where up to 50% should be expected from analysis of the isotherm. Further work is in progress to determine whether this apparent paradox is an indication that the model is incomplete.

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Spectroscopic and Magnetic Studies of Iron(III) Phosvitins†

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ABSTRACT: Studies of the binding of iron(III) by phosvitin, the phosphoglycoprotein of avian egg yolk, have led to the isolation of two derivatives, green and brown iron(III) phosvitins, that contain 6.9 (±0.2)% by weight of iron. The coordination structure of the bound iron(III) in each form has been investigated by infrared and visible absorption spectroscopy, electron paramagnetic resonance (epr), and the temperature dependence of the magnetic susceptibility. In the green form of iron(III) phosvitin the coordination structure of the great majority of the bound iron(III) ions is tetrahedral, $[Fe(III)O_4]_{tet}$. The available oxygen-donor ligands are most probably contributed by the numerous serine phosphate residues that are deduced to be arrayed in the β structure of pleated sheets. The magnetic and epr data indicate that antiferromagnetic coupling extends among several iron(III) ions in polynuclear clusters. The brown form of iron(III) phosvitin also binds iron(III) ions in large polynuclear clusters but, unlike the green form, the coordination structure of most of the iron binding sites is octahedral.

Phosvitin is a phosphoglycoprotein of mol wt 35,000 (Taborsky and Mok, 1967) containing 6.5% carbohydrate and a large number of phosphoserine residues (side chain: $-CH_2-OPO_3H_2$) arranged, at least in part, in linear sequences of up to eight residues uninterrupted by other amino acids (Joubert and Cook, 1958; Shainkin and Perlmann, 1971). The interaction of iron with phosvitin previously has been investigated with respect to a postulated rearrangement of the structure

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of the protein at alkaline pH (Taborsky, 1963). In the presence of oxygen and phosvitin, iron(II) chloride is rapidly oxidized to a bound form of iron(III), with accompanying release of phosphoryl groups from the protein (Rosenstein and Taborsky, 1970).

We wish to report the direct binding by phosvitin of iron(III) presented in chelate form both before and after heat treatment of the protein. The structures of both forms of iron(III) phosvitin have been investigated by a variety of magnetic and spectroscopic techniques.

Materials and Methods

Preparation of Iron(III) Phosvitin. Iron(III), as the $[FeNTA]^-$ chelate,[1] was presented to commercial preparations of phosvitin (Nutritional Biochemicals). The $[FeNTA]^-$ complex (Bates *et al.*, 1967) was initially formed by titrating iron(III), as $Fe(NO_3)_3 \cdot 6H_2O$, and NTA in a 1:1 molar ratio to pH 7.5 with dilute sodium hydroxide. The $[FeNTA]^-$ solution (*ca.* 0.12 M) in about 100-fold molar excess was mixed with phosvitin solution (50 mg/ml). The mixture was allowed to stand at room temperature for several hours and was then passed through a Sephadex G-25 column (2.5 × 40 cm) equilibrated with Tris buffer, 5×10^{-3} M, pH 7.5. This gel filtration step was effective in removing from phosvitin the low molecular weight contaminants, containing no phosphorus but absorbing at 280 nm, that have been reported to be present in commercial preparations of the protein (Ho *et al.*, 1969).

Iron(III) phosvitin was collected in the excluded green-yellow fraction, while the excess $[FeNTA]^-$ species remained in the included dark red-brown fraction. The labeling of the protein with ^{59}Fe was done in an identical manner using $[^{59}FeNTA]^-$. Fractions (2 ml) were collected and examined for radioactivity with a γ counter. This method of preparation yielded green iron(III) phosvitin that migrated as a single band on electrophoresis in polyacrylamide gels, pH 8.4.

The second form of iron(III) phosvitin was prepared by the above method with the modification that the solution of phosvitin was immersed in a boiling water bath for 10 min prior to the addition of iron(III) NTA. No precipitate was observed during this heat treatment nor during the subsequent incubation with the iron(III) NTA. Iron(III) phosvitin prepared in this manner was eluted at the void volume of the G-25 column as a rust-brown fraction that was heterogeneous, as determined by the absorption at 280 and 450 nm. Further fractionation on a P-60 column (100 × 2.5 cm) using the Tris buffer system yielded brown iron(III) phosvitin and a colorless component, presumably iron-free phosvitin.

The kinetics of the exchange between $[FeNTA]^-$ and phosvitin are important to the method of preparation. The half-life of the reaction, as measured by the drop in absorbance at 450 nm, is of the order of minutes. An equilibration time of several hours was, therefore, adequate to attain complete equilibrium. An extended incubation period complicated the preparation by allowing polymerization of iron(III) NTA. After an incubation period of 12 hr or more a brown polymeric fraction was excluded from the G-25 column along with iron(III) phosvitin. This polymerization is slow compared to that of iron(III) nitrate, which polymerizes within minutes after the addition of the appropriate amount of base (Spiro *et al.*, 1966).

All preparations of iron(III) phosvitin were analyzed for iron by atomic absorption. Phosphorus analyses were obtained from Galbraith Laboratories. Analytical determinations of protein-bound iron were complicated by the presence of an apparently essential amount of water in the freeze-dried material. Extensive drying led to irreversible denaturation, as determined by water insolubility. Samples equilibrated with the atmosphere were used for analysis. Analyses carried out on whole protein solutions and on acid-hydrolyzed (6 M HCl, 20 hr) samples gave equivalent results. The number of iron atoms was calculated using an assumed molecular weight for the iron-free protein of 35,000.

Spectroscopic Techniques. Absorption spectra in the near-infrared (near-ir), visible, and ultraviolet (uv) regions were recorded with Cary 14 RI, Cary 15, and Cary 17 spectrophotometers using quartz cells (1 cm). Low temperature spectra were obtained on a Cary 14 RI spectrophotometer in the visible–near-ir regions using a stable, clear glass of a 1:1 (v/v) mixture of ethylene glycol and Tris buffer (5×10^{-3} M, pH 7.5) in a Pyrex cell, suspended in a quartz dewar over liquid nitrogen. The all-quartz dewar was flushed with nitrogen before use and while cooling. Infrared spectra were recorded at room temperature on a Perkin-Elmer Model 621 grating spectrophotometer using samples in potassium bromide pellets.

A Varian E-3 spectrometer was used to obtain electron paramagnetic resonance (epr) spectra of aqueous solutions and freeze-dried powders at room and liquid nitrogen temperatures. Room temperature solution spectra were obtained using a special aqueous cell. The magnetic field was calibrated with a weak pitch standard provided by Varian and regulated by a field dial accessory. Typical operating conditions employed were: modulation amplitude, 25 G; microwave frequency, 9.15 GHz; microwave power, 20 W.

Magnetic Techniques. Magnetic susceptibility measurements on freeze-dried powders of the iron(III) proteins were carried out on a vibrating sample magnetometer (Princeton Applied Research, Model FM-1) which had been modified to allow continuous monitoring of susceptibility over the temperature range 300–85°K as described elsewhere (Schugar *et al.*, 1972). Samples used were freeze-dried preparations, 100–200 mg in mass, equilibrated with the atmosphere.

Room temperature data were calibrated with $HgCo(SCN)_4$ and a sample of high purity annealed nickel metal. The raw data at low temperatures were corrected for the diamagnetism of the sample holder and the effects of the density changes of the gaseous coolant and then calibrated with the room temperature values. The molar diamagnetic susceptibility used for the analysis of the iron(III) protein data was measured on the commercial preparation of the iron-free protein at room temperature, and found to be $-13{,}076 \times 10^{-6}$ cgs, using a mol wt of 35,000. The magnetic moment per iron (μ_{eff}/Fe) was calculated from the molar susceptibility per iron, using the measured iron analysis. For field strength dependence studies over the range 2–11 kG, magnetic field measurements were made using a transverse Hall probe (E. W. Bell 600 gaussmeter).

The solution susceptibility of green iron(III) phosvitin was determined over the temperature range 254–360°K by the method of Evans (1959) using a Varian HR-220 nuclear magnetic resonance (nmr) spectrometer, an instrument admirably suited for such studies (Live and Chan, 1970). The reference signal was provided by a *tert*-butyl alcohol (5%, v/v) in both the sample and reference compartments of coaxial tubes (Wilmed Glass Co.). The temperature of the sample was determined to an estimated accuracy of ±0.5°

[1] Abbreviation used is: NTA, nitrilotriacetic acid.

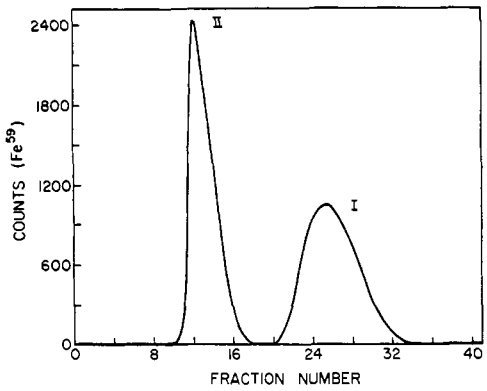

FIGURE 1: Separation of ^{59}FeNTA (peak I) and [^{59}Fe]phosvitin (peak II) from a mixture containing 10^{-2} M ^{59}FeNTA and 8.5×10^{-5} M phosvitin by Sephadex G-25 gel filtration. Fractions of 2 ml each were counted for 2 min in a γ counter.

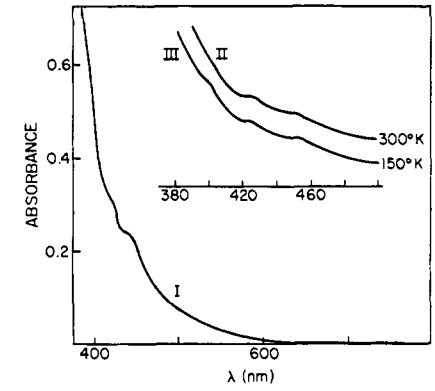

FIGURE 2: Visible and near-infrared absorption spectra of green iron(III) phosvitin (8.675 mg/ml) in Tris buffer (5×10^{-3} M, pH 7.5) at room temperature (curve I) and in a 1:1 v/v ethylene glycol–Tris solvent mixture at room temperature (curve II) and low temperature, ~150° K (curve III).

from the resonance frequencies of hydroxyl groups of ethylene glycol for temperatures from ambient to 360°K and from that of the methanol hydroxyl for temperatures from ambient to 254°K. Low temperature measurements were made using a solvent mixture of 1:1 (v/v) ethylene glycol and Tris buffer (5×10^{-3} M, pH 7.5). The contribution to the shift observed in the methyl resonance of *tert*-butyl alcohol from the diamagnetic protein was calculated from the molar diamagnetic susceptibility determined in the solid state.

Results

Iron(III) Binding to Phosvitin. The exchange of iron(III) from [FeNTA]$^-$ to phosvitin was studied by gel filtration. The elution pattern of a mixture of [^{59}FeNTA]$^-$ and phosvitin, prepared as described above for green iron(III) phosvitin, is shown in Figure 1. Approximately half of the iron(III) appears in the excluded protein fraction as expected if each protein molecule binds about 50 iron(III) ions. No more iron(III) binds to phosvitin by providing twice as much [FeNTA]$^-$. For both green and brown iron(III) phosvitins the analytical data show that 47 (±2) iron atoms are bound, and the P:Fe ratio is 2.0 (±0.1).

Visible, Near-Infrared, and Infrared Spectra. The visible and near-ir spectra of green iron(III) phosvitin are shown in Figure 2. Two distinct shoulders are observed in the aqueous solution spectrum recorded at room temperature. The peak positions indicated are 447 and 426 nm (22,400 and 23,500 cm^{-1}, respectively). The room temperature spectrum in a 1:1 (v/v) ethylene glycol–Tris (5×10^{-3} M, pH 7.5) mixture is the same as that in the Tris buffer alone. On cooling this mixture to about 150°K, a third shoulder is observed, at 400 nm (25,000 cm^{-1}). Extinction coefficients for the three weak bands were estimated by subtraction of a visual extrapolation of the intense uv band from the experimental trace over this region of the spectrum. The positions of the maxima corresponding to these shoulders were estimated from this extrapolation using a set of parallel rules. The absorption of the iron-free protein in this region is negligible. The visible and near-ir spectra of brown iron(III) phosvitin at room temperature show only one weak absorption peak, with λ_{max} 820 nm (12,200 cm^{-1}). This band occurs at the end of a strong absorption system that extends throughout the visible. The band positions and extinction coefficients per iron (ϵ/Fe values) for the two preparations of iron(III) phosvitin together with the assignments discussed below are listed in Table I.

The ir spectra of phosvitin and green iron(III) phosvitin were examined carefully in the region (800–1000 cm^{-1}) where bands characteristic of dimeric oxo- and hydroxo-bridged iron(III) complexes are found (Schugar *et al.*, 1972) and also around 1200 cm^{-1} where bands attributable to phosphorus–oxygen stretching vibrations have been observed (Nakamoto, 1970). However, except for the shift of one band at 1114 cm^{-1} in phosvitin to 1070 cm^{-1} in green iron(III) phosvitin, no significant differences were observed between the complex infrared spectra of the two compounds.

Magnetic Susceptibility Studies. A solution of green iron(III) phosvitin (1.61×10^{-3} g of Fe/ml) in Tris buffer (5×10^{-3} M, pH 7.5) caused the methyl resonance of *tert*-butyl alcohol, the reference material, to shift 260 Hz downfield. From this shift the molar susceptibility was calculated (Evans, 1959) to be 8979×10^{-6} cgs units, corresponding to a magnetic moment per iron of 4.56 BM. The μ_{eff}/Fe was found to be independent of temperature, within the experimental error of at least 3%, over the temperature range 254–360°K.

Susceptibility measurements as a function of temperature were carried out on dry samples of both green and brown iron(III) phosvitins. At 300°K green iron(III) phosvitin behaves as a simple paramagnet, *i.e.*, there is no field strength dependence of the susceptibility. For each protein the temperature dependence of the μ_{eff}/Fe from 300 to 85°K is shown in Figure 3. The moment for green iron(III) phosvitin drops from 4.45 to 3.39 BM over this temperature range. For brown iron(III) phosvitin, the room temperature μ_{eff}/Fe of 1.56 BM decreases to 1.18 BM at 85°K.

Epr Spectra. The epr spectrum of a frozen aqueous solution

TABLE I: Electronic Spectral Data for Iron(III) Phosvitins.

$^6A_1 \rightarrow$	λ_{max} (nm)	$\bar{\nu}$ (cm^{-1})	ϵ/Fe
	Green Iron(III) Phosvitin		
$^4T_1$	447	22,400	5.5
$^4T_2$	426	23,500	5.0
($^4A_1$, ^4E)	400	25,000	
	Brown Iron(III) Phosvitin		
$^4T_1$	820	12,200	~0.3

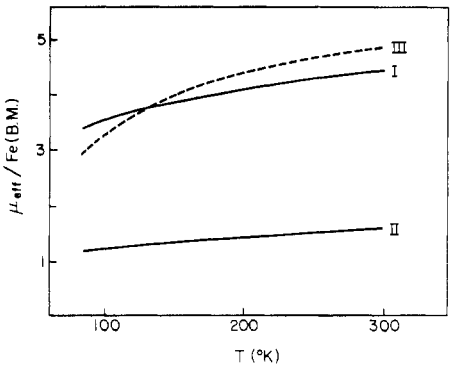

FIGURE 3: Temperature dependence of the magnetic moment per iron over the range 300–85°K for powder samples of green iron(III) phosvitin (curve I) and brown iron(III) phosvitin (curve II). Curve III is calculated for a ($^5/_2$, $^5/_2$) dimeric system with $J = -12$ cm^{-1}.

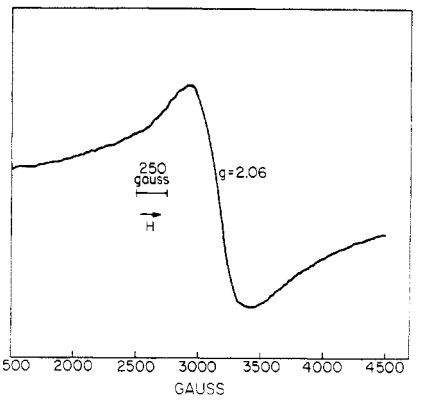

FIGURE 4: Electron paramagnetic resonance spectrum of a frozen aqueous solution of green iron(III) phosvitin at ~120°K.

of green iron(III) phosvitin at ~120°K is shown in Figure 4. The band is broad, with a peak-to-peak width of 500 G. The g value is 2.06. The spectrum is also observable at room temperature without additional broadening. Quite similar spectra were recorded with brown iron(III) phosvitin, although the low temperature spectrum was partly resolved in the low-field region. The g values at low temperatures are 2.04 and 2.13, as compared with 2.03 at room temperature. At both temperatures the peak-to-peak band width is 800 G.

Discussion

Green Iron(III) Phosvitin. Both the temperature dependence of the magnetic moment (Figure 3) and the epr spectrum (Figure 4) suggest that the iron(III) bound to phosvitin is polynuclear. The room temperature μ_{eff}/Fe of 4.45 BM (4.56 BM in solution) is substantially reduced from the high-spin ($S = {}^5/_2$) value of 5.92 BM. Furthermore, the magnetic moment is not temperature independent, as would be expected for a mononuclear iron(III) species at each binding site or for a discrete micellar particle below 100 Å in diameter (Neél, 1962), but decreases with temperature, indicating some antiferromagnetic interaction.

This general behavior is similar to that observed for iron(III) in simple dimeric systems which can be adequately described by a model in which spin–spin interaction ($\mathcal{H} = -2JS_1S_2$, with $J < 0$ in the antiferromagnetic case) takes place between two high-spin iron(III) ions (Schugar *et al.*, 1972). Curve III in Figure 3 illustrates the case for $J = -12$ cm^{-1} ($S_1 = S_2 = {}^5/_2$), assuming $g = 2.0$ and neglecting temperature-independent paramagnetism. Although it may be concluded from this comparison that some of the iron(III) ions are antiferromagnetically coupled, a model consisting of an array of dimeric units obviously does not account quantitatively for the data. As might be anticipated from this treatment and the parameters employed, it is possible to fit the magnetic behavior of green iron(III) phosvitin shown in Figure 3 by a suitable combination of high-spin monomers and spin–spin coupled dimers. The actual mixture required for an excellent fit (calculated and experimental values agree to better than 3% over the range 300–85°K) is 25% high-spin monomers and 75% dimers, assuming that the dimers have $J = -25$ cm^{-1} and a room temperature μ_{eff}/Fe of 3.88 BM. However, we note that the epr spectrum (Figure 4) fails to support the presence of significant numbers of monomeric iron(III) ions in green iron(III) phosvitin, and it is probable that most of the iron(III) is bound in fairly large ($n > 3$) polynuclear clusters. The broadening of the epr signal is much greater than has been observed in dimeric and trimeric complexes (Harris and Owen, 1965; Ball, 1969). In fact, it is of the same magnitude as that observed for the protein ferritin, where several thousand iron(III) ions are bound in a polynuclear cluster (Boas and Troup, 1971). The bandwidth appears to be independent of temperature, suggesting that it is not due to slow tumbling of the large protein molecule nor to spin–lattice relaxation effects but rather to magnetic dipole–dipole interaction that alters the total magnetic field at the positions of neighboring spins, broadening their resonance lines. This interaction results from the physical proximity of the coupled dipoles and unless some structural changes occur, it remains effectively temperature independent.

Information concerning the coordination geometry of individual iron(III) centers in the polynuclear complex may be derived from an analysis of the absorption spectrum in the visible and near-ir regions. The two common geometrical structures for $^6A_1$ iron(III) are octahedral and tetrahedral. Reference spectra of iron(III) coordinated to oxygen-donor ligands in both of these geometries have been studied fairly extensively. Although the spectrum of the octahedral species $[Fe(H_2O)_6]^{3+}$ has long been obscured by the absorptions of the hydrolysis and polymerization products of iron(III), the ligand field bands for this species have now been observed in large single crystals of iron(III) ammonium sulfate (Rossman, 1971) and in perchloric acid solutions of iron(III) perchlorate (Webb, 1972). A carefully studied absorption spectrum of a $[Fe(III)O_4]_{tet}$ system is that of an iron(III)-doped sample of orthoclase feldspar, ideally $KAlSi_3O_8$ (Faye, 1969; C. Cowman, 1972, unpublished data).

The reference spectra show clearly that the first two spin-forbidden transitions, $^6A_1 \rightarrow {}^4T_1$, and $^6A_1 \rightarrow {}^4T_2$, occur at much lower energies (12,600 and 18,200 cm^{-1}) in the $[Fe(III)O_6]_{oct}$ complexes than in the tetrahedral system. The iron(III)-doped orthoclase feldspar sample shows an extremely broad, weak system in the region of 20,000 cm^{-1} and bands at 22,500, 23,900, and 26,500 cm^{-1}. There is some uncertainty as to the exact assignment of this spectrum, depending on whether the $^6A_1 \rightarrow {}^4T_1$ transition is responsible for the very weak 20,000-cm^{-1} system or for the peak at 22,500 cm^{-1} (Thibeault, J., Powers, D., and Cowman, C., 1972, personal communication). The former assignment leads to ligand-field parameter values of $10Dq = 5200$ and $B = 740$ cm^{-1} ($C/B =$

4.33), whereas the latter gives $10Dq$ = 7400 and B = 330 cm^{-1} (C/B = 13.9). However, for either choice $10Dq$ is substantially lower than the 13,700-cm^{-1} value calculated from the octahedral reference spectra, as expected from theoretical considerations (Gray, 1971).

In the green form of iron(III) phosvitin the three observable ligand field bands are at 22,400, 23,500, and 25,000 cm^{-1}. Assignment of these bands as $^6A_1 \rightarrow {}^4T_1$, $^4T_2$, ($^4A_1$, ^4E) gives a $10Dq$ value of about 5200 cm^{-1}. The relatively low $10Dq$ value and, in particular, the close similarity to the spectrum of iron(III) orthoclase feldspar strongly suggest that the binding sites in green iron(III) phosvitin are tetrahedral. The absence of peaks in the region 800–1000 nm rules out the possibility that a significant fraction of the iron(III) sites are octahedral. Additional evidence for a single, presumably tetrahedral, type of iron(III) binding site is derived from the 77°K Mössbauer spectrum of a frozen solution of green iron(III) phosvitin, which consists of a simple resonance doublet with a quadrupole splitting of 0.42 mm sec^{-1} and an isomer shift, relative to ^{57}Co in platinum, of 0.21 mm sec^{-1} (Bearden, A., 1971, personal communication).

The intensities of the first two electronic absorption bands in green iron(III) phosvitin are almost an order of magnitude greater than those measured for analogous bands in iron(III)-doped orthoclase feldspar. Such intensity enhancement of spin-forbidden bands, however, is to be expected in a polynuclear complex featuring antiferromagnetically coupled iron(III) centers (Schugar *et al.*, 1972, and references therein).

It is highly likely that in green iron(III) phosvitin the oxygen-donor atoms are furnished by the phosphate groups of the phosphoserine residues. Other potential oxygen-donor ligands, as determined by the amino acid composition (Allerton and Perlmann, 1965), are those of aspartic and glutamic acids, each of which is only 10% as abundant as serine phosphate. Nitrogen-donor histidine residues and the single oxygen-donor carbohydrate moiety (Shainken and Perlmann, 1971) are also not sufficiently abundant to be cast in a prominent iron(III) binding role. Many serine phosphate residues have been reported to be arranged in continuous sequences of up to eight residues (Shainken and Perlmann, 1971; Joubert and Cook, 1958). This close proximity is expected to be quite suitable for binding a polynuclear cluster of iron(III) ions. Recent detailed studies of optical rotatory dispersion and circular dichroism spectra have shown that at low pH or in suitable organic solvents the conformation of the polypeptide chain of phosvitin is predominantly that of a β-type pleated sheet (Taborsky, 1968; Perlmann and Grizzuti, 1971). These conditions reduce the electrostatic repulsions between the highly acidic phosphate side chains, allowing the more ordered conformation to be established. The binding of iron(III) ions to the serine phosphate side chains is expected also to reduce these electrostatic repulsions at physiological pH, inducing a conformational change toward the β structure. The sequentially arranged phosphate groups oriented alternately on opposite sides of the sheet structure could act as bridging groups between adjacent iron(III) ions in tetrahedral coordination, as depicted schematically in Figure 5.

Brown Iron(III) Phosvitin. The temperature-dependent μ_{eff}/Fe of brown iron(III) phosvitin is depressed far below that of high-spin monomeric iron(III) and of the polynuclear iron(III) in green iron(III) phosvitin. The epr signal is even broader, 800 G peak-to-peak, than that of the green form, and is effectively temperature dependent. As in the green form, the magnetic susceptibility and epr data indicate that the iron(III) is bound in some type of polynuclear cluster.

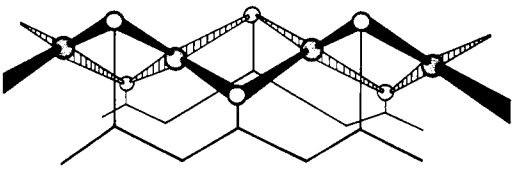

FIGURE 5: Schematic representation of the polynuclear, tetrahedral iron(III) coordination structure proposed for the green form of iron(III) phosvitin. Shaded spheres are iron atoms, open spheres oxygen atoms.

The ligand field band at 12,200 cm^{-1} in brown iron(III) phosvitin is attributable to the $^6A_1 \rightarrow {}^4T_1$ transition of octahedral iron(III). Although no bands are observed which may be assigned to tetrahedrally coordinated iron(III), a small number of such sites could easily escape detection because of the strong background absorption in the 20,000–25,000-cm^{-1} region. It is probable that the heating procedure incorporated in the method of preparation modifies the protein such that a significant fraction of the iron(III) binding sites are opened to hydrolytic polymerization, thereby leading to some octahedral coordination. The proposed modification does not appear to be phosphate loss, however, as a P:Fe ratio of 2.0 was obtained for both the green and brown forms of iron(III) phosvitin. Further characterization of brown iron(III) phosvitin is obviously needed.

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Isolation of a Protein Containing Tightly Bound 5-Methoxybenzimidazolylcobamide (Factor IIIm) from *Clostridium thermoaceticum*†

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ABSTRACT: A corrinoid–protein has been purified from *Clostridium thermoaceticum*. It has a mol wt of about 27,000 and contains 1 mol of tightly bound 5-methoxybenzimidazolylcobamide/mol of protein. The protein is likely a single polypeptide chain but also exists as a dimer of mol wt 55,000. Spectrophotometric and electron paramagnetic resonance (epr) studies indicate that in the native protein the corrinoid exists in a reduced form corresponding to cob(II)alamin that is stable to oxygen. After precipitation with ammonium sulfate the corrinoid–protein is oxidized by air to the cob(III)alamin form. The oxidized corrinoid–protein reacts with cyanide at alkaline pH to form the monocyano derivative Under strong acid conditions the corrinoid–protein does not undergo a spectral change indicating a split of the coordinate bond between the base and the cobalt atom as does the free 5-methoxybenzimidazolylcobamide. The oxidized corrinoid–protein is reduced by extracts of *C. thermoaceticum* with pyruvate as a source of electrons. It is suggested that the corrinoid–protein functions in the synthesis of acetate from 5-methyltetrahydrofolate, which is catalyzed by extracts from *C. thermoaceticum* in the presence of pyruvate.

Acetate is synthesized from CO_2 by *Clostridium thermoaceticum* and it has been shown that methylcorrinoids are intermediates in this synthesis (Ljungdahl *et al.*, 1965; Poston *et al.*, 1966). Thus, in pulse labeling experiments with $^{14}CO_2$, (5-methoxybenzimidazolyl)-*Co*-methylcobamide (methyl factor IIIm) and *Co*-methylcobyric acid are formed labeled with ^{14}C exclusively in their *Co*-methyl groups. When these compounds or [$^{14}CH_3$]cobalamin is incubated with extracts from *C. thermoaceticum* in the presence of pyruvate, acetic acid is formed containing label in the methyl group (Ljungdahl *et al.*, 1965). The methyl group of 5-methyltetrahydrofolate is also labeled in pulse experiments with $^{14}CO_2$ (Parker *er al.*, 1971) and is converted to acetate (Ghambeer *et al.*, 1971). It has been postulated that a protein-bound corrinoid serves as carrier of the methyl group between 5-methyltetrahydrofolate and acetate (Ghambeer *et al.*, 1971). In this paper we wish to report the isolation and some properties of a protein containing tightly bound 5-methoxybenzimidazolylcobamide. Although a role for this protein has not been established in the synthesis of acetate from CO_2 we would like to suggest that it functions in this synthesis.

Experimental Section

Bacterial Culture. C. thermoaceticum was grown in a medium of the following composition in grams per liter: glucose, 18; yeast extract, 5; tryptone, 5; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.25; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.392; $Na_2MoO_4 \cdot 2H_2O$, 0.024; $Co(NO_3)_2 \cdot 6H_2O$, 0.029; $MnCl_2$, 0.0013; Na_2SeO_3, 0.0017; $NaHCO_3$, 16.8; K_2HPO_4, 7; KH_2PO_4, 5.5; sodium thioglycolate, 0.5. This medium differs from that used by Ghambeer *et al.* (1971) by the addition of ferrous ammonium sulfate, manganous chloride, sodium molybdate, and sodium selenite. Andreesen *et al.* (1973) have found that addition of these compounds greatly increases the growth yield. The organism was grown for 48 hr at 55° under CO_2 in carboys containing 18 l. of medium. The cells were harvested in a Sharples centrifuge at room temperature and were stored frozen at −20°.

Materials. Diethylaminoethylcellulose (DE-23) was obtained from Whatman. Before use it was washed with 0.5 M HCl, water, 0.5 M NaOH, and water, and after the pH had been adjusted to 7 by the addition of 1 M KH_2PO_4 it was washed with 0.005 M potassium phosphate, pH 7.0. DEAE-cellulose from Schleicher and Schuell was used in the later steps of the purification of the corrinoid–protein. It was found that the corrinoid–protein eluted more quantitatively from the DEAE-cellulose from Schleicher and Schuell. Aluminum oxide, 0537, was purchased from J. T. Baker. It was soaked with 12 N hydrochloric acid overnight and washed with water; the pH was then adjusted to 7.6 by the addition of 1 N

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