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EXAFS study of the active site of alkaline phosphatase from *E. coli*

Stefano Mangani*
Department of Chemistry, University of Siena, Siena (Italy)

Paolo Carloni, Maria Silvia Viezzoli
Department of Chemistry, University of Florence, Florence (Italy)

and Joseph E. Coleman
Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06510 (USA)

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Abstract

The structure of the active site of the enzyme alkaline phosphatase (AP) from *E. coli* has been investigated by EXAFS spectroscopy. The study used two derivatives of the enzyme, $Zn_{2_A}E_{2_B}E_{2_C}AP$ and $Zn_{2_A}Co_{2_B}E_{2_C}AP$, the symbols referring to the fact that each active center of the enzyme dimer, consisting of two identical subunits, contains three metal binding sites (E refers to empty). Thus EXAFS has examined the environment of the Zn(II) ion in the A site of the enzyme in the absence and presence of a metal ion in the B site, 3.9 Å away. The analysis of the data has been performed by comparison with model compounds among which $Zn(imidazole)_2(acetate)_2$ shows a very close approximation to the structure of the zinc environment in the enzyme. From these findings the coordination of the metal at site A in the active site of AP appears to be composed of two histidine nitrogens plus four oxygen donors at an average distance of 2.04(2) Å from the Zn(II) ion. The oxygen donors appear to be made up of a carboxylate group acting as a bidentate ligand and the oxygens of two coordinated water molecules. Neither the ligands nor the coordination geometry around the Zn(II) ion at the A site change upon binding of a metal ion to the B site.

Introduction

Alkaline phosphatase (AP) from *E. coli* is a non-specific phosphomonoesterase of $MW \simeq 94\,000$, which catalyzes the hydrolysis of phosphate monoesters through the formation of a phosphoseryl intermediate with Ser 102 in the sequence of 449 amino acids [1–4]. AP is a dimeric metalloenzyme composed of identical subunits each of which contains three metal binding sites. In the native enzyme, two of them, A and B, are occupied by Zn^{2+} and the third site, C, by Mg^{2+}. From the crystal structure of the non-covalent phosphate complex of $Zn_{2_A}Zn_{2_B}Mg_{2_C}AP$, usually named E·P, at 2.0 Å of resolution and that of the $Cd_{2_A}Cd_{2_B}Cd_{2_C}AP$ at 2.5 Å resolution, the catalytic site A metal ion has been shown to be coordinated by four donor atoms from the protein, the NE2 of His 331, the NE2 of His 412 and both oxygens of the carboxyl group of Asp 327 [5]. In E·P, one of the phosphate oxygens is a fifth donor atom to Zn_A with a Zn–O bond length of 1.97 Å and a normal Zn–O–P bond angle of $\approx 120°$ [5].

The coordination of Zn_A in the E·P complex can best be described as pseudo tetrahedral with both carboxyl oxygens of Asp 327 occupying one of the apices. His 372 which was originally believed to be one of the A site ligands is not a ligand; instead the NE3 of His 372 is hydrogen bonded to one of the carboxyl oxygens of the ligand Asp 327 [5]. It is not possible to exclude an additional exchanging water molecule as a ligand to the Zn^{2+} at the A site on the E·P complex, whose presence is suggested by some NMR data [6].

Zinc ion in site B is coordinated by the NE3 atom of His 370, one carboxyl oxygen from Asp 51 and one carboxyl oxygen of Asp 369. The carboxyl group of Asp 51 forms a bridge between Zn_B and the magnesium ion in site C. In the E·P complex, a second oxygen of the phosphate is 1.97 Å from Zn_B completing a tetrahedral array of ligands. The Zn_B–O–P bond angle, however, is a highly unusual one of almost 180°.

The Mg^{2+} coordination at the C site can be described as a slightly distorted octahedron made up of the second carboxyl oxygen of Asp 51, one of the carboxyl oxygens of Glu 322, the hydroxyl of Thr 155 and three slowly exchanging water molecules. Asp 153 is not a direct

*Author to whom correspondence should be addressed.

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ligand as previously thought [7], but forms hydrogen bonds with two intervening water molecules which are the direct ligands [5].

Because of the apparent differences between the coordination of the Zn_A in the E·P complex, for which the high resolution crystal structure is known, and that of the native enzyme, for which only the crystal structure at 2.8 Å is available, we have investigated the structure of the Zn_A binding site present in the active site of alkaline phosphatase from *E. coli* by EXAFS spectroscopy. EXAFS is now a 'mature' spectroscopic technique able to provide structural information on the local environment of metal ions in metalloproteins [8]. First shell metal–donor atom distances are typically determined to an accuracy of ± 0.02 Å and coordination numbers to ± 20–35%. Furthermore EXAFS can detect histidine binding because of the characteristic enhancement of the backscattering from the atoms of the histidine imidazole ring due to a multiple scattering effect [9, 10].

Experimental

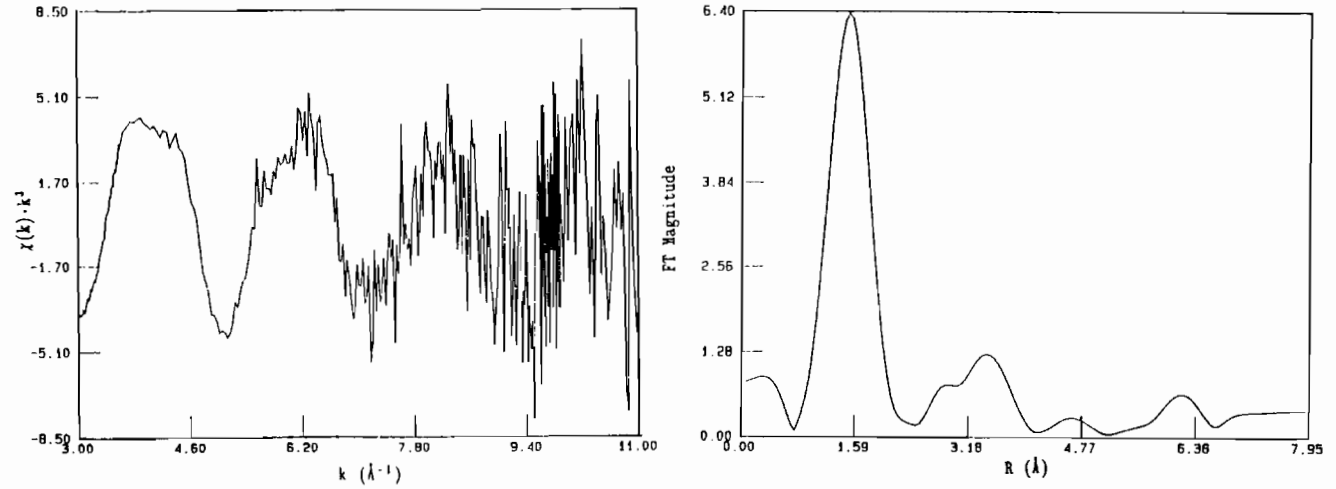
Native alkaline phosphatase has the metal ion composition $Zn_{2_A}Zn_{2_B}Mg_{2_C}AP$ reflecting occupancy of three sites, A, B and C, at each active center. The structure of the site A metal complex has been investigated by EXAFS in two different derivatives, $Zn_{2_A}E_{2_B}E_{2_C}AP$ (I) and $Zn_{2_A}Co_{2_B}E_{2_C}AP$ (II) (E refers to empty). AP was prepared from the CW 3747 strain of *E. coli* transduced with plasmid pH1 carrying the pho A gene. Isolation and purification followed literature procedures [11]. The two derivatives were prepared from the apoenzyme by adding stoichiometric amounts of aqueous solutions of the metal ions [12]. When two Zn^{2+} or Cd^{2+} are added to ApoAP the metal ions are preferentially bound to the A site as has been demonstrated by ^{31}P and ^{113}Cd NMR [4]. The presence of the metal ion at the B site is not absolutely required for hydrolysis activity, but the B site metal ion has a dramatic effect on the rate of phosphorylation of Ser 102 [4]. Hence the B site metal ion participates in the overall mechanism.

The samples used were aqueous solutions of the two derivatives 1.5 mM in protein at pH 6. Protein concentration was determined spectrophotometrically [13]. Model compounds $Zn(im)_2(ac)_2$ [14], $Zn(im)_6Cl_2 \cdot 4H_2O$ [15] and $Zn(acac)_2 \cdot H_2O$ [16] were prepared following literature methods (im = imidazole; ac = acetate; acac = acetylacetonate). Room temperature X-ray data on the protein samples were recorded at HASYLAB (DESY, Hamburg) as a series of 12–13 fluorescence spectra at the Zn and Co edges on the EXAFS spectrometer of the EMBL [17] equipped with a Si[220] double crystal monochromator [18]. The DORIS II storage ring was operating at 3.6 GeV in dedicated mode with current ranging from 35 to 90 mA. The monochromator energy was brought to an absolute scale by a calibration technique [19]. The energy resolution of the monochromator was 1.5 eV. No sign of sample denaturation was detected during data collection. Absorption data on model compounds were recorded at INFN (Adone, Frascati) using the experimental setup of the PULS laboratory [20]. Analysis of the data was carried out as previously reported [21] comparing the coordination shells of site A with the corresponding shells of model compounds by a curve-fitting procedure and the ratio method [22, 23].

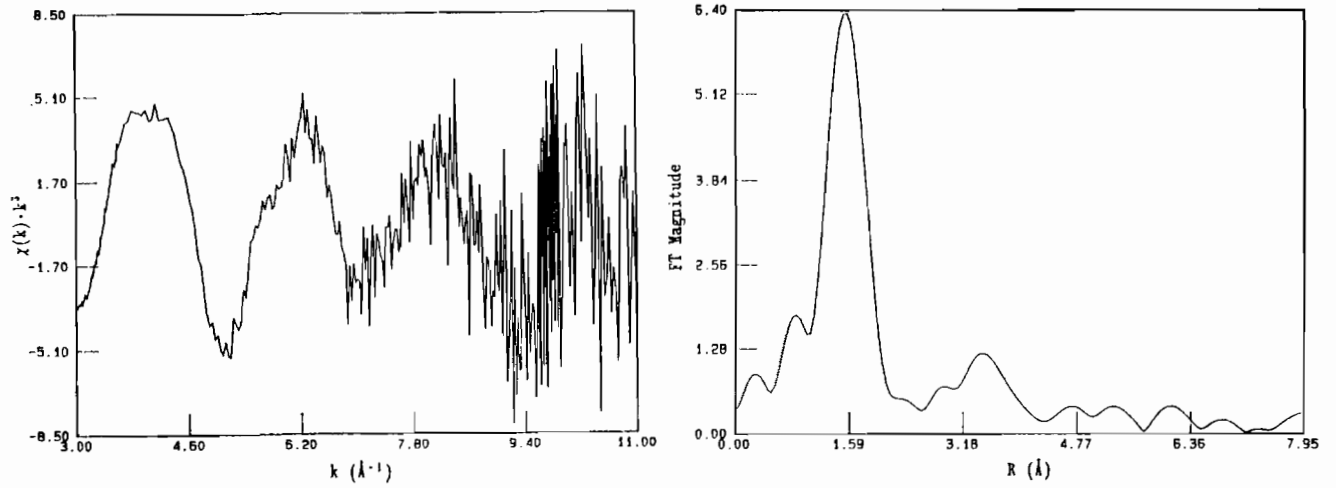
Results and discussion

EXAFS spectra obtained at the cobalt edge on sample II were affected by a high noise level which prevented any satisfactory analysis. The EXAFS spectra of the A site, taken at the zinc edge, for samples I and II are reported in Fig. 1 together with their Fourier transforms. In Fig. 1 are also shown the EXAFS and the Fourier transform of the model compound $Zn(im)_2(ac)_2$ for comparison. The EXAFS spectra taken at the zinc edge of samples I and II are almost identical confirming the preference of the Zn^{2+} ion for the A site.

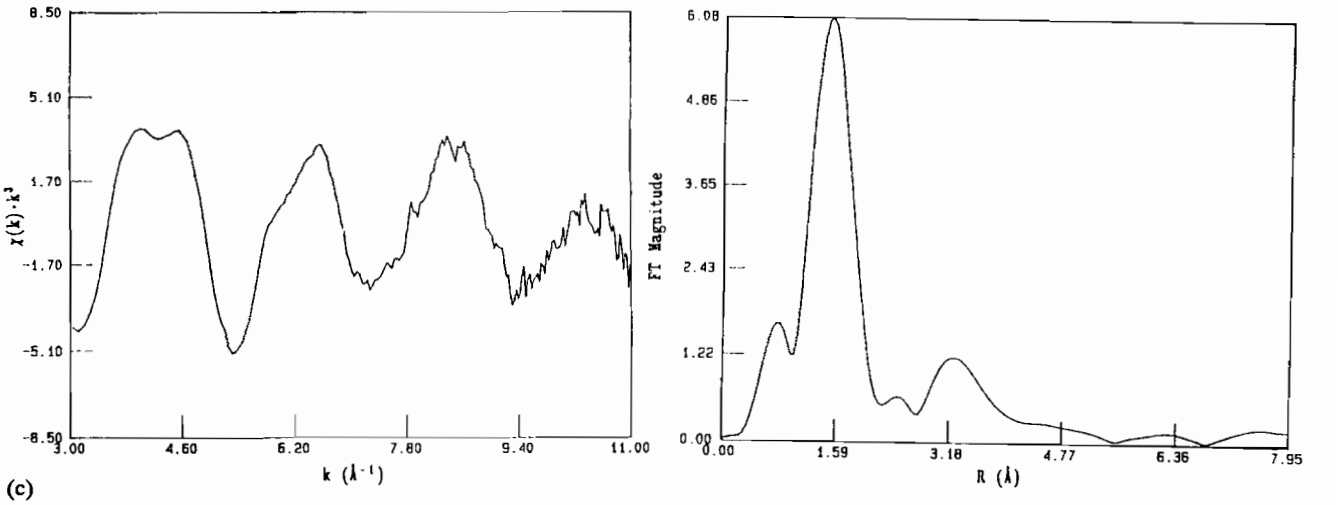
The structural parameters obtained from the analysis of the Fourier filtered first and second shell data for the A site compared with analogous data of the model compound $Zn(im)_2(ac)_2$, are reported in Table 1. The excellent quality of the fit obtained for the first shell data of $Zn_{2_A}Co_{2_B}E_{2_C}AP$ and $Zn_{2_A}E_{2_B}E_{2_C}AP$ with this model compound indicates a great similarity in the amplitude and phase functions of the enzyme Zn and that of the model and hence one can conclude that the nature and arrangement of the ligands in the model must approximate those found at the A site of AP. The ratio method analysis on the first shell confirms these findings (Table 1). Attempts to reproduce the first coordination shell of Zn(II) in the A site of AP with a two-shell curve fitting using $Zn(im)_6Cl_2 \cdot 4H_2O$ and $Zn(acac)_2 \cdot H_2O$ to obtain the Zn–N (imidazolic) and Zn–O (carbonylic) functions always resulted in worse fits in spite of the higher number of parameters used ($F^2 \simeq 10^{-2}$, for F^2 definition see Table 1). This can be explained by observing that in the model compound $Zn(im)_2(ac)_2$ a carboxylate group acts as a bidentate ligand with the second oxygen atom at 2.645(2) Å from Zn^{2+} [14]. The contribution from this oxygen atom would be present in the Fourier filtered first shell peak, backtransformed for the analysis. This suggests that at the A site of AP in solution a similar situation



(a)



(b)



(c)

Fig. 1. k^3 weighted EXAFS spectra and their Fourier transforms uncorrected for the phase shift of $Zn_{2A}E_{2B}E_{2C}AP$ (a), $Zn_{2A}Co_{2B}E_{2C}AP$ (b) and $Zn(im)_2(ac)_2$ (c). Transforms are calculated by using k^3 weighting and Gaussian windows for $3.0 < k < 11.0$ $Å^{-1}$.

TABLE 1. EXAFS results for $Zn_{2A}E_{2B}E_{2C}AP$ and $Zn_{2A}Co_{2B}E_{2C}AP$, zinc edge, from curve fitting and ratio method, using $Zn(im)_2(ac)_2$ as model compound[a]

	N	R (Å)	$\Delta\sigma^2$ (Å$^2\times10^3$)	ΔE_0 (eV)	F^2
1st shell (1.1–2.1 Å backtransform window)					
$Zn_{2A}Co_{2B}E_{2C}AP$					
Curve fitting	1.2(5)	2.04(2)	1.4(7)	2.2(3)	6.7×10^{-4}
Ratio method	1.2	2.04	3.5	2.3	
$Zn_{2A}E_{2B}E_{2C}AP$					
Curve fitting	1.1(5)	2.03(2)	0.8(5)	3.6(3)	2.7×10^{-3}
Ratio method	1.2	2.04	0.0	3.0	
2nd shell (2.4–4.2 Å backtransform window)					
$Zn_{2A}Co_{2B}E_{2C}AP$					
Curve fitting	0.9	3.52	0.0	0.6	1.0×10^{-2}
$Zn_{2A}E_{2B}E_{2C}AP$					
Curve fitting	1.0	3.52	0.1	−5.7	1.1×10^{-2}

[a]N is the ratio between coordination numbers of model and unknown compounds, R is the bond length from the zinc ion, σ^2 is the Debye–Waller factor, F^2 is a fit index defined as $\Sigma[k^3\chi_m(k)-k^3\chi_u(k)]^2/\Sigma[k^3\chi_u(k)]^2$, χ_m and χ_u being the EXAFS function of model and unknown compounds.

exists in which a carboxylate group acts as bidentate ligand to the Zn(II) ion, as the crystal structure indicates for the carboxylate of Asp 327 [5].

The outer shell peak present in the FT of both derivatives of AP at about 3.5 Å (Fig. 1) is compatible with the major contribution to this peak coming from the backscattering of the imidazole side chains of the histidine ligands present in the A site. However a contribution from the metal atom in the B site could not be ruled out. In order to obtain an estimate of the M(A)–M(B) distance in solution, if it were detectable, and to examine whether it could affect the EXAFS of site A, we have computed the difference spectrum between $Zn_{2A}Co_{2B}E_{2C}AP$ and $Zn_{2A}E_{2B}E_{2C}AP$. These spectra might be expected to differ only by the contribution of the cobalt ion in the B site, i.e. if no reorganization of the A site is induced by binding of the B site metal ion. The resulting spectrum was featureless indicating that the contribution of a heavy atom at 4.0 Å or more is negligible in complex systems like metalloproteins [24]. On the other hand, this analysis allowed us to obtain a rough estimate of the number of imidazole ligands by comparing the backtransforms of compounds I and II in the range 2.4–4.2 Å with the analogous data from the $Zn(im)_2(ac)_2$ model by curve fitting. The curve fitting was performed giving to the imidazole shell a starting single variable distance of 3.5 Å (an approximate average of the second and third shell distances) [25] while $\Delta\sigma^2$, E_0 and N were allowed to vary. The results of this analysis are reported in Table 1. The similar backscattering in both $Zn_{2A}E_{2B}E_{2C}AP$ and $Zn_{2A}Co_{2B}E_{2C}AP$ strongly supports the presence of only two imidazole ligands contributing to the EXAFS at the A site.

From the above results obtained for the two derivatives, it is possible to outline a quite clear picture of the coordination chemistry of the A site of alkaline phosphatase. The Zn(II) ion has a first coordination shell of nitrogen and oxygen atoms at an average distance of 2.04(2) Å. A precise statement on the number of ligand donor atoms and the identity of the amino acid residues or external ligands contributing them is of course more speculative. For both AP derivatives, the EXAFS data for the zinc edge are best approximated by the data for the model compound, $Zn(im)_2(ac)_2$. Thus the first coordination sphere for the A site zinc ion in AP appears to be made up of five to six light scattering atoms. The 2nd shell analysis (Table 1) provides some independent support for the conclusion that two of these are nitrogens of the two imidazole side chains of histidine residues.

The probable presence of four oxygen donors at site A, as the comparison of the model compound suggests, can only be interpreted in light of what is known from the high resolution crystal structure. A reasonable picture that satisfactorily explains the EXAFS data in solution in relation to the findings from the crystal structure is that two of the oxygen atoms are contributed by the bidentate carboxylate of Asp 327 and that two additional oxygen donors must come from coordinated water molecules. One water molecule is almost certainly displaced from the A site Zn by the phosphate oxygen observed in the 2 Å crystal structure of the E·P complex of $Zn_{2A}Zn_{2B}Mg_{2C}AP$ [5]. A number of NMR experiments, including ^{35}Cl line broadening experiments on the zinc enzyme [26] and studies measuring nuclear magnetic resonance dispersion (NMRD) of bulk solvent in the presence of the manganese enzyme [6] suggest

that there are two coordinated water molecules at the A site of alkaline phosphatase in the absence of external ligands. Both the present EXAFS data and the high resolution crystal structure would be compatible with two water ligands.

The EXAFS data show that it is very likely that the A site Zn(II) ion of alkaline phosphatase has the same coordination geometry and ligands in solution as it does in the crystal structure. The EXAFS data also strongly support the reinterpretation of the crystal structure, originally based on 3 Å data, and now based on 2.0 Å data which shows that only His 331 and His 412 are direct ligands to the A site Zn, with the additional protein ligand being the carboxyl of Asp 127 acting as bidentate ligand [5]. An additional conclusion made possible by the EXAFS data on two different derivatives, $Zn_{2_A}E_{2_B}E_{2_C}AP$ and $Zn_{2_A}Co_{2_B}E_{2_C}AP$, is that occupancy of the B site by a metal ion does not significantly change either the geometry or the ligands to zinc at the A site. Not only does this support what has been suspected for some time on the basis of ^{115}Cd NMR [4] and the low resolution crystal structure [7], namely, that the sites A and B share no ligands despite their separation by only 3.94 Å, but EXAFS also supports the conclusion that binding of the B site metal ion does not cause any significant change in the conformation of the active center. In other words most of the metal-ion-induced organization of the active center must be brought about by occupancy of the A site. This is compatible with the observation that the $Zn_{2_A}E_{2_B}E_{2_C}AP$ demonstrates significant catalytic activity.

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