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EXAFS studies of Fe(III)–phosvitin at high metal to protein ratios

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The stereochemistry of the Fe(III) binding sites in chicken egg phosvitin (PST) at very high iron content, in solution and as a powder, has been investigated through EXAFS spectroscopy. We found that the EXAFS spectra obtained for aqueous PST solutions at metal:protein ratios of 20:1 and 40:1 are very similar to those previously obtained by us on a Fe_{10}PST sample. In all cases the iron ions are octahedrally coordinated by oxygen atoms of the serine-bound phosphate groups and by other ligands from either the protein or the solvent. The average metal–donor atom distance is 1.94 Å. At variance, the EXAFS results for a Fe_{50}PST powder sample suggest the occurrence of a switch in iron coordination from octahedral to lower coordination numbers (5,4). The average iron–oxygen distance is virtually unchanged; apparently, four iron ligands are provided by four different coordinate phosphate groups from the phosphorylated serine residues abundant in the protein. This finding contains interesting implications for the structure–function relationships of this intriguing protein.

Keywords: EXAFS spectroscopy, iron stereochemistry, phosvitins

Introduction

Phosvitins are a group of relatively small phosphoglycoproteins (molecular weight = 35 000) that are found in the vertebrate egg yolk (Grogan & Taborsky 1987). Yet, their biological role is not fully understood; however, since essentially all tripositive and bipositive metal ions present in the yolk are bound to these proteins, they probably act as metal depositories for the embryo (Grogan & Taborsky 1987).

The primary structure of hen phosvitin (PST) consists of a single chain of 216 amino acids among which 123 are phosphorylated serines (Byrne *et al.* 1984). The three-dimensional structure is as yet unknown. Peculiarities of phosvitins are the virtual lack of non-polar amino acid residues, and the almost total absence of secondary and tertiary structure elements (Taborsky 1983). Moreover, these proteins are characterized by high hydrophilicity, high solubility at neutral pH and by a unique ability to bind a large number of metal ion equivalents through the several phosphate groups (Taborsky 1983). In solution PSTs essentially behave like large free polyelectrolytes. Only at acidic pH, with the protonation of the phosphate groups, PSTs rearrange toward more ordered structures (Taborsky 1983).

Because of the unordered conformation of PST in solution, the consequent difficulty to crystallize and the strong tendency to bind metal ions at multiple sites, it proves rather difficult to get detailed structural information on this protein through conventional spectroscopies. For the above reasons EXAFS spectroscopy has been of great help in elucidating the nature and the stereochemistry of the metal binding sites of the protein under various solution conditions. Some insight into the structural properties of copper–PST (Kozlowsky *et al.* 1988) and iron–PST (Mangani *et al.* 1990) in solution was already gained in previous EXAFS investigations. In particular, the EXAFS study of iron–PST, carried out on a Fe_{10}PST sample at neutral pH, indicated that the iron ions are octahedrally coordinated by four oxygen atoms of serine-bound phosphate groups and by two additional light atoms which may

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come either from water molecules or from other protein residues. On the basis of the similarity of the EXAFS spectra and derived parameters of iron–PST to those of iron–phosphate model complexes, it was also inferred that the iron centers of the protein could be arranged in polynuclear clusters with O–P–O bridges (Figure 1).

On the other hand, early spectroscopic and magnetic investigations on the green form of Fe(III)–PST, in the solid state, at an iron:protein ratio around 50:1, had provided evidence for the presence of antiferromagnetically coupled polynuclear iron centers, linked by μ-oxo bridges, with tetrahedral Fe(III) coordination (Webb *et al.* 1973).

To better elucidate the relationship between the iron content of PST, the resulting stereochemistry and the overall conformation, we decided to carry out an EXAFS investigation on iron–PST samples at high metal:protein ratios.

Materials and methods

Two aqueous solutions of iron–PST, 1 mM in protein and at iron:PST ratios of 20:1 and 40:1, have been prepared following the reported procedure (Mangani *et al.* 1990). For higher metal:protein ratios the solubility of iron–PST samples markedly decreases and the protein eventually precipitates. A powder sample, with a metal:protein ratio of 50:1, was prepared by recovering the precipitate and drying it under vacuum at room temperature.

The EXAFS spectra of protein samples have been collected at the PULS X-ray beam time at the Frascati SR facility. The experimental procedure and data analysis has been carried out as previously reported (Mangani *et al.* 1990).

Twelve EXAFS spectra of the two samples in solution (Fe_{20}PST and Fe_{40}PST) were collected and averaged; because of restriction in beam time, only one spectrum could be collected for the powder sample. The first shell of Fe(*acac*)$_3$ and the second shell of $FePO_4$ model compounds were used as standards in both the curve-fitting and the ratio method analysis as in our previous work (Mangani *et al.* 1990). These two compounds were chosen since they already proved particularly appropriate in modeling the iron environment in Fe_{10}PST.

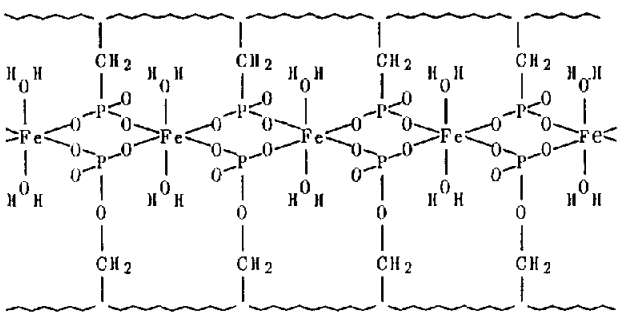

Figure 1. Proposed structure for the iron binding sites of Fe_{10}PST in solution.

Results and discussion

Figures 2 and 3 show the EXAFS data and their k-weighted Fourier transforms for the three iron–PST samples. The two largest peaks are attributed to the first and second coordination shells of the Fe(III), respectively.

In the case of Fe_{20}PST and Fe_{40}PST, the transforms do not differ significantly from those obtained for Fe_{10}PST. The structural parameters obtained for these two samples (Table 1), both for the first and the second coordination shell, are very similar to those previously found. These data are suggestive of a first coordination sphere with six light ligand atoms, four of which are phosphate oxygen atoms, as indicated by the presence of four phosphorus atoms in the second shell. Six coordination is also suggested by the characteristic pre-edge feature observed in the experimental spectra (Heald *et al.* 1979, Roe *et al.* 1984), which is identical in the two samples. Figure 4, for instance, shows the K-edge spectrum of Fe_{40}PST. The resulting picture strictly resembles that already proposed for Fe_{10}PST.

A visual inspection of the most important features in the Fourier transform and in the K-edge spectrum of powdered Fe_{50}PST (Figures 3 and 4, respectively) predicts substantial differences in the Fe(III) stereochemistry with respect to the previous cases. Indeed, in the Fourier transforms the ratio between the amplitudes of the peaks corresponding to the first and the second shells is considerably smaller than those obtained in the previous cases. Consistently, both the ratio method and the fitting analysis applied to the first coordination sphere indicate a lower coordination number of the iron ions, 3.9 and 4.5, respectively (Table 1). However, because of the intrinsic uncertainty on coordination number associated with EXAFS measures of protein samples (typically 20%), we cannot definitively state if the iron ions are four or five coordinated in Fe_{50}PST. However, the lowering in the coordination number is also confirmed by the presence of the diagnostic 1s–3d pre-edge peak (Figure 4) in the K-edge spectrum, which is indicative of a significant deviation from centrosymmetry in the metal stereochemistry (Heald *et al.* 1979, Roe *et al.* 1984). The peak is indeed absent in the other samples (see, for instance, that of Fe_{40}PST in Figure 4). This feature is, however, much less intense than in a typical tetrahedral iron complex, such as that of $FePO_4$, as

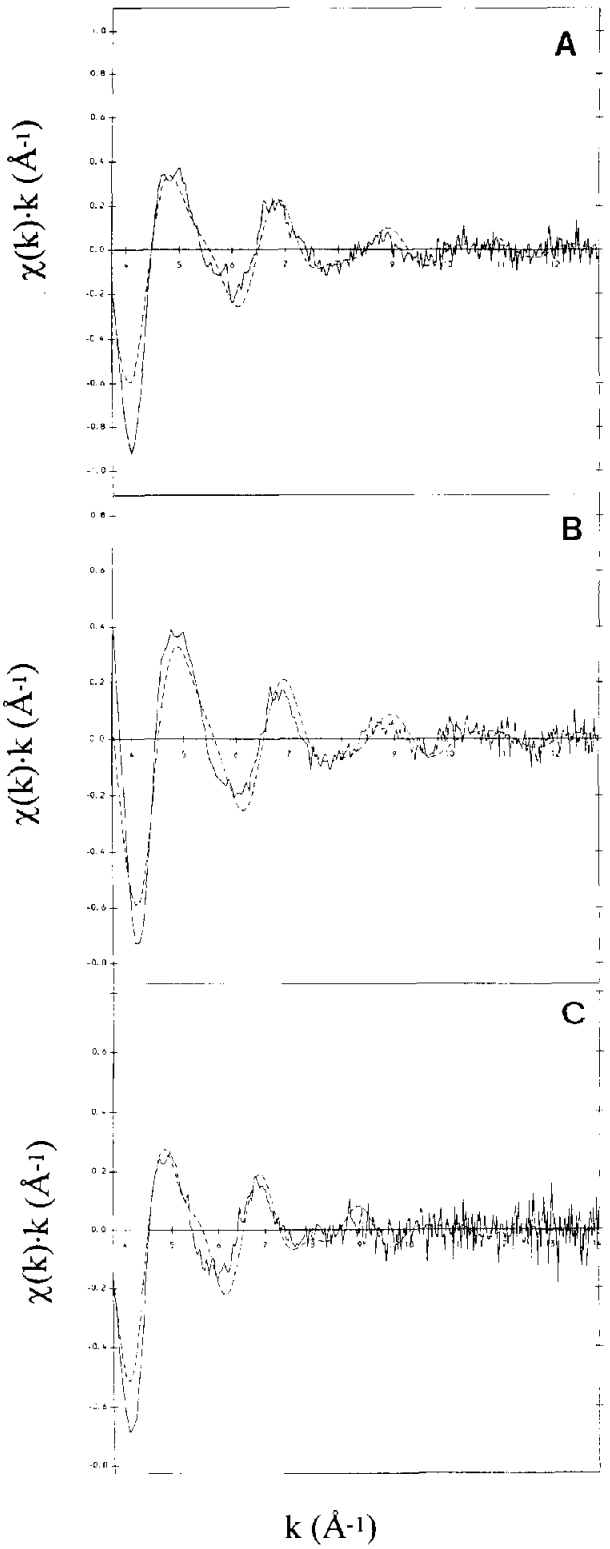

Figure 2. The k-weighted EXAFS spectra (continuous line) of Fe_{20}PST in solution (**A**), Fe_{40}PST in solution (**B**) and Fe_{50}PST as a powder (**C**). The dashed line is the simulation of the whole EXAFS spectrum obtained using the parameters from the first and second shell fits reported in Table 1.

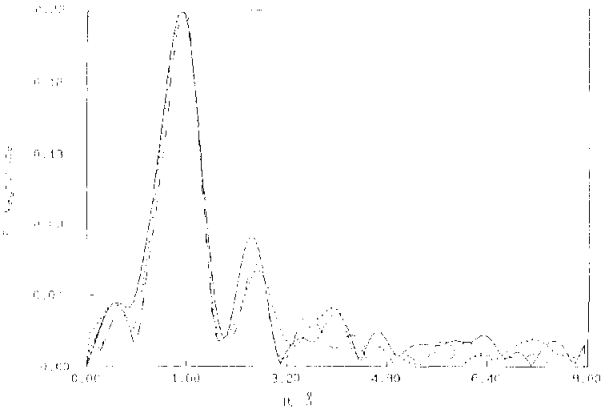

Figure 3. Fourier transform of the k-weighted EXAFS spectra of Fe_{20}PST in solution (dotted line), Fe_{40}PST in solution (dashed line) and Fe_{50}PST as a powder (continuous line, multiplied by 1.4 for normalization). All the transforms are uncorrected for their phase shift.

shown in Figure 4, suggesting the probable formation of pentacoordinated adducts in Fe_{50}PST. We conclude that our data strongly indicate a change of the iron coordination towards lower coordination numbers, namely four or five, upon increasing the Fe(III) content.

Interestingly, the atom–donor distance is similar in the three samples. Indeed, whereas in a simple complex one may expect an increase of the average metal–donor atom distance with the number of ligands, the correlation is not straightforward in the case of a metalloprotein, since, as has already been pointed out (Mangani *et al.* 1992), other important constraints, originating from the protein structure, act on the ligands, competing with the metal coordination effects.

The analysis of the second shell suggests that since four donor atoms of the ferric ions are provided by four phosphate oxygen atoms the second shell of the sample could be reproduced using backscattering amplitude and phase functions from the analogous shell of the $FePO_4$ model compound. It may also be noticed that attempts to simulate the second shell EXAFS with contributions from ferric ions were unsuccessful indicating the absence of any Fe–Fe short interactions through μ-oxo type bridges (Table 1). Thus, our EXAFS results suggest for the Fe_{50}PST sample a rearrangement of the polynuclear structure in which the individual iron centers, coordinated by four phosphate oxygen atoms, are linked by P–O–P bridges. Eventually, a fifth ligand is provided from solvent or from other protein residues.

The change in coordination number from 6 to lower values might be accounted for in terms of

Table 1. EXAFS results of Fe_{20}PST (a), Fe_{40}PST (b) and Fe_{50}PST as powder (c) from both the curve fitting and ratio methods[a]

	N	R (Å)	$\Delta\sigma^2$ (Å^2)	ΔE_0 (eV)	χ^2
First shell oxygen					
(a)					
curve fitting	6.5(6)	1.93(2)	0.004	−1.1	5.0×10^{-3}
ratio method	6.0	1.95	−0.003	−2.9	
(b)					
curve fitting	6.3(6)	1.94(2)	0.004	1.1	1.8×10^{-3}
ratio method	6.0	1.94	−0.005	0.0	
(c)					
curve fitting	4.5(6)	1.95(3)	0.005	−1.6	5.0×10^{-3}
ratio method	3.9	1.95	0.000	−2.0	
Second shell phosphorus					
(a)					
curve fitting	4.6(9)	3.15(4)	0.001	−1.0	1.9×10^{-3}
ratio method	4.0	3.11	−0.022	0.04	
(b)					
curve fitting	4.2(9)	3.15(4)	0.003	−1.4	2.1×10^{-3}
ratio method	4.2	3.12	0.002	−1.5	
(c)					
curve fitting	4.2(9)	3.13(4)	0.004	−1.8	6.4×10^{-3}
ratio method	3.7	3.00	0.000	−4.1	

[a] N is the number of atoms contributing to the shell, R is the distance of the shell from the metal ion, $\Delta\sigma^2$ is the difference between the Debye–Waller factors of the protein and of the model compounds, ΔE_0 is the change in the threshold energy with respect to the experimental threshold chosen at the inflection point of the edge jump at the energy of 7121.0 eV, χ^2 is a fit index defined as $\Sigma[k\chi_m(k) - k_u(k)]^2/\Sigma[k\chi_u(k)]^2$, χ_m and χ_m being the EXAFS function of model and unknown compounds.

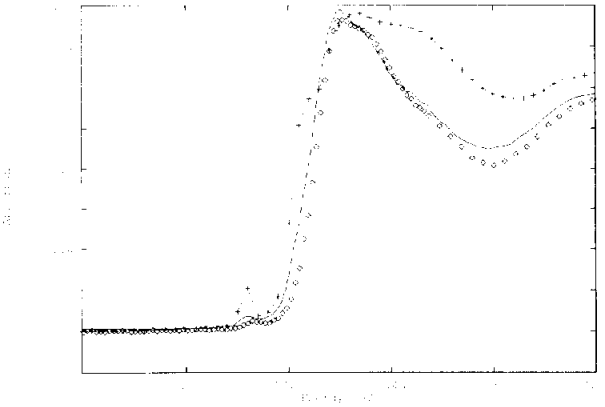

Figure 4. The k-edge spectra of Fe_{40}PST in solution (dotted line), of Fe_{50}PST as a powder (continuous line) and $FePO_4$ (dashed line).

conformational rearrangements of the protein occurring at high iron:PST ratios such that ligands other than phosphate oxygen atoms are excluded from coordination in some sites and cannot access anymore to the bound metals.

In a recent study Taborsky has reported on the solution properties of iron–PST samples at different degrees of iron saturation under acidic conditions (Taborsky 1991). It was shown that the solubility of the iron-phosvitin complex is drastically dependent on the iron:protein ratio; with iron:PST ratios around 60:1 the iron-PST complex is poorly soluble but resolubilization can be attained with higher metal:protein ratios. This means that the protein conformation is extremely sensitive to iron concentration in the medium and reacts to changes of the latter; in other words phosvitin acts somehow as a *biosensor* of Fe(III) concentration.

Our results, even if conducted under different experimental conditions, lead to similar conclusions. Indeed, small changes in iron concentration bring about a conformational change of the protein and a reorganization of the metal clusters. Further investigations are now needed to elucidate the functional implications of these findings.

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