Electron-Paramagnetic-Resonance Spectroscopy of Iron-Binding Fragments of Hen Ovotransferrins

By R. MICHAEL BUTTERWORTH, JOHN F. GIBSON and JOHN WILLIAMS
Inorganic Chemistry Laboratories, Imperial College of Science and Technology, London SW7 2AY, U.K.,
and Molecular Enzymology Laboratory, Department of Biochemistry, University of Bristol,
Bristol BS8 1TD, U.K.

(Received 1 April 1975)

1. It is confirmed that there are two e.p.r. (electron-paramagnetic-resonance) signals associated with fully loaded ovotransferrin, which has two iron-binding sites. 2. Through experiments in which either of the two sites of whole ovotransferrin is occupied, the other being empty, the first occupied site is shown to belong to the N-terminal region of the protein; the second occupied site is in the C-terminal region. 3. When the protein is cleaved with trypsin or subtilisin, the N-terminal and C-terminal fragments are spectroscopically similar to the monoferric ovotransferrin complexes in which the iron atom occupies the N-terminal or C-terminal site respectively. Each fragment displays the same two e.p.r. signals, though not in the same proportions. 4. Computer summations of the e.p.r. spectra confirm that there is no iron–iron interaction which affects the spin Hamiltonian parameters at the iron-binding sites.

A major point of interest in the study of the transferrins is the nature of iron uptake to the two metal-binding sites. After various proposals that this may be pairwise or random, it is generally thought that there is little to distinguish between the two sites, at least on the basis of their iron-binding capacities (Aisen, 1973; Morgan, 1974), and that binding is not pairwise. That it is actually sequential rather than random, however, in hen ovotransferrin at least, is evident because monoferric ovotransferrin with iron in the N-terminal half may be formed by incomplete saturation of ovotransferrin with iron (Williams, 1974, 1975). Also, measurements have shown that the decrease in tryptophan fluorescence on binding of Fe^{3+} ions was biphasic, the first site being occupied immediately, the second site being occupied over a period of about 1 min (Evans & Holbrook, 1973).

E.p.r. (electron paramagnetic resonance) had already shown that in ovotransferrin there were great differences between the two centres, because two e.p.r. spectra could be recognized (Aasa, 1972). Price & Gibson (1972), however, deduced that both spectra were exhibited by each site, and proposed that a difference between the sites arose through a shift in the equilibrium between two possible environments at one site, relative to a similar equilibrium at the other site. Aisen et al. (1973) also recognized the two e.p.r. spectra, and noted that the observed non-equivalence after binding does not imply non-equivalence before binding.

In the present paper it is shown, by comparisons of e.p.r. spectra, that the first iron atom bound goes to the site at the N-terminal end of the protein, where it displays two different e.p.r. spectra, and that when this protein is cleaved by trypsin digestion, the iron-binding site remains essentially unchanged. The second iron atom bound goes to a site at the C-terminal end of the protein which also shows two e.p.r. spectra, though in different relative amounts; when the protein is cleaved by subtilisin digestion, this site also is unchanged.

The e.p.r. spectrum of diferric ovotransferrin is compared with a computer summation of those of equal amounts of the C-terminal and N-terminal fragments, in the search for any magnetic interaction between the iron-binding sites.

Materials and Methods

Nomenclature

Ovotransferrin (also known as conalbumin) is referred to as 'OT' or 'Fe–OT–Fe' according to whether it is devoid of iron or fully saturated with it. The monoferric ovotransferrins are referred to as 'Fe–OT' or 'OT–Fe' according to whether the bound iron occupies predominantly the N-terminal or the C-terminal binding site. The iron-binding fragments are referred to as 'CS' or 'NT', according to whether a subtilisin or a trypsin digestion was used. Thus 'CS–Fe' is the C-terminal fragment, prepared by using subtilisin and loaded with one Fe atom; 'Fe–NT' is the N-terminal fragment, prepared by using trypsin, and also loaded with one iron atom.

Reagents and proteins

Hen ovotransferrin was prepared as described by Williams (1968). The monoferric proteins and the
proteolysis fragments which contain iron were prepared as described by Williams (1974, 1975), after which final traces of chelating agents were removed by passage of the iron–protein complexes through a column (90cm × 4.5cm) of Sephadex G-100 in 0.1M-NH₄HCO₃. The iron contents of the fragment preparations were determined by the method of Pollard et al. (1957). Fragments Fe–NT and CS–Fe were dissolved in 0.1M-NaHCO₃ to give 0.225 µg-atom of Fe/ml. The fully loaded diferric ovotransferrin was dissolved in 0.1M-NaHCO₃ to give the same iron concentration. The monoferric ovotransferrin complexes were dissolved at a concentration of 10% (w/v) in 0.1M-NaHCO₃.

E.p.r. spectroscopy

A Varian E-12 spectrometer was used. Samples (about 0.5ml) were placed in quartz tubes, frozen, and kept at constant temperature by gaseous N₂ by using the Varian variable-temperature accessory. Where relative intensities were to be measured, use was made of only one sample tube, placed at the same position in the cavity and kept at the same temperature, for all samples. Selected spectra were measured by hand and the data were transferred via punched cards to a CDC 6400 computer for summation spectra, either graphical or microfilm output being produced.

Results

Monoferic ovotransferrins

Monoferic ovotransferrin, prepared by treating OT with ferric nitrilotriacetate to 30% saturation at pH8.5, and subsequently purified by isoelectric focusing, contains iron predominantly in the N-terminal region (Williams, 1974). A sample of this, Fe–OT, gave an e.p.r. spectrum containing the broad and sharp features marked 'B' and 'S' in Fig. 1; these have been described earlier (Price & Gibson, 1972; Aisen et al., 1973). However, the broad features were enhanced relative to a sample of Fe–OT–Fe. Monoferic ovotransferrin prepared by partially removing iron from Fe–OT–Fe with 0.1M-citrate at pH5, and subsequently purified by isoelectric focusing, contains iron predominantly in the C-terminal region (Williams, 1975). A spectrum of this, OT–Fe, likewise showed the broad and sharp features, but this time the broad features were diminished, relative to Fe–OT–Fe. Spectra from samples containing 10% (w/v) protein are compared in Fig. 1, from which we calculated that the sharp signal is about 2.16 times more intense in OT–Fe than in Fe–OT for comparable amounts of iron.

Iron-binding fragments of ovotransferrin

A trypsin digest of 30% iron-saturated ovotransferrin produced a pink N-terminal fragment
E.P.R. SPECTRA OF OVOTRANSFERRIN FRAGMENTS

consisting of a single polypeptide chain of mol.wt. about 35000, which contained iron apparently bound in much the same way as it is in native ovotransferrin (Williams, 1974). The e.p.r. spectrum of a solution of this material, fragment Fe-NT, is distinctly similar to the monoferric ovotransferrin, Fe-OT, showing enhanced broad features. Fragment CS-Fe, which is produced by subtilisin digestion of OT-Fe, is believed to be the C-terminal half of ovotransferrin; like Fe-NT it is pink, and reversibly binds iron. Its e.p.r. spectrum is like that of OT-Fe in that the broad features are diminished. The e.p.r. spectra of these two fragments, CS-Fe and Fe-NT, containing similar amounts of iron are compared in Fig. 2; calculation here shows that the sharp signal is about 2.29 times more intense in CS-Fe than in Fe-NT.

**Effect of perchlorate on the fragments**

It has been established that ovotransferrin may be reversibly altered by addition of perchlorate ions, and that in the presence of perchlorate both of the iron atoms in the molecule are similarly bound, because the sharp e.p.r. signal grows at the expense of the broad one as the perchlorate concentration is increased (Price & Gibson, 1972). Therefore it was decided to add perchlorate to the CS-Fe and Fe-NT fragments. The results were as for the whole protein: addition of NaClO₄ to 0.7M to a solution of fragment CS-Fe in 0.1M-NaHCO₃ caused the sharp signal to grow and the weak broad signal to diminish further; a similar addition to a solution of fragment Fe-NT in 0.1M-NaHCO₃ caused a marked decrease in the broad signal, and a further increase to 1.4M-NaClO₄ gave a spectrum which was almost indistinguishable from that of fragment CS-Fe in 0.7M-NaClO₄. In each case, the broad signal virtually disappears in the presence of perchlorate, leaving only the sharp e.p.r. spectrum, which is typical of non-interacting high-spin Fe³⁺ ions in only one type of environment. The effect was shown to be reversible by removing the perchlorate by dialysis against several changes of water, followed by membrane-filtration concentration and the re-running of the e.p.r. spectra.

**Summation of spectra**

When equal volumes of fragments CS-Fe and Fe-NT, which contained 0.225µg-atom of Fe/ml, were mixed, the e.p.r. spectrum was recorded and found to be almost indistinguishable from that of the whole ovotransferrin Fe–OT–Fe. Similarly, when the separate e.p.r. spectra of CS-Fe and Fe-NT fragments were summed by computer, the result was

---

Fig. 2. *E.p.r. spectra of the isolated fragments of hen ovotransferrin*

Solutions contain 0.225µg-atom of Fe/ml in 0.1M-NaHCO₃. Solutions and e.p.r. conditions were as described in Fig. 1. See the text for nomenclature. ——, Fe-NT; · · · ·, CS-Fe.
indistinguishable from that for native Fe-OT-Fe; this latter comparison is shown in Fig. 3.

Discussion

There appear to be two explanations of the B and S signals which are observed in the e.p.r. spectra. Aasa (1972) imagined that they represented the two environments which were allocated, one to each of the two iron-binding sites, and that the first bound iron atom went to the site which gave the broad signal. Aisen et al. (1973) accorded with this notion, but from their experiments deduced that although the first iron atom bound might not go to a specific site, it gave the B signal on binding. The alternative description (Price & Gibson, 1972) requires that each site can give both signals, which represent two environments in equilibrium with each other. The difference between the two sites is shown by a shift in the equilibrium at one site relative to the other. The experiments described in the present paper are relevant to this discussion in two ways.

First, the enhanced B signal may be associated with a specific site, namely the N-terminal site of the protein, because it is Fe-OT, the monoferric transferrin with iron at the N-terminal end of the protein, which gives an enhanced B signal (see Fig. 1). That this is also the first-occupied site is evident from the way Fe-OT is formed, namely by the incomplete saturation of OT with the ferric nitrilotriacetate at pH8.5 (Williams, 1974, 1975).

Secondly, the observation of both e.p.r. signals in both monoferric transferrins and in both isolated fragments is taken as evidence to support the proposal that at each site there is an equilibrium between two environments. In Fe-OT and in OT-Fe, B and S signals both appear, but with different relative magnitudes. The fact that neither e.p.r. signal is seen without the other is easily explained if exchange is possible between the two sites, or if the iron-binding constants for each site are not grossly different, and if separation of the monoferric ovo-transferrins by isoelectric focusing is incomplete. However, it is most interesting also to observe both B and S signals in the fragments CS-Fe and Fe-NT, because here, exchange between the two sites is impossible, and although it is true that some contamination of a 'pure B or S signal' could arise from undigested protein which might be present in the fragments, such contamination is thought to be small and is considered unimportant, since the enhancement of the sharp signal of OT-Fe relative to Fe-OT (2.16) is strikingly similar to that of fragment CS-Fe relative to fragment Fe-NT (2.29). It seems more likely to us that in the fragments, as in the whole protein (Price & Gibson, 1972), both signals are associated with each iron atom which may find itself in one of two ligand fields, according to the conformation of the polypeptide chain in its

Fig. 3. E.p.r. spectra of a 0.1 m-NaHCO₃ solution containing 0.225 µg-atom of Fe/ml of diferric hen ovotransferrin compared with the computer summation of the two spectra shown in Fig. 2.

No computer normalization or adjustments of e.p.r. conditions have been made. ——, Fe-NT+CS-Fe; ·····, Fe-OT-Fe.
E.P.R. SPECTRA OF OVOTRANSFERRIN FRAGMENTS

immediate vicinity. Thus we write an equilibrium for each iron atom whether it is in one of the two iron-bearing fragments or in the native protein:

\[ B \rightleftharpoons S \]

The sharp signal S is fairly well understood (Aasa, 1972; Price, 1972; Pinkowitz & Aisen, 1972) in terms of a high-spin Fe\(^{3+}\) ion in a near-rhombic ligand field with large zero-field-splitting parameters. The broad signal B has so far proved difficult to interpret in terms of the parameters of a static Hamiltonian, but preliminary results (Aasa, 1972; R. M. Butterworth & J. F. Gibson, unpublished work) show that a slight variation in the zero-field-splitting parameters which describe the S signal, to a less-rhombic ligand field, is all that is needed to produce the dramatic change to an e.p.r. signal similar to B.

Aisen et al. (1973) put forward another hypothesis, that the same static Hamiltonian could apply to both sites, but that the first-occupied site was different in some dynamic respects. We do not support this view, because we find more compelling the evidence above that both spectra can occur from iron in one site, and also because no change in the e.p.r. line-shape was observed down to 1.7\(^\circ\)K (Pinkowitz & Aisen, 1972), at which temperature most dynamic effects are largely frozen out. Further, our experiments with the chaotrophic agent, perchlorate, are easily understood in terms of a conformational change, which shifts the above equilibrium to the right whether we consider the whole protein (Price & Gibson, 1972) or the fragments, and it is difficult to see how this anion could destroy the proposed, but unexplained, dynamic mechanism.

Previous experiments with whole ovotransferrin have shown that the two iron-binding sites do not interact so as to affect the ligand fields at the iron atoms (Luk, 1971). This has now been strikingly confirmed by showing that the computer summation of the spectra of the Fe-NT and CS-Fe fragments, where interaction is out of the question, is very similar to that of fully loaded Fe-OT-Fe (see Fig. 3). We further deduce from this comparison that if our proposal of an equilibrium between states B and S for each site is correct, then this equilibrium is not disturbed by separating the two regions of the protein which separately carry one iron atom. Thus although the protein is a single polypeptide, it possesses two regions which function similarly to, but apparently quite independently from, each other. The difference which can be observed by e.p.r. spectroscopy is that the equilibrium at the N-terminal end is shifted towards signal B, whereas that at the C-terminal end is shifted towards signal S.

We thank the Medical Research Council for financial assistance and the Science Research Council for a grant to R. M. B.

References

Williams, J. (1975) Biochem. J. 149, 237–244