR spectra were

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‡ The abbreviation used is: EPR, electron paramagnetic resonance.
obtained as with the other methods of freezing. Thus we have found no evidence that the rate or method of freezing in any way affected the resultant EPR spectra. Any one method gave reproducible results.

RESULTS

Conalbumin—The EPR spectrum of a 10% solution of Fe₂-conalbumin in water at pH 7.3 is shown in Fig. 1A. Addition of NaClO₄ to this sample caused the features at b and B to diminish while the features at s and S grew larger and better resolved; see the spectrum shown in Fig. 1B corresponding to 0.4 M perchlorate. Further addition of NaClO₄ up to 3 M caused no further change. The features marked S and s increased by a factor of 3.4 from the sample with no perchlorate to the sample containing 0.4 M perchlorate; in the same sequence, the features marked B and b disappeared. Small but reproducible changes in the spectra at fields greater than 4 kilogauss were also observed; these are shown in Fig. 2. Clearly the broad upward-going absorption between 4 and 6.5 kilogauss is associated with B and b while the shallow depression at about 7 kilogauss is associated with S and s.

EPR spectra for the fully loaded Fe₂-conalbumin, recorded as a function of increasing NaClO₄ concentration are shown in Fig. 3. Isosbestic points are observed, the lower one being expanded in the inset.

If the conalbumin was less than fully loaded with iron, the increase in the sharp signal on adding perchlorate was greater. For example, Fig. 4 shows the S signal in 0.4 M perchlorate to be about 5.5 times greater than in water when the iron present corresponds to 3/4 of that required for saturation.

The broad signal was always present in the absence of perchlorate whether the conalbumin was dissolved in water, Tris buffer, or phosphate buffer (I = 0.1, pH = 7.6) and whether nitrilotriacetate was present or not. That this broad signal does represent specifically bound iron is shown by the fact that in all these cases, in the presence or in the absence of perchlorate, there was no change in the visible or ultraviolet spectrum.

Transferrin—Similar experiments were performed with transferrin; the effect of perchlorate, however, is the reverse of that on conalbumin. The EPR spectrum of a 10% solution of Fe₂-transferrin in water at pH 7.3 is shown in Fig. 5A. The EPR spectrum of the same transferrin solution containing 0.4 M NaClO₄ is shown in Fig. 5B. Taking account of the gain settings of the spectrometer, it can be seen that the height of the sharp signal labeled S has in this case decreased and by a factor of 2 on the addition of 0.4 M NaClO₄; also the S peak has become less well resolved. At the same time, a broad shoulder (B) has appeared on the low field side of the sharp signal and a more intense low field absorption may be observed (Feature b). The shoulder marked s in Fig. 5B is part of the peak labeled s in Fig. 5A. Changes in the region between 4 and 10 kilogauss were also observed with transferrin, the absorption positions corresponding closely to those for conalbumin. Addition of NaClO₄
FIG. 4. EPR spectra recorded at 103° K of the half-loaded iron-conalbumin. $A$, in water at pH 7.6, gain 500; $B$, in 0.4 M perchlorate, gain 100.

FIG. 5. EPR spectra recorded at 103° K of the fully loaded Fe$_2$-transferrin. $A$, in water at pH 7.3, gain 40; $B$, in 0.4 M perchlorate, gain 80.

Changes induced in the EPR spectrum by addition of NaClO$_4$ were studied for transferrin solutions which were fully, $x$, and $>$ sat. with iron and at values of pH in the range 7 to 9. In all cases, the height of signal $S$ decreased at the same rate as the NaClO$_4$ concentration increased. Under no conditions, however, was its height less than $\frac{1}{2}$ that observed in the absence of NaClO$_4$. Also, iron incorporated in the presence of strong perchlorate gave an equal distribution between the $B$ and $S$ EPR signals.

The same results were obtained when the transferrin was buffered with phosphate buffer ($\rho = 0.1$, pH 7.6), but the broad signal was not observed when Tris buffer was used nor when nitrolactate was present in the phosphate-buffered solutions. Again, in none of these cases, was there a change in the visible or ultraviolet absorption spectrum.

The EPR spectrum of transferrin as it occurs naturally in blood serum was also recorded. In this sample which was approximately half-saturated with iron, no evidence for any broad signals was observed. No change occurred when either we added more iron to this sample to saturate the transferrin, or when we added perchlorate to it.

Both Proteins—That the effect of the sodium perchlorate is reversible and not harmful to the proteins was shown as follows. A 25% solution of protein, which contained 0.4 M NaClO$_4$, a portion of which was diluted 10 times, and a 2.5% protein solution which was made 0.04 M in NaClO$_4$ all give identical EPR spectra. If added perchlorate was removed either by passing the protein solution down a column of Sephadex G-25 equilibrated with water and reconcentration by membrane filtration, or by dialysis against two changes of water, the EPR spectrum characteristic of the protein in water was obtained.

**INTERPRETATION AND DISCUSSION**

That there is an equilibrium involving at least two paramagnetic high spin ferric protein species, and which is affected by perchlorate concentration, is clearly shown by the isosbestic points in the EPR spectra. That there is a third species detectable by EPR is most unlikely since (a) its molar extinction coefficient at the isosbestic points would have to be the same as that of the other two species and (b) a careful search over a wide field range at temperatures down to 2° K revealed no new features.

The effect of the perchlorate is most interesting. A change in the ligand binding the iron is thought to be most unlikely since ClO$_4^-$ is a very weak complexing agent and also because there is no change in the ultraviolet/visible spectrum. Admit-
tedly, there is a change in the EPR spectrum, but this is known
to be a change to small changes in the strength and sym-
metry of the ligand field (13). Addition of any substance to
an aqueous solution may alter the hydrogen-bonding properties
of the medium, the term chaotropic having been coined for
this effect. Perchlorate is a well known chaotropic anion (14, 15)
and the conformation adopted by a protein is well known to be
sensitive to the hydrogen-bonding properties of the solvent. We
suggest, therefore, that the EPR changes we have recorded are
carried by conformational changes which result in slightly dif-
ferent ligand fields at the ferric ion sites.

Both conalbumin and transferrin contain two independent
iron-binding sites with remarkably similar properties (16) and,
therefore, we seek a single scheme for the two equilibria. There
has been some discussion as to whether transferrin has a subunit
structure (17) or whether there is only one polypeptide chain
(18, 19). However, it seems fairly clear that at least some dup-
lication occurs (19, 20) and, in this sense, the protein may be
thought to consist of two halves (whether or not these are linked
in a common conformation) each of which binds one iron atom.
Furthermore, a conformational change in the protein has been
proposed (8) to account for the ease with which transferrin re-
leases iron in vivo compared with the difficulty of doing this in
vitro at physiological pH. With these facts in mind we depict
the equilibria for the two proteins as follows.

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     S
    a1 ---- b1 ---- c1
     S
    a2 ---- b2 ---- c2
     S
    a3 ---- b3 ---- c3
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The implications of this diagram are (a) in one conformation
the iron atoms are bound identically to give the sharp EPR sig-
nal (SS) as in c1; (b) a conformational change in one half causes
just that iron to give the broad EPR signal (SB) as in c2; (c) a
conformational change in the other half causes the other iron
also to give the broad EPR signal (BB) as in c3.

Consider first conalbumin. In water the equilibrium SS ⇌
SB = BB is balanced towards the right but perchlorate drives
it fully to the left. If the protein is less than fully loaded with
iron the B site is preferred (b2); and when a second iron atom is
added, the B site is again preferred (c3). Thus we account for
the greater-than-2-fold drop in the S signal when perchlorate is
removed. This is an alternative description to Woodworth's crevice model (21) and does retain the different iron-binding
affinities for the two sites, the B site being more strongly binding,
as well as incorporating our new data. That there should be a
preference for the more firmly bound B site is in keeping with
the apparent function of conalbumin, to bind metal firmly with
little or no provision for later release.

For transferrin, the S signal dropped no more than 2-fold on
addition of perchlorate; therefore, in our experiments, only the
equilibrium SS ⇌ SB is important; this was true whether the
protein was fully loaded or not.

Aisen et al. (2), like us, found that transferrin in isolated human
blood serum gave an EPR spectrum identical with that of the
commercial sample of pure transferrin. We further found our
sample to be half-loaded and unaffected by perchlorate, there-
fore, as in at, the conformation in which iron is in the weaker
binding site. We envisaged, therefore, that when fully loaded,
the conformation of the protein might change, under the in-
fluence of the red cell precursor, from SS to SB. This would
then give up 1 iron atom from the weaker S site in preference
to 2, in accord with the findings of Fletcher and Huehns (8).
Thus the important distinction between conalbumin and trans-
ferrin is upheld, the function of the latter being to bind and later
release the iron it is carrying.

Of course our measurements on frozen solutions provide no
direct evidence of two conformations under physiological con-
ditions. However, we have found artificial conditions under
which the two sites differ and make the points that the sites
could differ in vivo probably as a result of conformational changes.

It is known that protein conformations can alter markedly
with salt concentration gradients set up during the freezing
process (22) and it could be argued that such changes are being
induced by perchlorate in our experiments. This does not
affect our interpretation in terms of two conformations but it
does imply that the room temperature concentrations of such
conformers might be different from those of the frozen solutions.
Salt gradient effects presumably do not affect conalbumin where
the two EPR signals are seen in the absence of added perchlorate
since it is hard to see how acid or salt concentration gradients
can be set up in the simple aqueous protein solution. Trans-
ferrin may be affected in this way but we think this is unlikely
because: (a) the same spectra resulted from different rates and
conditions of freezing; (b) its two EPR spectra are so similar
to those of conalbumin in the absence of added salt; (c) the in-
tensity of the sharp signal dropped by just 35, strongly indicating
that just one of the two binding sites was being affected.

Ultracentrifuge experiments have shown the presence of an
equilibrium (23) when conalbumin is exposed to certain buffer
solutions but that the forms in equilibrium may be monomeric
or aggregated according to the prior absence or presence of sul-
phate or perchlorate (24).

Other experiments (25) have shown that EPR spectra may
be affected by dipolar broadening through solute aggregation
on freezing. This is expected to be important for small mole-
cules but not for proteins, unless the paramagnetic site is on
the outside of the molecule, because the broadening being propor-
tional to 1/r^3 diminishes rapidly with increasing distance
between the paramagnetic sites. We know of no evidence to
suggest that the iron-binding sites are near the surface of the
molecule in transferrin or conalbumin; indeed the EPR spectra
of the lyophilized proteins are not significantly different from
those of the frozen solutions and this suggests that they are not.

Aggregation in either of these two senses is not expected to
affect our interpretations.

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