FEBS Letters 358 (1995) 278-282 FEBS 15061

Zinc co-ordination in the DNA-binding domain of the yeast transcriptional activator PPR1

Linda J. Ball[a], Gregory P. Diakun[b], Paresh L. Gadhavi[a], Nigel A. Young[c], Elaine M. Armstrong[c], C. David Garner[c], Ernest D. Laue[a,]*

[a]*Department of Biochemistry, Tennis Court Road, Cambridge CB2 1QW, UK*
[b]*DRAL, Daresbury Laboratory, Warrington WA4 4AD, UK*
[c]*Department of Chemistry, University of Manchester, Manchester M13 9PL, UK*

Received 21 December 1994;

Abstract The structure of the native zinc form of the DNA binding domain in the yeast transcriptional activator PPR1 was investigated by extended X-ray absorption fine structure (EXAFS). By carrying out the EXAFS measurements at 11k we were able to demonstrate explicitly the proximity of the two zinc ions (Zn–Zn distance = 3.16 ± 0.03 Å) and the presence of bridging cysteine ligands. The results show that the six cysteine residues co-ordinate two zinc ions in a two-metal ion cluster. PPR1 is the first member of this class of protein for which such information has been obtained.

Key words: PPR1; Structure; DNA binding domain; Extended X-ray absorption fine structure; EXAFS; Metal ion cluster

1. Introduction

The PPR1 (pyrimidine pathway regulatory 1) protein activates the expression of the URA1 and URA3 genes involved in the pyrimidine biosynthetic pathway of the yeast *Saccharomyces cerevisiae* [1–3]. PPR1 recognises and binds co-operatively to a specific 12 base pair DNA sequence present within the upstream activating sequence for uracil (UAS_u) [4]. It belongs to a class of gene regulatory proteins that all contain a highly conserved cysteine-rich sequence ($Cys\text{-}X_2\text{-}Cys\text{-}X_6\text{-}Cys\text{-}X_6\text{-}Cys\text{-}X_2\text{-}Cys\text{-}X_6\text{-}Cys$) within their DNA binding domains [5,6]. In GAL4, the most highly studied of these proteins, this region has been shown to bind two atoms of either zinc or cadmium per molecule of protein [7,8]. Furthermore, the cadmium substituted form of GAL4 has been shown by two-dimensional $^1H\text{-}^{113}Cd$ NMR experiments to contain a two metal ion cluster of the type Cd_2Cys_6 [9]. Subsequent structure determination and comparisons between the NMR spectra of Cd(II) and Zn(II) GAL4 suggested that the native zinc form contained an analogous cluster [10,11]. However, NMR spectroscopy cannot readily be used to determine direct connectivities to zinc, so unambiguous proof of these structures using NMR is difficult. In principle EXAFS should allow such proof, but our previous work on Zn(II) GAL4 yielded ambiguous results [8].

Here we report the results of both electrospray ionisation mass spectrometry (EI-MS) and low temperature EXAFS studies on the native zinc form of a fragment of PPR1, Zn(II)-PPR1(1–118), which contains the UAS_u DNA binding domain. After purification of the DNA binding domain of PPR1 from an over-expressing strain of *E. coli*, EI-MS studies showed that it contains two zinc ions per protein molecule. EXAFS studies confirmed that the native zinc form of PPR1 contains a Zn_2Cys_6 two metal ion cluster, of the type reported for Cd(II) GAL4 [9–12].

2. Materials and methods

2.1. Protein purification

The Zn(II)-PPR1(1–118) fragment was purified using a modified version of the procedure described by Gadhavi et al. [13]; improved resolution was obtained using a Superdex 30 gel filtration column (Pharmacia) instead of the S-100 gel filtration column for the second step of the purification. Protein for EXAFS was concentrated to ~1 mM in the presence of 80 μM $ZnCl_2$. All protein concentrations were determined by amino acid analysis.

2.2. Gel retardation assays

Gel retardation assays were carried out on an 8% acrylamide gel, using 45 mM Tris-borate (pH 8.3) as the running buffer. The PPR1 UAS_u binding site was labelled at the 5′ end using $\gamma\text{-}^{32}P$-labelled ATP [14]. Varying concentrations of PPR1(1–118) were incubated with the labelled binding site in a buffer containing 10 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 20 mM $ZnCl_2$ and 10% (v/v) glycerol. All incubations were carried out in the presence of a ten-fold excess of poly(dI-dC).

2.3. Electrospray ionisation mass spectrometry (EI-MS)

EI-MS was carried out using a Bio-Q quadrupole mass spectrometer (VG Mass Lab Ltd.). The delivery solvent consisted of a mixture of 50% (v/v) acetonitrile in water; the flow was 2–4 μl/min and positive ions were detected. Calibrations were performed on myoglobin for the mass/charge (*m/z*) range 500–2000. The cone extraction voltage (B1) was set to 80 V. Each sample (~10 μl) contained 50% (v/v) methanol and 20–50 pmol/μl of protein.

Samples of the metallo form of the protein, Zn(II)-PPR1(1–118), were prepared as stated above. To obtain apo-PPR1(1–118), the metal ions were removed by reversed-phase HPLC on a C_{18} column using a water/acetonitrile gradient containing 0.1% TFA. All spectra were recorded in multiple scan acquisition mode, and molecular weights were determined using the manufacturer's software.

2.4. EXAFS studies

X-Ray absorption spectra were recorded over the energy range corresponding to the Zn K-edge, as fluorescence excitation spectra. The data were obtained on a Wiggler beamline using station 9.2 of the Synchrotron Radiation Source at Daresbury Laboratory. The electron storage ring operated at 2 GeV with an average current of 150 mA. Harmonic contamination was minimised by employing a double crystal Si (220) monochromator [15]. An array of 12 germanium solid state detectors with individual electronics [16] were used to monitor the X-ray fluorescence excitation spectra. A total of 20 one-hour scans were recorded for a 1 mM sample of PPR1(1–118) in a solution of 80 μM $ZnCl_2$ at pH 5.5 which was frozen and maintained at 11k by an APD DE204SL closed cycle cryostat. Prior to averaging the data, spectra from each germanium detector were weighted according to the edge height. Any spectrum showing irregularities was omitted from the averaging process.

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*Corresponding author. Fax: (44) (223) 33-3345.

SSDI 0014-5793(94)01448-5

The isolated EXAFS data for PPR1 were analysed over the range $2.75 < K < 12.75$ Å^{-1} using EXCURVE90 [17], employing the single scattering spherical wave method for EXAFS calculations. Phase shifts were obtained from ab initio calculations as described previously [18,19]. As an aid to the interpretation of the PPR1 data, corresponding X-ray absorption spectra were recorded in transmission mode at 11k for model compounds of known structure, $[PPh_4]_2[Zn_2(SPh)_6]$ [20] and $[NMe_4]_2[Zn_4(SPh)_{10}]$ [21], from powdered samples diluted with graphite.

3. Results

We initially used limited proteolysis of a fragment of the PPR1 protein, residues 1–143 (PPR1(1–143)), in an attempt to define the domain structure within the DNA binding region (Griffiths and Gadhavi, unpublished results). Gel retardation assays of several smaller fragments of the DNA binding domain showed that a fragment consisting of residues 1–118 bound specifically to a consensus 12 base pair DNA binding site, whereas smaller fragments did not. However, the larger fragment PPR1(1–143), was seen to bind somewhat more strongly and at lower protein concentrations (data not shown). Fig. 1 shows a gel retardation assay of Zn(II) PPR1(1–118) in the presence of its cognate DNA and an excess of poly(dI-dC); the PPR1-UAS_u complex can be seen at a protein concentration of 2.5 μM. Mass spectra of the apo and the metallo forms of Zn(II) PPR1(1–118) showed a mass difference between the two forms equivalent to that of two zinc atoms, confirming that this DNA binding domain binds two molecules of zinc (see Fig. 2).

EXAFS was employed to probe the immediate environment of the zinc ions in PPR1 and the two model compounds. This technique provides information on the number and types of ligands and their distances from the atom being probed. The measurements were carried out at a temperature of 11k so as to minimise thermal disorder in the sample, thereby enhancing the EXAFS signal. Phase shifts and the calculation procedures were checked using the model compounds. From the EXAFS data of the model compounds and their Fourier transforms (see Fig. 3) it is clear that scattering from multiple shells is responsible for the data, which are best fitted with a combination of Zn–S and Zn–Zn components. The first shell, due to Zn–S, was further sub-divided for both model compounds into two shells, thus providing an improved simulation of the experimental data. When the data were fitted to a single shell of sulphur atoms a large Debye-Waller type factor was obtained. As the EXAFS data were collected at a temperature of 11k, the atoms should have little thermal motion, so the higher than expected value for the Debye-Waller type factor could be attributed to static disorder. The final theoretical EXAFS simulations agree well with the crystal structures of the two model compounds and also reveal different Zn–S bond distances for the terminal (shorter distance) and bridging Zn–S bonds (see Table 1), thus providing us with confidence in the refinement procedure.

For Zn(II) PPR1, the EXAFS data and subsequent Fourier transforms (see Fig. 3) also show that there are contributions from two shells of atoms. The initial fit was to four sulphur atoms at 2.32 Å and a zinc atom at 3.16 Å. Again the Debye-Waller type factor for the single shell of sulphur atoms was large and it was consequently split into two shells each containing two sulphur atoms. The bond distances were allowed to float and the best fit was found with two sulphur atoms at 2.28 Å and two at 2.41 Å (the estimated error in the distances determined by analysis of the EXAFS data is ±0.03 Å). The second shell was fitted with a zinc atom at 3.16 Å. This was confirmed by Fourier filtering the peak in the transformed data and then fitting it with both zinc and sulphur. The fit index for zinc was 0.038 whilst for sulphur it was 0.090, suggesting that the second shell was indeed due to a zinc atom. The results are in agreement with the structure of the cadmium substituted form of the sister protein GAL4 as determined by NMR spectroscopy and X-ray diffraction [10–12].

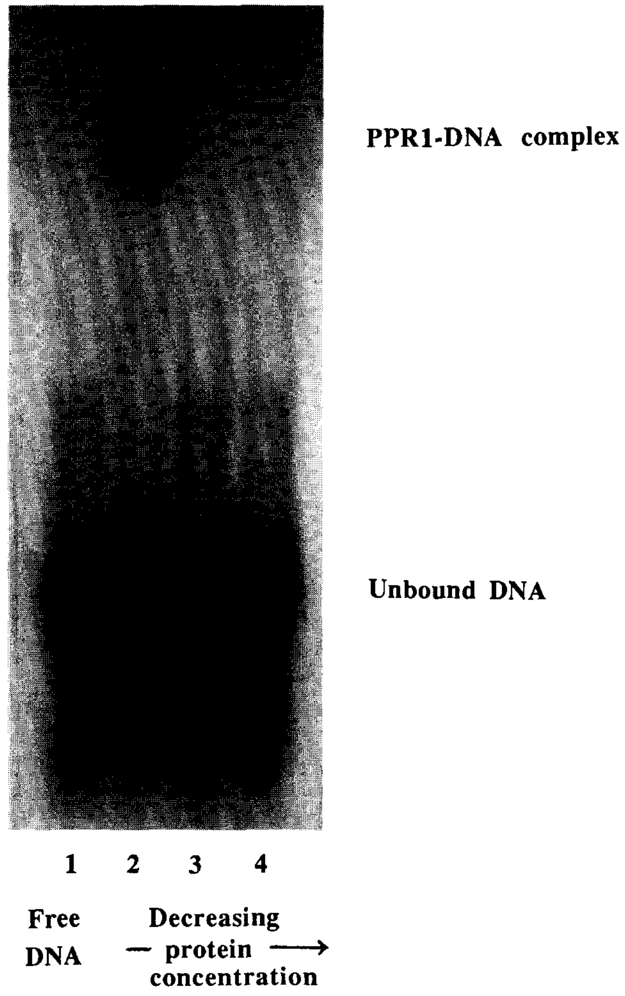


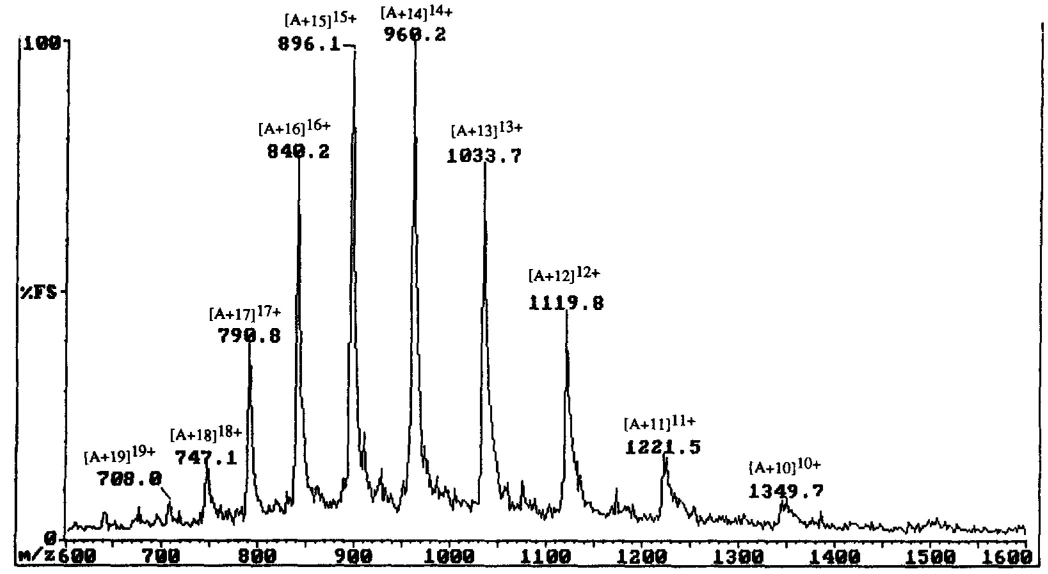
Fig. 1. Gel retardation assay showing that PPR1(1–118) binds sequence specifically to the UAS_u binding site, in the presence of an excess of non-specific poly(dI-dC). Lane 1 contains free DNA and lanes 2–4 contain DNA plus PPR1(1–118) at concentrations of 2.5 μM, 0.25 μM and 0.025 μM respectively. At the protein concentration of 2.5 μM, a single band due to complex formation is clearly visible.

4. Discussion

A combination of deletion analysis and probing of the domain structure using limited proteolysis experiments was used to define the minimal DNA binding domain of PPR1. Gel retardation analysis showed that the native Zn(II) form of residues 1–118 of PPR1 binds specifically to DNA and EI-MS showed that it contains two zinc ions per molecule of protein.

In our original studies of Cd(II) GAL4 we were able to show, by 2D ^1H-^{113}Cd NMR spectroscopy, that the six cysteine resi-

(a) Protein : Apo-PPR1(1-118)
Observed Mr. = 13,428 ± 1.38
Calculated Mr. = 13,429.70



(b) Protein : Zn(II)-PPR1(1-118)

Apo-protein	[A] :	13,433.10 ± 1.43
Metallo-protein	[M] :	13,560 ± 0.81
Mass difference	[Δ] :	127.38 (2 Zn)

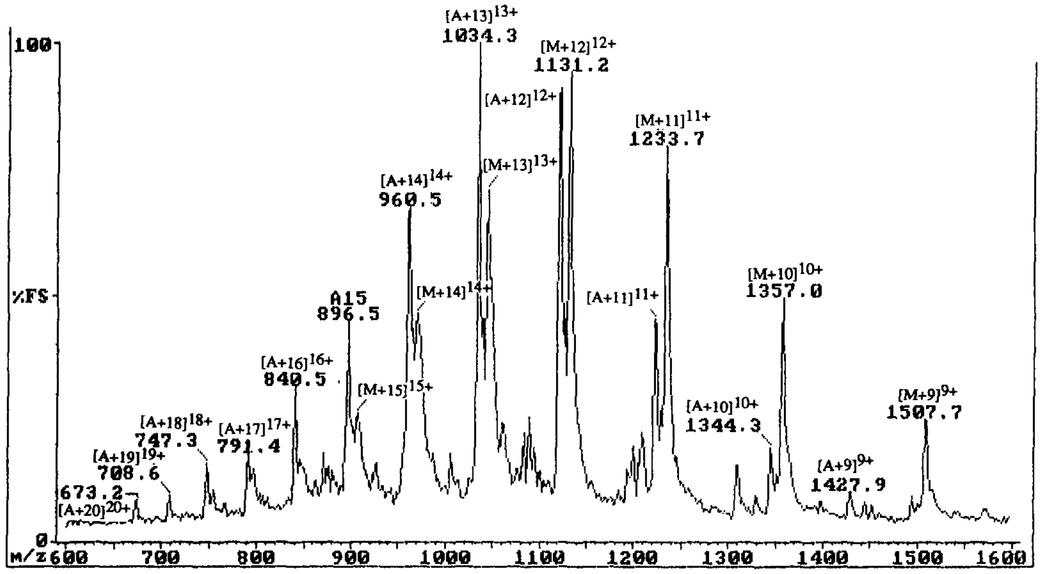


Fig. 2. Mass spectra of (a) apo PPR1(1–118) purified by reversed-phase (C_{18}) HPLC, to remove the metal ions, and (b) native Zn(II)-PPR1(1–118). The peaks labelled $[A+n]^{n+}$ denote the net charge (n) of the apo (A) form and $[M+n]^{n+}$ the net charge of the metallo (M) form of the protein. The spectra show clearly that two zinc ions are present per molecule of protein in the native metallo form.

dues co-ordinate the cadmium ions in a two metal ion cluster. Given the similar DNA binding affinities and ^{1}H NMR spectra of the Cd(II) and Zn(II) forms of GAL4, it was possible to infer that the native zinc form of GAL4 contained the same structure. The results reported here from the EXAFS studies of the closely homologous PPR1 protein show that this is correct. We propose that the two zinc ions are each co-ordinated by four sulphurs, two from terminal and two from bridging cysteines, in an arrangement (see Fig. 4) which corresponds to that identified for the $[Zn_2(SR)_6]^{2-}$ (R = Et [22] or Ph [20,23]) anions. The

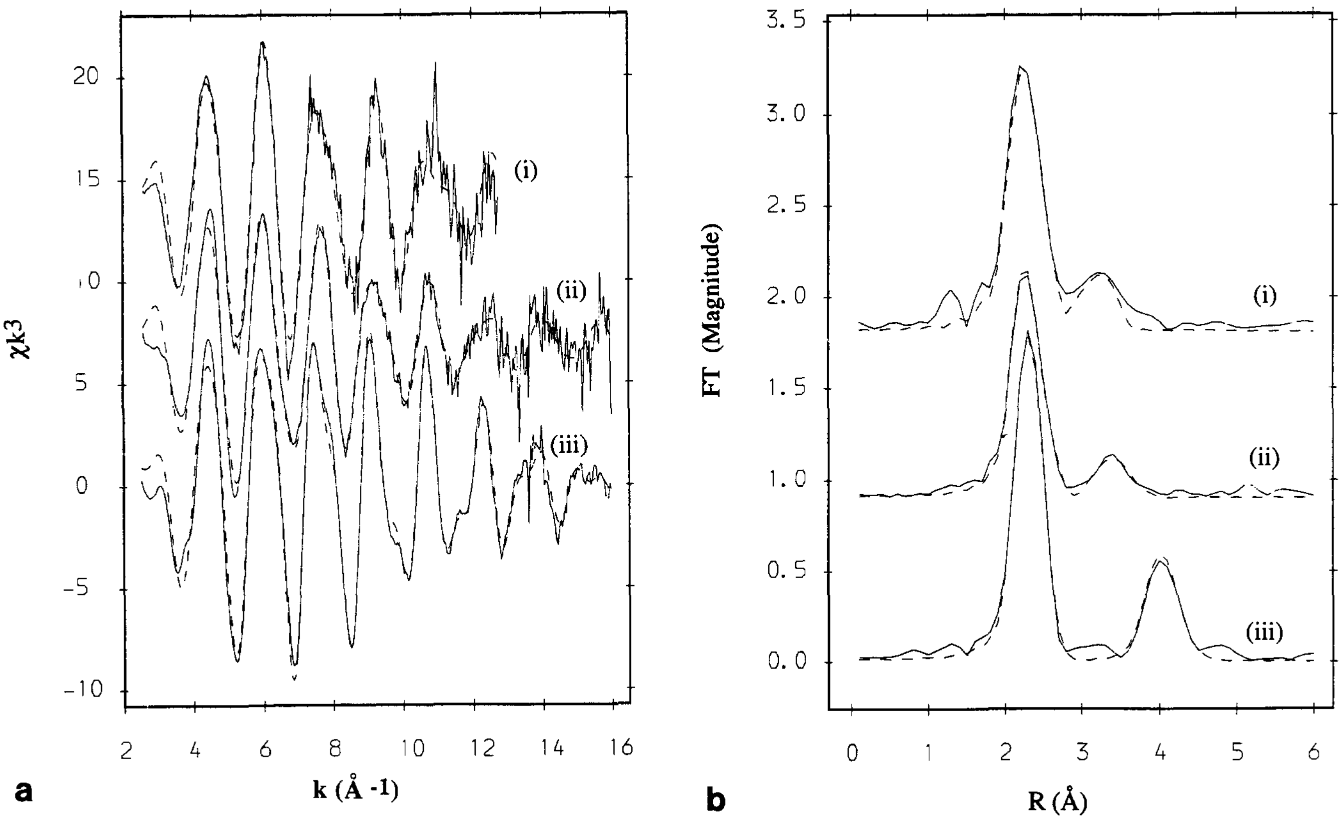


Fig. 3. (a) Zn-K edge EXAFS of (i) PPR1 (ii) $[Zn_2(SPh)_6][PPh_4]_2$ and (iii) $[Zn_4(SPh)_{10}][NMe_4]_2$. Continuous lines represent the experimental spectra, broken lines the simulated spectra. (b) Fourier transforms of the EXAFS data from Fig. 3a, where continuous lines represent experimental spectra and broken lines simulated spectra, of (i) PPR1, (ii) $[Zn_2(SPh)_6][PPh_4]_2$ and (iii) $[Zn_4(SPh)_{10}][NMe_4]_2$. The Fourier transform ranges were 2.5–12.75, 2.5–16.0 and 2.5–16.0 $Å^{-1}$, respectively.

Zn–Zn separation identified for PPR1 of 3.16 Å is in particularly good agreement with that of 3.18 Å in $[Zn_2(SEt)_6]^{2-}$, consistent with the zinc ions being bridged by two alkyl thiolates in each case. Also, the pattern of the Zn–S distances obtained for PPR1 is the same as that obtained for the $[Zn_2(SR)_6]^{2-}$ anions, in which the terminal and bridging Zn–S bonds are ca. 2.30 and 2.43 Å respectively [20,22,23]. These results clearly suggest that each monomer of the Zn(II) form of PPR1 contains a bimettalic cluster, in which each zinc ion is tetrahedrally co-ordinated by four cysteine residues, two of which act as bridging ligands between the two neighbouring zinc atoms (see Fig. 4).

Acknowledgements: We thank A. Roy for the cDNA encoding PPR1, Mrs. J. Jacoby for amino acid analyses and Paul Skelton for help with mass spectrometry. This work was supported by the SERC and its Cambridge Centre for Molecular Recognition. We also thank the SERC for an Advanced Fellowship (N.A.Y.).

Table 1

Samples and methods	No. of atoms	Zn–S distance (Å)[a]	No. of atoms	Zn–S distance (Å)[a]	No. of atoms	Zn–Zn distance (Å)[a]
PPR1						
EXAFS[b]	2	2.28 $\sigma^2 = 0.001$	2	2.41 $\sigma^2 = 0.002$	1	3.16 $\sigma^2 = 0.005$
$[(PPh_4)]_2[Zn_2(SPh)_6]$						
EXAFS[b]	2	2.29 $\sigma^2 = 0.025$	2	2.41 $\sigma^2 = 0.004$	1	3.35 $\sigma^2 = 0.007$
X-ray diffraction [21]	2	2.30	2	2.43	1	3.42
$[NMe_4]_2[Zn_4(SPh)_{10}]$						
EXAFS[b]	1	2.28 $\sigma^2 = 0.0005$	3	2.37 $\sigma^2 = 0.0025$	3	3.97 $\sigma^2 = 0.0055$
X-ray diffraction [21]	1	2.29	3	2.37	3	3.98

[a] For the EXAFS data the Debye Waller type factor σ^2, obtained in the final fit, is also quoted.
[b] The estimated error in the distances determined by analysis of the EXAFS data is ±0.03 Å.

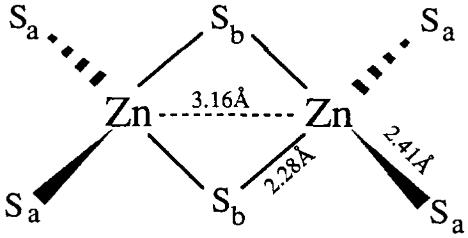


Fig. 4. Schematic representation of the zinc cluster in the DNA binding domain of PPR1. Bond lengths to terminal (a) and bridging (b) sulphur atoms (obtained from the EXAFS data) are marked on the diagram and the metal–metal separation is 3.16 Å. These values are in good agreement with those obtained for the two metal ion cluster observed in Cd(II) GAL4 [21].

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