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Spectroscopic and Magnetic Studies of Iron(III) Gastroferrin[†]

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ABSTRACT: The spectroscopic and magnetic properties of samples of iron(III) gastroferrin (mol wt 2.6×10^5) containing 180–190 iron atoms have been investigated. The observed positions of the ligand field absorptions of iron(III) gastroferrin indicate that the vast majority of iron(III) coordination sites are octahedral, $[Fe^{III}O_6]$. Magnetic susceptibility studies have further characterized these sites as antiferromagnetically coupled. A structural model featuring a polynuclear iron(III) cluster bound to the hydroxyl groups of the glycoprotein is proposed.

Gastroferrin is an iron(III)-binding glycoprotein that can be isolated from human gastric juice and from pig gastric mucin (Davis *et al.*, 1969; Multani *et al.*, 1970). The glycoprotein, which is 85% carbohydrate and 15% polypeptide by weight and has a molecular weight estimated by a variety of techniques as 2.6×10^5, is of particular interest because of its possible role in the regulation of intestinal absorption of iron (Spiro and Saltman, 1969).

Recent studies in our laboratories have been concerned with the nature of the bound iron(III) in the glycoprotein. In this paper we report the results of spectral and magnetic studies of iron(III) gastroferrin and discuss their structural implications.

Materials and Methods

Preparation. Gastroferrin was prepared from pooled human gastric juice by the procedure of Multani *et al.* (1970). Iron(III) gastroferrin was prepared by the addition of 54.1 g (0.2 mol) of $FeCl_3 \cdot 6H_2O$ in 200 ml of water to gastroferrin (120 mg in 100 ml, pH 2.0). The solution was dialyzed for 7 days against Tris buffer (0.001 M, pH 8.0) to remove salt. Any suspension was removed by centrifugation (10,000 rpm, 30 min) and the clear supernatant retained as the soluble iron(III) gastroferrin complex. Iron was determined by atomic absorption.

Spectroscopic Studies. Absorption spectra in the near-infrared and visible regions were recorded with Cary-14RI and Cary-17 spectrophotometers fitted with the absorption scales 0–0.2 and 0–2.0. Solutions of iron(III) gastroferrin prepared as described above were used to record the spectra at ambient temperature in quartz cells of 1, 5, and 10 cm path lengths.

Magnetic Susceptibility Studies. The magnetic susceptibility of iron(III) gastroferrin was determined in solution and in the solid state. Solution measurements employing the Evans method (1959) were performed on a Varian HR-220 nuclear magnetic resonance (nmr) spectrometer (Live and Chan, 1970). The sample and reference solutions were contained in the outer and inner compartments, respectively, of coaxial nmr tubes (Wilmad Glass Co.). The reference signal was provided by *tert*-butyl alcohol present in 5% concentration (v/v).

Solid state measurements on freeze-dried powders of iron(III) gastroferrin (100–200 mg) were carried out over the temperature range 300–80°K on a vibrating sample magnetometer (Princeton Applied Research, Model FM-1) that had been modified as described elsewhere (Schugar *et al.*, 1972). The raw data were corrected for the diamagnetism of the sample holder and of the iron-free gastroferrin, both measured directly, and of the gaseous coolant. Room temperature data used to calibate the variable temperature data were themselves calibrated with mercury(II) tetrathiocyanatocobaltate(II) and a sample of high purity annealed nickel metal. Magnetic field measurements were made using a transverse Hall probe (E. W. Bell 600 gaussmeter).

Results

Electronic Absorption Spectroscopy. The absorption spectrum of a solution of iron(III) gastroferrin from 1050 to 450 nm is shown in Figure 1. Three weak shoulders, at 490, 620, and 960 nm, are observed on the side of an intense absorption that extends from the ultraviolet into the near-infrared. No other spectral peaks or shoulders have been resolved down to 300 nm. Iron-free gastroferrin exhibits a featureless,
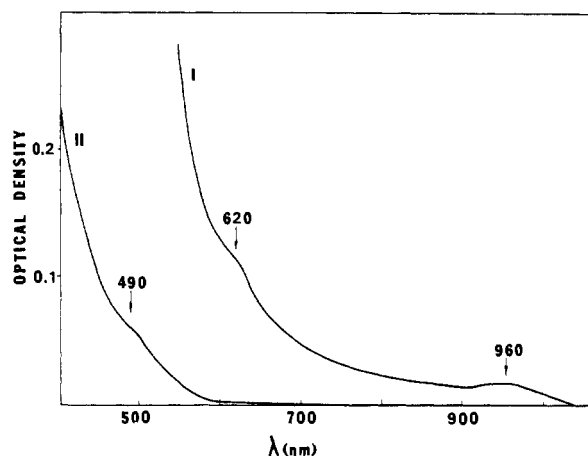
FIGURE 1: Visible and near-infrared absorption spectrum of iron(III) gastroferrin (1.1 × 10⁻³ M in iron) at ambient temperature: 10-cm cell (curve I) and 1-cm cell (curve II).

intense absorption in the ultraviolet which tails into the visible with rapid attenuation ($E_{500}^{1\%} = ca.$ 0). The molar extinction coefficients of the shoulders were corrected by visual extrapolation and subtraction of the underlying intense band. The positions of the maxima corresponding to these shoulders were estimated from this extrapolation using a set of parallel rules. Of course, there is some uncertainty in the extinction coefficients and absorption maxima because of the low intensities of these shoulders. The spectral data and the assignments discussed below are collected in Table I.

Magnetic Susceptibility Studies. A solution of iron(III) gastroferrin that is 0.064 × 10⁻³ M in iron produces a 5.65-Hz shift in the *tert*-butyl alcohol signal at 290°K when the reference solution is made equimolar in glycoprotein to the sample solution by the inclusion of iron-free gastroferrin. This shift corresponds to a magnetic moment per iron of 3.5 ± 0.05 BM. A lyophilized preparation was used for the study of the temperature dependence of the magnetic moment in the solid state. From the measured iron content of 3.95 ± 0.15% it is determined that 180–190 iron atoms are bound to each molecule of mol wt 2.6 × 10⁵, which corresponds to about 70% of the total iron-binding capacity of the glycoprotein. The molar diamagnetic correction for the protein was determined directly to be −155,200 × 10⁻⁶ cgs units. The room temperature magnetic moment per iron for this iron(III) gastroferrin powder is 3.45 (±0.05) BM, consistent with the value determined in solution. The moment increases in an almost linear fashion with decreasing temperature, rising to 3.65 (±0.05) BM at 90°K. The iron(III) gastroferrin sample was studied further by determining the dependence of the magnetic susceptibility on the strength of the applied field at 299°K. At low-field strengths (below 3 kG) the susceptibility appears to deviate from the linear behavior expected of a simple paramagnet.

TABLE I: Spectral Data for Iron(III) Gastroferrin.

λ (nm)	ν (cm⁻¹)	ε (approx)	Assignment
960 ± 5	10,400 ± 50	5	$^6A_1 \rightarrow {}^4T_1$
620 ± 10	16,200 ± 200	3	$^6A_1 \rightarrow {}^4T_2$
490 ± 10	20,400 ± 400	2	$^6A_1 \rightarrow {}^4A_1, {}^4E$

Discussion

The molecular composition of gastroferrin (Multani *et al.*, 1970) indicates that almost all of the potentially significant ligand atoms are oxygens. Calculated on the basis of a molecular weight for gastroferrin of 2.6 × 10⁵, the sulfur-containing amino acids cysteine and methionine are present at the low levels of 11 and 3 mol/mol of protein, respectively. The nonpeptide nitrogens of histidine and tryptophan are available for metal binding, but are present also in small amounts—9 and 4 mol/mol of protein. In contrast to the situation for sulfur and nitrogen, there is an abundance of potential oxygen-donor ligands provided by the large number of carbohydrate residues (about 1000 mol/mol of protein), some of which are sulfated, and by glutamic and aspartic acids (19 and 16 mol/mol of protein, respectively). Hence the ligand environments of the bound iron(III) ions are expected to be comprised almost exclusively of oxygen-donor atoms.

Comparison of the positions and intensities of the absorption bands in iron(III) gastroferrin with reference spectra of iron(III) complexes containing oxygen-donor ligands establishes the great predominance of octahedral iron(III) binding sites in the glycoprotein. For example, absorption bands of low intensities (ε <1) attributable to spin-forbidden ligand field transitions from a $^6A_1$ ground state to $^4T_1$, $^4T_2$, [$^4A_1$, ^4E], and $^4T_2$ excited states of the octahedral $Fe(H_2O)_6^{3+}$ complex have been observed in a large single crystal of iron(III) ammonium sulfate (Rossman, 1971; Gray, 1971) and in perchloric acid solutions of iron(III) perchlorate following multiple recrystallizations from perchloric acid (Webb, 1972). The first three ligand field absorptions in these spectra occur at 12,600, 18,200, and 24,300 cm⁻¹, which are only slightly higher energies than the corresponding bands in iron(III) gastroferrin (Table I). Furthermore, several other octahedral [$Fe^{III}O_6$] reference spectra match the band maxima in the spectrum of iron(III) gastroferrin quite closely (Webb, 1972).

The high carbohydrate content of gastroferrin raises the possibility of oxidation–reduction between the carbohydrate and bound iron(III). There is no evidence from the spectrum for any iron(II) present in the complex. Both high-spin and low-spin complexes of iron(II) exhibit spin-allowed ligand field spectra that differ greatly in band positions and intensities from those observed here.

There is also no evidence of bands attributable to iron(III) in tetrahedral coordination to four oxygen atoms, [$Fe^{III}O_4$]. Reference spectra for this coordination mode show a cluster of prominent sextet → quartet bands between 22,000 and 27,000 cm⁻¹ (Faye, 1969; Gray, 1971), a region in which the spectrum of iron(III) gastroferrin shows only the $^6A_1 \rightarrow$ $^4A_1$, ^4E octahedral band. While a few tetrahedral sites could escape spectral detection, there is little doubt that most of the iron(III) ions are bound octahedrally to gastroferrin.

The observed intensities of the ligand field transitions are higher in iron(III) gastroferrin than in the mononuclear reference compounds, but are still less than those characteristic of spin-allowed transitions. This increase in intensity can be attributed to the polynuclear nature of the iron bound to gastroferrin, which is discussed in more detail below. Several cases have been reported where the intensities of spin-forbidden bands are increased by factors of 10–50 in antiferromagnetically coupled systems (Lohr and McClure, 1968; Schugar *et al.*, 1972, and references therein).

The magnetic moment per iron at room temperature is 3.5 BM, both in solution and in the solid state. This value is

depressed from the spin-only value of 5.92 BM for $^6A_1$ iron(III), strongly suggesting the presence of antiferromagnetic coupling among the iron atoms. Coupling of this nature in small polynuclear clusters of iron(III) ions creates a characteristic temperature dependence of the magnetic moment, which decreases with decreasing temperature. If the cluster attains the dimensions of a small particle, 30–100 Å in diameter, the magnetic interactions among the paramagnetic ions within the cluster are expressed coherently as an intrinsic property of the whole particle rather than of the constituent ions (Néel, 1962). This effect has been observed experimentally as the temperature-independent magnetic moment of iron(III), ~3.5 BM, in fine particles of several minerals, e.g., α- and β-FeOOH (Takada and Kawai, 1962), and with the iron core of the storage protein ferritin (Schoffa, 1965; Blaise et al., 1967). The observed slight increase in the magnetic moment per iron in iron(III) gastroferrin from 3.45 BM at 300°K to 3.65 BM at 90°K, together with the results of the field strength study, suggests the presence of a small magnetic remanence.

The Mössbauer spectrum of a frozen solution of iron(III) gastroferrin at 77°K consists of a well-formed doublet characterized by a quadrupole splitting of 0.71 mm sec^{-1} and an isomer shift, relative to ^{57}Co in platinum, of 0.19 mm sec^{-1} (Bearden, 1972).[1] These values are consistent with high-spin (or antiferromagnetically coupled high-spin) iron(III). The comparatively small quadrupole splitting indicates that the symmetry of the charge about the iron nucleus is not far from spherical. There is no evidence for any hyperfine splitting or line broadening at 77°K, indicating that any intrinsic ordering present is small. The weak magnetic remanence was observed in magnetic susceptibility studies of several preparations of iron(III) gastroferrin, suggesting that it is an intrinsic molecular property. Inclusion of a trace ferromagnetic impurity could account for this ordering, but such an impurity would be expected to exhibit a hyperfine Mössbauer spectrum. The spectrum of iron(III) gastroferrin at 77°K shows no evidence for this impurity.

The above evidence confirms that most of the 180-190 octahedral iron(III) ions bound to gastroferrin form a polynuclear cluster, although a small number of monomeric iron(III) ions may be present as well. It is likely that the $[Fe^{III}O_6]$ octahedra are coupled together by means of oxo and possibly hydroxo bridging groups, with the remaining coordination sites filled by oxygen-donor atoms furnished by the glycoprotein. As there are almost 200 bound iron(III) ions, most of the ligand oxygens must necessarily come from the hydroxyl groups of the large number of carbohydrate residues. Many sugars and polyols are known to complex iron(III) ions and in some instances to form polynuclear iron(III) species (Spiro and Saltman, 1969). An example is the polynuclear iron(III) fructose complex system, which exhibits a magnetic moment of 3.4 BM per iron (Aasa et al.,

1964) and from which an iron(III) polymer of mol wt 65,000, containing about 300 iron atoms, has been isolated (Renner, 1972).[2] Gastroferrin probably functions in a manner analogous to the discrete sugars, but more efficiently, as a large excess ($\times$20) of carbohydrate is usually required to prevent the precipitation of iron(III) hydroxide at physiological pH (Charley et al., 1963). The molar ratio of carbohydrate:iron in the gastroferrin preparations used in this study was about 6:1, which is much less than the 20:1 of the simple carbohydrate system. We suggest that this increased efficiency results from the high local concentrations and suitable orientation of the hydroxyl and other oxygen-donor groups available on the glycoprotein to bind to the polynuclear iron(III) cluster.

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