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EXAFS studies on the active site of purple acid phosphatase from sweet potato *Ipomoea batatas*

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XAS investigations on the active site of the purple acid phosphatase isolated from the sweet potato *Ipomoea batatas* provided first information about the structure. Data analysis reveal a dinuclear active site containing an iron and a zinc atom. The first coordination sphere of each metal in the active site consists of six nitrogen/oxygen donor atoms, resulting in a distorted octahedral coordination sphere very similar to the active site of the enzyme isolated from the red kidney bean *Phaseolus vulgaris*. The XAS spectra were recorded in fluorescence mode at 18 K using a Canberra 13-element germanium solid state detector.

Keywords: EXAFS; zinc; purple acid phosphatase; sweet potato; *Ipomoea batatas*

Abbreviations

PAPs (purple acid phosphatases); spPAP (purple acid phosphatase isolated from sweet potatoe *Ipomoea batatas*; kbPAP (purple acid phosphatase isolated from red kidney bean *Phaseolus vulgaris*); MALDI-MS (matrix assisted laser desorption/ionisation mass spectrometry; SDS (sodium dodecylsulfat); SRXRF (synchrotron radiation excited X-ray fluorescence).

Introduction

PAPs with a Fe(III)-Me(II) center in their active site catalyse the hydrolysis of activated phosphoric acid esters and anhydrides in the pH range from 4 to 7. These metalloenzymes show a characteristic Tyr→Fe(III) charge transfer band responsible for the characteristic purple colour. Unlike the monomeric mammalian PAPs the plant enzymes have been reported to be homodimeric glycoproteins with a molecular weight of approximately 110 kDa.

MALDI-MS analysis of spPAP revealed a molecular mass of 112 kDa for single charged molecules and the corresponding peaks at 56031 and 28080 Mr/z for multiple charged molecules. The amino acid sequence alignment indicates that the metal-ligating residues in the active site of spPAP (accession number AJ006870) are identical to those in kbPAP (accession number P80366).

The pH-optimum was found at pH 6.0 and two pK_A values have been estimated at 4.6 and 7.4 from the Gaussian curves. A pK_A of 4.7 can also be derived from the pH dependency of the UV/Vis-spectra of the native enzyme. The absorption maximum (Tyr→Fe(III) charge transfer band) shifts from 514 nm at pH 3.25 to 547 nm at pH 6.5. (Durmus *et al.*, 1998)

Experimental

Sample preparation

The enzyme was isolated from sweet potato tubers according to a modified procedure by ammonium sulphate precipitation, acid treatment, gel filtration, anion exchange chromatography and carbohydrate affinity chromatography on Con A-Sepharose. The purity of the enzyme was shown by SDS polyacrylamide gel electrophoresis and MALDI-MS analysis (Durmus *et al.*, 1998).

The liquid enzyme solution samples (0.68 mM/subunit in 140 µl of 0.5 M $LiNO_3$) were adjusted to pH 7.0, filled in capton foiled cells and shock frozen in liquid nitrogen.

Data aquisition

The SRXRF, XANES and EXAFS data were recorded using a Canberra 13-element germanium solid state detector at the European Molecular Biology Laboratory (EMBL) at beamline D2 at the DORIS III storage ring at DESY, Hamburg. The data aquisition was carried out at 18 K in fluorescence mode with an electron beam energy of 4.5 GeV and an average current of 80 mA over the energy range from 9200 to 11000 eV. A Si(111) double crystal monochromator was used with a measured energy resolution better than 2.5 eV at the Zn-K-edge. Harmonic rejection was accomplished by detuning the first monochromator crystal to 50% of its peak intensity.

The metal ratio of spPAP was determined and quantified by SRXRF. This multi-element analysis is a very sensitive, non-destructive analytical method to determine and quantify elements (Sparks *et al.* 1983). In order to quantify the metal content of spPAP the peak area of the Fe K_α signal at 6404 eV was normalized to the analogous peak area of FeZn standard samples. The fluorescence energies are Fe (K_α = 6404 eV, K_β = 7058 eV) and Zn (K_α = 8639 eV, K_β = 9572 eV). The accuracy of the procedure is established by comparing the data with a model compound, showing a close agreement between our experimental data (Fe : Zn = 1 : 0.96) and the theoretical value of Fe : Zn = 1 : 1. The calculated values for the Fe : Zn ratio are 1 : 0.77 ± 0.04 for spPAP and 1 : 0.76 ± 0.04 for kbPAP (Durmus *et al.*, 1998).

Results and Discussion

For the XAS analysis 46 individual scans were recorded. Each scan had a runtime of about 50 min and consisted of 13 single channels. Every channel was sighted and only the channels without artificial contributions were averaged for analysis. The background subtraction, the normalization of the spectra, the determination of the edge position and the extraction of the fine structure have been performed as described earlier (Priggemeyer *et al.*, 1995). In this work we used 9660.0 eV for the Zn-K-edge to define E_0 for the data reduction. The $\chi(k)$ values were multiplied by k^3 and transformed by Fourier procedures. The white line intensity of 1.4 and the position of the Zn-K-edge of 9663.6 eV suggest a five- to six-coordination by nitrogen and oxygen (Priggemeyer *et al.*, 1995).

The wide-shell filtered data were fitted using a curved wave approach with the program EXCURV92 (Binstedt *et al.*, 1992; Binstedt *et al.* 1992; Binstedt & Hasnain 1996) and constrained refinement of the imidazole groups including their multiple scattering contributions as described earlier (Zippel *et al.*, 1996).

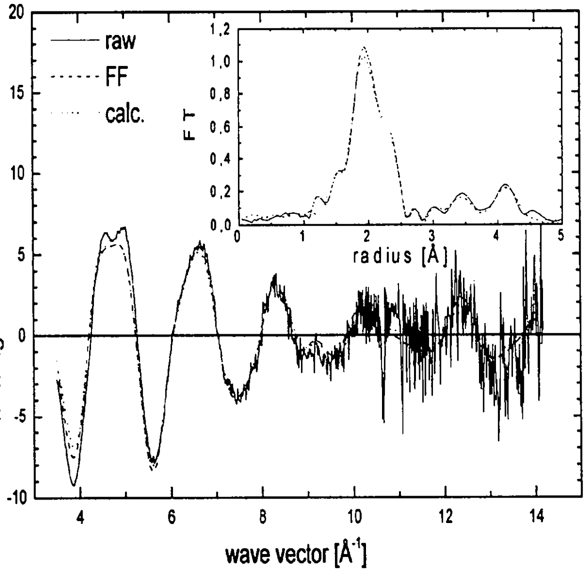

Figure 1

k^3-weighted fine structures of spPAP Zn-K-edge, the inset shows the FT of the spPAP Zn-K-edge.

The E_0 value is refined for all shells together, a value of 0.8 was used for the amplitude reduction factor and a value of −1.0 eV for the constant imaginary potential, which describes the lifetime of the photoelectrons. The maximum number of parameters allowed to vary simultaneously in the curve fitting analyses were estimated by $N_{free} = 2\Delta R_{FF}\Delta k_{fit}/\pi$ where ΔR_{FF} is the width of the filter window and Δk_{fit} is the length of the data set in k space. Using a window width of 1.0-3.9 Å and a k range of 12 Å^{-1} the estimated value for N_{free} is 22. The quality of the fit is represented by the R-factor which is given as

$$R = \sum_{i=1}^{N} \frac{k_i^n}{\sum_{j=1}^{N} k_j^n \left| \chi_j^{exp}(k_j) \right|} \left| \chi_i^{exp}(k_i) - \chi_i^{th}(k_i) \right| \times 100\%$$

where $\chi^{exp}(k)$ and $\chi^{th}(k)$ are the experimental and theoretical EXAFS (Binstedt et al., 1992).

EXAFS analysis using x-alpha phase shifts revealed a dinuclear active site containing an iron and a zinc atom coordinated by nitrogen and oxygen very similar to the active site of kbPAP. The k^3-weighted fine structures and Fourier transforms of the Zn-K-edge are shown in Fig. 1.

The zinc is coordinated by 6 nitrogen/oxygen donor atoms in the first shell. The first shell can be divided into 3 subshells consisting of 2 N/O donor atoms at a distance of 1.90 Å, 3 N/O donor atoms at a distance of 1.96 Å and 1 N/O donor atom at a distance of 2.42 Å that causes a distortion in the coordination geometry of the zinc. This distortion of the Zn-center is also indicated by the high values for the mean square deviations of the first coodination sphere. The analysis of the higher shells reveals one iron at a distance of 3.92 Å. Fits with the iron at a shorter distance of 3.26 Å or 3.57 Å lead to a increase of 50-150 % for the values of the R-factor and the mean square deviations. The fit results for the main backscatterers are summarized in Table 1.

Table 1

Curve-fitting results for Zn-K EXAFS of spPAP

N and scatterer	R, Å	$2\sigma^2$, Å^2	E_0, eV	Φ, deg	R-factor, %
3, O	1.955(2)	0.012(1)	18.2(3)		10.2
1, O	2.418(4)	0.002(1)			
2, N(imi)	1.901(8)	0.013(1)		121.9(3)	
2, C(imi)	2.815(8)	0.006(2)			
2, C(imi)	2.991(8)	0.006(2)			
2, C(imi)	3.997(8)	0.019(3)			
2, N(imi)	4.101(8)	0.019(3)			
1, Fe	3.924(6)	0.015(5)			

[a] N is the number of scatterers per metal, R is the metal-scatterer distance,

$2\sigma^2$ is a mean square deviation in R. The fit was performed over the k = 2-14 Å^{-1} range. The high values for the mean square deviations of the first coodination sphere indicate the distorted Zn-center.

The results are in good agreement with those found for frozen solutions of kbPAP (Priggemeyer *et al.*, 1995; Sift, 1996). The main difference can be found in the first coordination sphere. In contrast to kbPAP one of the six coordinating N/O is found to be in a longer distance at 2.42 Å. This result can explain the XANES results suggesting a five- to six-coordination instead of a six-coordination as found for kbPAP (Priggemeyer *et al.*, 1995; Sift, 1996).

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