from Ca lead to greater Zn binding.

We also evaluated two dihydroxyphenol ligands, the bidentate tiron and the hexadentate MECAMS. The results for MECAMS were similar to those for HBED. MECAMS was clearly superior to Tf in its ability to bind Al, but it also bound significant amounts of Zn. Tiron was considered next because it was expected to have lower absolute binding affinities than MECAMS and thus should complex less zinc. A 10 μM concentration of tiron complexed about 30% of the Al, but did not bind either Ca or Zn. Thus it appeared that one should be able to synthesize catecholate ligands which would bind a large fraction of serum Al without disturbing the metabolism of either Zn or Ca. We would note that replacement of desferrioxamine B by its bidentate analogue acetohydroxamic acid gave quite different results. In this case, the loss in absolute binding affinity was enough to eliminate completely the binding of Al.

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Registry No. DFO-B, 70-51-9; NTA, 139-13-9; EDTA, 60-00-4; HBED, 35998-29-9; MECAMS, 71353-06-5; Tiron, 149-45-1; AHA, 546-88-3; Al, 7429-90-5; Fe, 7439-89-6; Zn, 7440-66-6.

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EXAFS Investigation on the Iron(III) Binding Sites of Hen Phosvitin

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EXAFS spectroscopy has been applied to investigate the nature and the stereochemistry of the iron(III) binding sites in the protein phosvitin from chicken eggs in water solution with an iron/protein molar ratio of 10/1 at pH 7.2. The main result is that the iron atoms are bound to the protein in an octahedral environment and that the main binding sites are provided by the oxygen atoms of serine-bound phosphate groups at 1.93 (2) Å from the metal. The average number of bound phosphate groups is 4.4 (9) per iron atom, hexacoordination being achieved upon binding of other possible donors from the protein or solvent water molecules. No evidence has been found of short Fe-Fe interactions, so that, on the basis of our data, it can be stated that iron is not essentially involved in a polynuclear structure with Fe-O-Fe bridges.

Introduction

Phosvitins are a group of small phosphoglycoproteins that are the major components of the highly structured granules of the vertebrate egg yolk. All the phosvitins from different ovarian species share a very unusual amino acid composition characterized by an extraordinarily high serine content (about 50%) with extensive phosphorylation of these residues and by the relatively dominant presence of basic amino acids such as lysine and arginine, while those with nonpolar or sulfur-containing side chains are nearly or completely absent.[1] The biological role of phosvitins is not yet fully understood, although in view of the fact that essentially all the yolk metal is bound by this protein, it has been proposed that they can act as metal depositories for the embryo.[2] However, it is possible that they play a role as metal carriers in the blood of the maternal organism in the form of their precursors, the vitellogenins.[1,2]

Their ability to bind metals is certainly connected with the large number of serine-bound phosphoryl groups; however, in certain circumstances the imidazole nitrogens of the histidine residues and the peptide amide nitrogens can also act as donor atoms.[3]

The primary structure of hen phosvitin (PST) consists of a single chain of 216 amino acids for a molecular weight of ~35 000. There are 123 serine residues, of which 80 are located in a core region (residues 56-154) in blocks of up to 14 alternated with arginines, lysines, and more rarely asparagines. The C-terminal portion (residues 155-216) is particularly rich in histidines and contains another 27 serines.[4] A total of 98% of the serine residues are phosphorylated.[1]

Hen phosvitin in solution appears to be extremely flexible and unfolded, behaving essentially like a polyelectrolyte, the appearance of some secondary structure depending on the protonation of the phosphate groups. In fact only at very acidic pH (<3) does an extensively ordered conformation of a β-sheet type appear,[5] which is due to the presence of hydrogen bonds and salt linkages between the phosphate groups and the positively charged groups of basic amino acids. In the pH range 7-10, ORD[6,7] and resonance Raman studies[8] indicate the presence of an unordered structure. The binding of Ca^{2+}, Mg^{2+}, or Fe^{3+} does not induce a transition toward a β-type structure.[9,10]

Several reports on the interaction of phosvitin with metal ions such as Fe(II), Fe(III), Co(II), Mn(II), Ca(II), and Mg(II) have appeared in the literature;[1,10-12] however, the structure of the metal chromophore and the nature of the metal ligands remain to be fully understood. Early spectroscopic and magnetic studies on two forms of Fe(III) phosvitin at an Fe/protein ratio of 50/1 showed the iron atom coordinated by the oxygen atoms of the serine phosphate groups. It was suggested that in the green form the bound iron(III) ions have a tetrahedral coordination and magnetic susceptibility data indicated the formation of polynuclear iron clusters in which the metal ions are antiferromagnetically coupled.[13]

Because of the unordered conformation of phosvitin and its unique ability to bind metal ions, EXAFS spectroscopy seems to be exceptionally well suited to provide detailed structural information on the metal binding sites of this protein. We have recently investigated by CD and EXAFS techniques the interaction of

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Table I. Mean Distances from Fe(III) for Model Compounds (X-ray Data)[a]

	1st shell			2nd shell			3rd shell		
	N	type	*R*, Å	*N*	type	*R*, Å	*N*	type	*R*, Å
$Fe(acac)_3$[14]	6	O	1.95 (2)	6	C	2.90 (5)	3	C	3.18 (8)
$FePO_4$[25]	4	O	1.85 (1)	4	P	3.16 (1)	4	O	3.57 (2)
$FePO_4$[26]	4	O	1.83 (1)	4	P	3.15 (1)	4	O	3.57 (2)
$FePO_4 \cdot 2H_2O$[22]	6	O	1.98 (6)	4	P	3.30 (1)	2 + 2	O	3.58 (4)
									3.83 (3)

[a] Esd's are given in parentheses.

Cu(II) ions with phosvitin.[3] We wish to report here an EXAFS investigation on iron(III) hen phosvitin.

Experimental Section

Sample Preparation. Hen phosvitin (PST) was purchased from Sigma as the metal-free protein and used without further purification. The protein concentration was determined through UV measuring of the 275-nm band (ϵ = 17 800 M^{-1} cm^{-1}) or by weighing the protein and assuming a molecular weight of 35 000. The two methods are found to agree within 10%. The Fe(III) derivative was prepared by autoxidation in air of an iron(II) sulfate solution added to the phosvitin solution at slightly acidic pH.[2] The pH was subsequently adjusted to 7.2 with NaOH, and the resulting filtered solution was green-yellow. The sample used for the EXAFS measurement was 1 mM in protein and 10 mM in metal. No buffer was used in order to avoid possible competition in metal binding. The model compound $Fe(acac)_3$ (acac = acetylacetonate), in which the iron atom is coordinated by six oxygen atoms in a regular octahedral geometry (see Table I), was prepared by following literature methods.[14]

EXAFS Measurements. X-ray absorption measurements on the protein sample and on the standard were performed at the NSLS in Brookhaven on beamline X11A.[15] During data collection the synchrotron was operating at 2.5 GeV with ring current between 110 and 160 mA. A Si(111) double-crystal monochromator with energy resolution ($\delta E/E$) of 2×10^{-4} detuned by 50% for harmonic rejection was used. The protein spectra were recorded from 6950 to 7950 keV in steps of 1 eV in the edge region and 2 eV in the EXAFS region. The X-ray absorption of the phosvitin sample was monitored by fluorescence with a solid-state detector equipped with Soller slits and a Mn foil 1 μM thick to reject background photons.[16] The phosvitin solution was contained in a Teflon cell covered with Kapton windows. A series of 10 scans were collected at room temperature for a total of 5×10^5 counts per point. No damage of the protein sample was observed during the exposure to the X rays. In fact, no detectable difference in the X-ray absorption spectrum occurred between the first and last scan. Furthermore, the solution exhibited its normal color after the exposure with no sign of denaturation. The model compound $Fe(acac)_3$ was measured as a powdered solid in the transmission mode. The X-ray absorption data for anhydrous $FePO_4$ were kindly provided by Dr. Le Bail (Université du Maine, Maine, France).[17] Crystallographic data for the model compounds are reported in Table I.

Data Analysis. The data were processed by following standard procedures.[18,19] The 10 scans were averaged, and the EXAFS spectrum was extracted by subtracting the slowly varying atomic background and normalizing by the edge step. The experimental energy threshold was chosen at the inflection point of the edge jump at the energy of 7121 eV. The EXAFS data, weighted by k (Figure 1a), were Fourier-transformed over the range k = 3–12.5 $Å^{-1}$, to obtain the radial distribution function (Figure 1b). The k weight was chosen because it allows a better resolution of the first and second coordination shells with respect to the k^3-weighted data (see Figure 1b). The two shells were filtered by using smooth windows from 0.8 to 2.0 Å and from 2.1 to 3.1 Å and back-transformed. The data were subsequently used for a curve-fitting analysis in which the four parameters r (neighbor distance), N (number of neighbors), $\Delta\sigma^2$ (difference in the Debye–Waller factors), and ΔE_0 (change in the threshold energy) were allowed to vary for both coordination shells. The first shell of $Fe(acac)_3$ and the second shell of the $FePO_4$ model compounds were used as standards in the curve-fitting procedure. The ratio method[19,20] was also employed to compare the first coordination shells of Fe phosvitin and $Fe(acac)_3$ and the second coordination shells of Fe phosvitin and $FePO_4$.

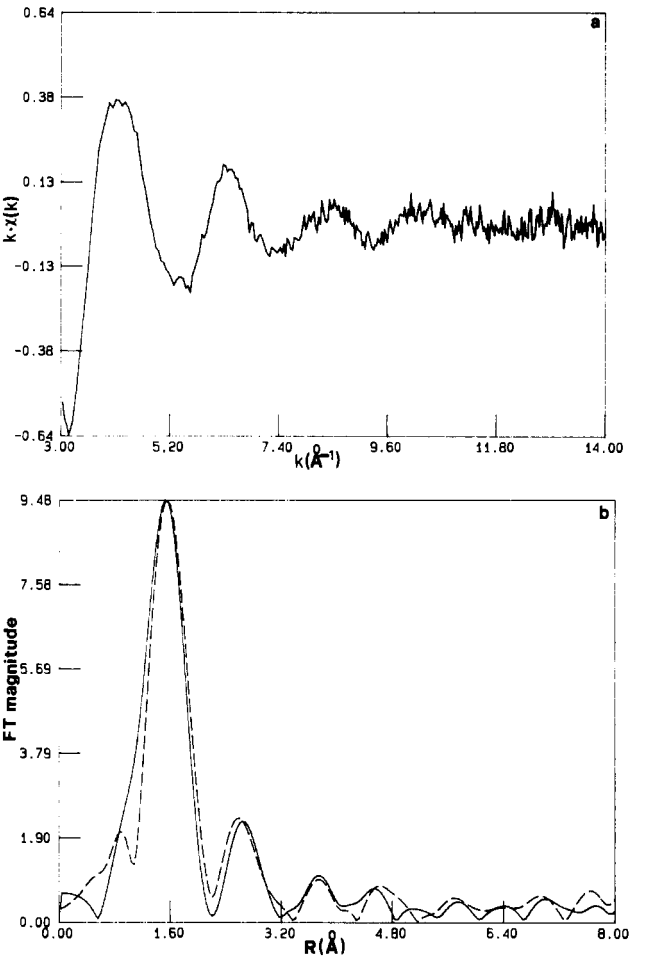

Figure 1. (a) k-weighted EXAFS spectrum of Fe phosvitin. (b) Fourier transform of the k-weighted (continuous line, multiplied by 43.09 for normalization) and of the k^3-weighted EXAFS spectra for Fe phosvitin (dashed line) uncorrected for the phase shift.

An estimate of the errors introduced by the experimental measurements and by the data analysis was obtained by analyzing separately four sets of data obtained by collecting the 10 scans in groups of two or three. For each set the EXAFS spectrum was extracted and Fourier-transformed and the first and second shells were isolated and analyzed in the same way as for the complete set. The standard deviation among the four sets was calculated and reported in the results of the analysis.

Results and Discussion

In Figure 1b are reported the Fourier transforms of the Fe–PST EXAFS data weighted by k and k^3 (solid and dashed lines, respectively) in which the two major peaks correspond to the first and second coordination shells of the iron atoms in the protein. The lower weighting scheme reduces the interference between the peaks, while their relative heights remain unchanged. Since the k^3 weighting should enhance the contribution of heavier atoms

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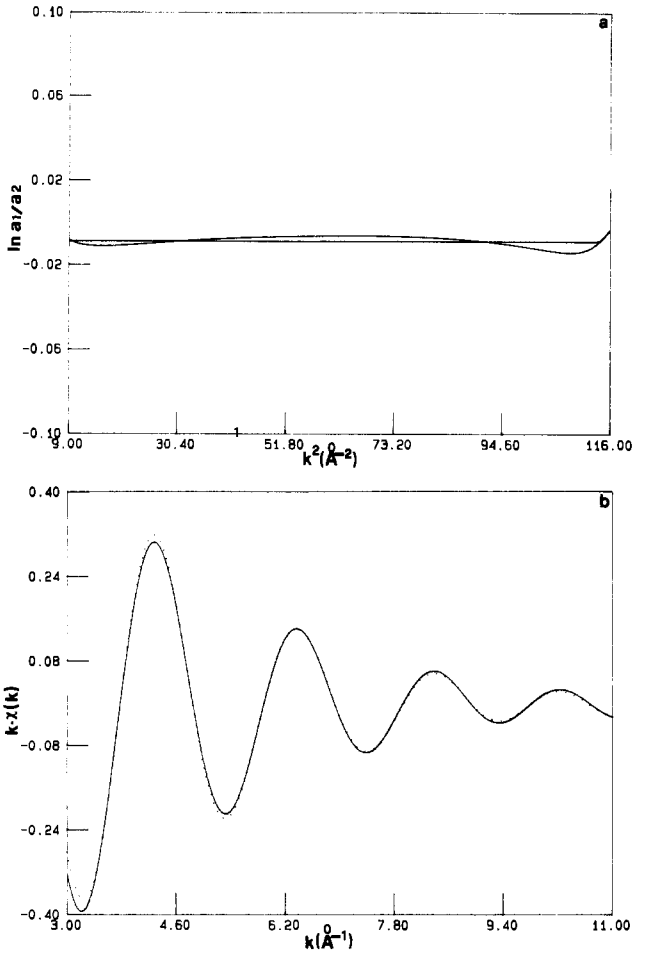

Figure 2. (a) Plot of the logarithm of the ratio of the envelope functions vs k^2 for the first shells of Fe–PST and Fe(acac)$_3$ and its least-squares line. (b) Best fit of the back-transformed first shell of Fe–PST with the amplitude and phase from the Fe(acac)$_3$ model compound: (continuous line) experimental data; (dotted line) best fit.

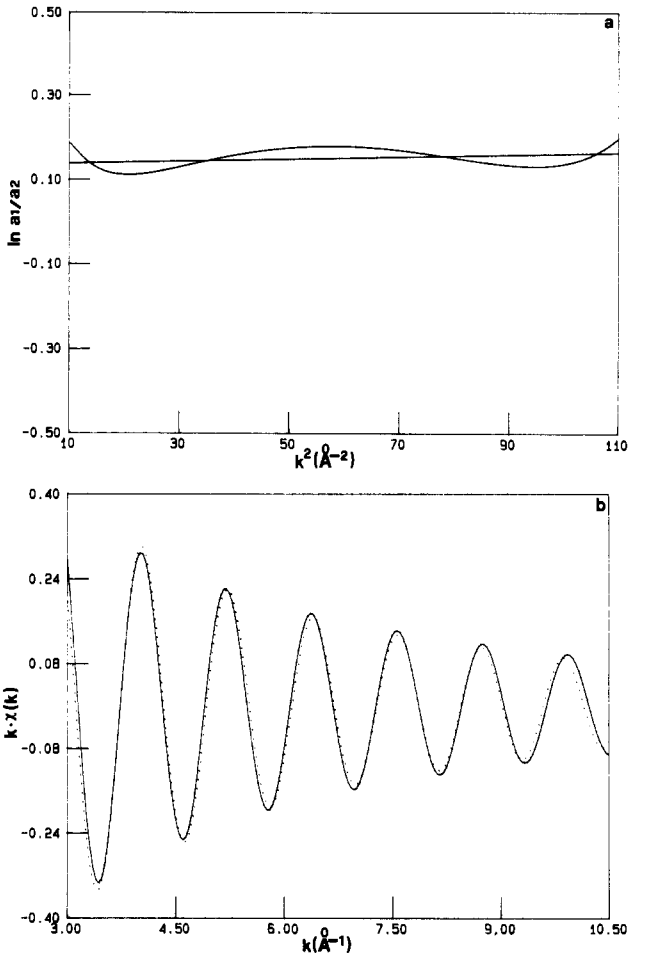

Figure 3. (a) Plot of the logarithm of the ratio of the envelope functions vs k^2 for the second shells of Fe–PST and $FePO_4$ and its least-squares line. (b) Best fit of the back-transformed second shell of Fe–PST with the amplitude and phase from the $FePO_4$ model compound: (continuous line) experimental data; (dotted line) best fit.

Table II. EXAFS Results for Fe(III) Phosvitin from Curve Fitting and the Ratio Method

	N	R, Å	$\Delta\sigma^2$, Å^2	ΔE_0, eV
	First-Shell Oxygen			
curve fitting	6.2 (6)	1.93 (2)	0.004 (4)	−1.1 (3)
ratio method	6.0	1.92	0.000	−2.0
	Second-Shell Phosphorus			
curve fitting	4.4 (9)	3.31 (4)	0.001 (8)	6.1 (5)
ratio method	4.9	3.30	−0.0003	4.0

to the transform, one should expect an increase of the amplitude if iron neighbors were to provide a predominant contribution to the second coordination shell.[21]

The symmetrical and narrow shape of the first peak suggests a quite narrow distribution of distances within this shell, indicating a possible homogeneity of the Fe binding sites in spite of the high Fe/PST molar ratio.

This is supported by the comparison of the first-shell back-transforms of Fe–PST and Fe(acac)$_3$ by the ratio method, in which the logarithm of the ratio of the envelope functions for the unknown and the standard is plotted against k^2 (Figure 2a). If the backscattering amplitude $B(k)$ is the same for the two compounds, the plot should yield a straight line with an intercept corresponding to the ratio of the coordination numbers and distances and a slope that depends on the difference in σ^2.

The good linear dependence obtained (see Figure 2a) shows that the first shell of Fe–PST is composed of oxygen/nitrogen

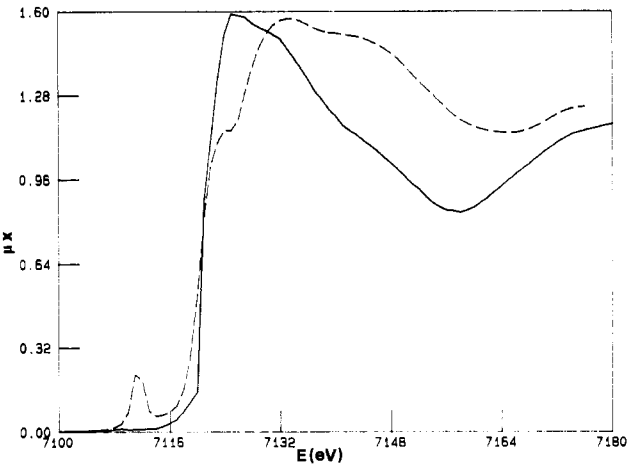

Figure 4. Near-edge structure of Fe–PST (continuous line) and of $FePO_4$ (dashed line) plotted at the same resolution (1 eV).

atoms, and the intercept near the origin indicates that the coordination number of this shell is nearly the same as that of the standard.

The curve-fitting analysis reported in Table II confirms these results, giving a first coordination shell of six light atoms at 1.93 (2) Å from the iron. This distance is in good agreement with the value expected for an Fe^{3+} ion in an octahedral oxygen environment[14,17,22] (see Table I). The six-coordinate iron stereochemistry

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is also supported by the K-edge structure (Figure 4), which shows no 1s–3d pre-edge feature in agreement with the behavior of octahedral iron(III) complexes.[21,23] Visual inspection of the Fourier transform determines the absence of the typical features of imidazole binding[24] (i.e. a third shell peak due to the back-scattering of C(4) and N(5) atoms of the imidazole ring enhanced by a multiple scattering effect), which is even more evident if compared with the spectrum of Cu^{II}–PST[3] at the same molar ratio, in which an imidazole ligand participates in the copper first coordination shell.

The second-shell analysis performed by both the ratio method and curve fitting gives comparable results, which are reported in Table II. The plot of the natural logarithm of the amplitude ratio vs k^2 for this shell is shown in Figure 3a. The amplitude and the Debye–Waller factor obtained by this plot are affected by a higher error with respect to that for the first shell, and this is probably due to the fact that the window used to isolate the second shell of Fe–PST still includes some contribution from low-Z atoms which cannot be completely separated from the phosphorus. However, the consistency of the two methods used in the analysis and the fact that the second shell of Fe–PST can be easily fitted with only one distance by using amplitude and phase functions from the $FePO_4$ standard leave little doubt that the predominant contribution to the second coordination shell of the iron atoms in the protein is made by phosphorus atoms. The Fe–P distance of 3.31 (4) Å found here is longer than the 3.16 (1) Å crystallographically determined for anhydrous $FePO_4$[25,26] but is in agreement with the distance found in $FePO_4 \cdot 2H_2O$,[22] in which the iron atom lies at the center of an octahedron made of four oxygen atoms from phosphate groups and two oxygens from water molecules coordinated in a cis position.

From the present EXAFS results it appears that the Fe(III) binding sites in hen phosvitin are provided by the phosphate groups of the phosphorylated serine residues and that the stereochemistry of the iron atoms is most probably octahedral. The average number of 4.4 (9) for Fe–P second-shell interactions suggests that six-coordination is achieved upon binding of other groups from the protein or solvent water molecules.

Finally, the absence of any evidence for an Fe–Fe short interaction allows us to conclude that iron is not essentially involved in a polynuclear structure with oxygen bridges, while a polymeric structure with –O–P–O– bridges as found in several metal phosphinates,[27] where the metal–metal distances are about 5 Å, cannot be excluded.

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Registry No. Fe, 7439-89-6; $FePO_4$, 10045-86-0; $Fe(acac)_3$, 14024-18-1.

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