RESEARCH COMMUNICATION

Evidence for a conserved binding motif of the dinuclear metal site in mammalian and plant purple acid phosphatases: ^1H NMR studies of the di-iron derivative of the Fe(III)Zn(II) enzyme from kidney bean

Gianantonio BATTISTUZZI^{*†}, Markus DIETRICH[†], Renate LÖCKE[†] and Herbert WITZEL^{†§}

^{*}Department of Chemistry, University of Modena, Via Campi 183, 41100 Modena, Italy, and [†]Institute of Biochemistry, Westfälische Wilhelms-Universität, Wilhelm-Klemm Str. 2, D-48149 Münster, Germany

The di-iron core of mammalian purple acid phosphatases has been reproduced in the plant enzyme from kidney bean (M_r 111000) upon insertion of an Fe(II) ion in place of the native zinc(II) in the dinuclear Fe(III)Zn(II) core. The shortening of the electronic relaxation time of the metal centre allows detection of hyperfine-shifted ^1H NMR resonances, although severe broadening due to Curie relaxation prevents independent signal assignment. Nevertheless, comparison of the spectral features of

the structurally characterized plant enzyme with those of the mammalian species, which were previously extensively assigned, is consistent with a close similarity of the metal-binding sites, also suggested by previous sequence-alignment studies. Some differences appear to be mainly localized at the M(II) site. Spectral comparison was also carried out on the Fe(III)Co(II) derivatives.

INTRODUCTION

Purple acid phosphatases (PAPs) isolated from mammals and plants are enzymes which *in vitro* catalyse the hydrolysis of activated phosphoric acid esters and anhydrides over the pH range 4–7. They are characterized by dinuclear Fe(III)M(II) metal centres, and show a typical Tyr-Fe(III) charge transfer band around 560 nm, which is responsible for their purple colour [1–3]. The mammalian enzymes from pig uterus [uteroferrin (Uf)] and from bovine spleen (BSPAP) (M_r 35000–40000) contain an anti-ferromagnetically coupled di-iron centre, which *in vivo* exists in the reduced, catalytically active Fe(III)Fe(II) form, whose EPR spectrum presents a rhombic signal at $g_{av} = 1.75$, indicative of an $S = 1/2$ ground state. Oxidation by H_2O_2 or $\text{S}_2\text{O}_8^{2-}$ leads to an EPR-silent ($S = 0$), catalytically inactive Fe(III)Fe(III) species [1–3]. In contrast, the most studied plant enzyme, isolated from red kidney bean (KBAP), is a homodimeric protein (M_r 111000), containing an Fe(III)-Zn(II) centre per monomer (Figure 1) [4–8]. Although Uf and KBAP are scarcely sequence related (about 20 % identity), sequence-alignment studies suggest that the metal-binding residues should be conserved [9]. Moreover, the recently solved X-ray structure of the plant enzyme [10,11], which is the only three-dimensional structure of PAPs available to date, shows that striking analogies exist between the nature and arrangement of the metal-binding residues of the bimetallic core of the protein and those proposed for the mammalian PAPs on the basis of ^1H NMR measurements [12–15]. In order to probe for these similarities on spectroscopic grounds, we have hereby exploited the hyperfine-shifted ^1H NMR resonances [16,17] as a sort of spectral fingerprints of the active site. We have recorded the ^1H NMR spectrum of the Fe(III)Fe(II) derivative of KBAP, in which the native zinc has

been replaced by an Fe(II) ion [4,5], thus reproducing in the plant enzyme the di-iron core of the mammalian species. In the comparison of the spectral features of the structurally characterized plant enzyme with those of the mammalian enzymes, which were previously extensively assigned [12–15], we profit from the known three-dimensional structure of the former species to verify assignments and probe for conserved features of the metal-binding domains. Spectral comparison was also extended to the Fe(III)Co(II) derivatives [5,12].

EXPERIMENTAL

Isolation of the native form of KBAP has been carried out with a modification of the method of Beck et al. [6], based on salt extraction, ethanol fractionation, $(\text{NH}_4)_2\text{SO}_4$ precipitation and affinity chromatography on Blue Sepharose CL-4B (Pharmacia) [8]. Preparation of the Zn-depleted enzyme (semiapoenzyme) was achieved through incubation of the native enzyme with 0.1 M EDTA/0.5 M NaCl, pH 6.5, followed by extensive dialysis to remove the excess of chelating agent [4]. The Fe(III)Fe(II) and Fe(III)Co(II) derivatives were obtained by incubating the semiapoenzyme in 0.1 M Bistris/0.5 M NaCl/15 % (v/v) glycerol, pH 6.0, with an excess of Fe(II) (in the presence of β -mercaptoethanol as reducing agent) and Co(II) respectively [4]. Unbound metal ions were removed by ultrafiltration in a membrane cone (Amicon CF25). Protein concentration was determined by measuring the absorbance at 280 nm (ϵ_{280} 280 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ for the holo protein) [4]. ^1H NMR spectra were recorded on a Bruker AMX-400 spectrometer, operating at 400.13 MHz, using 16000 data points over a 125 kHz bandwidth.

Abbreviations used: PAP, purple acid phosphatase; BSPAP, bovine spleen PAP; KBAP, kidney bean PAP; Uf, pig uteroferrin.

[†] To whom correspondence should be addressed.

[§] Professor H. Witzel died in Münster on 1 September 1996.

Figure 1
Hydroxo and water ligands could not be unambiguously detected by X-ray analysis. Scheme of the dimetal site of KBPAP according to the X-ray structure [9,10].

Suppression of the water peak was achieved by the super-WEFT pulse sequence (180– τ –90–AQ + delay) [18] with recycle times of 65–70 ms and delay times (τ) of 60–65 ms. A typical spectrum run on a 1 mM protein sample required 65000–100000 scans. Spectra were recorded in water/glycerol [15% (v/v)] solution (100 mM Bistris and 0.1 M NaCl, pH 6.0), with 10% $^2\text{H}_2\text{O}$ for the lock, or in 100% $^2\text{H}_2\text{O}$ solution (100 mM Bistris and 0.1 M NaCl, pH 6.0) at 300.1 °C. Chemical shifts and linewidths of the hyperfine-shifted signals were unaffected by the presence of glycerol. Spectra on Uf and the di-iron-KBPAP derivative were run in the presence of a slight excess of a reducing agent (β -mercaptoethanol) under argon atmosphere. Chemical-shift values are referenced to tetramethylsilane after calibration against the water peak, set at 4.78 p.p.m. from tetramethylsilane.

RESULTS AND DISCUSSION

The ^1H NMR study of native KBPAP is hampered by the slow-relaxing properties of high-spin Fe(III) [16,17], which cause the resonances of the active-site protons to be broadened beyond detection. In Fe(III)Fe(II)–KBPAP the fast-relaxing Fe(II) ion inserted in place of the Zn(II) ion helps to render the above line broadening less severe, since in strongly coupled dinuclear centres the electronic relaxation time is dominated by the fast-relaxing ion [16,17]. A number of broad hyperfine-shifted ^1H NMR resonances is indeed obtained, two of which are solvent exchangeable, which are shown in Figure 2(a). Their spectral parameters are listed in Table 1. Since hyperfine shifts of protons in the surroundings of both Fe(III) and Fe(II) centres are mainly contact in origin [14–17], these resonances most probably arise from metal-co-ordinating residues. Incidentally, we note that the present protein derivative is the largest non-haem paramagnetic iron protein for which resolved hyperfine-shifted ^1H NMR signals have been obtained [16]. The signals are much broader than those of the ^1H NMR spectra of the reduced mammalian PAPs, most likely because of Curie relaxation, which is the dominant contribution to transverse relaxation time (T_2) in high- M_r paramagnetic metalloproteins [16,17]. The submillisecond T_1 values do not allow any successful one-dimensional nuclear Overhauser enhancement or two-dimensional experiments [2,5,6,12], nor, hence, independent signal assignment. In any case, the comparison of the spectral features arising from the Fe(III)Fe(II) centre in the present derivative with those of the homologous centre in mammalian enzymes is a meaningful tool to probe for conserved features in the active sites of PAPs. Moreover, it can

Figure 2
High-frequency region of the 400 MHz ^1H NMR spectra of (a) Fe(III)Fe(II)–KBPAP in 100 mM Bistris buffer, containing 0.1 M NaCl, in 15% (v/v) glycerol/water solution, pH 6; (b) reduced native Uf in 50 mM Bistris buffer in water, pH 6 (peak lettering is taken from [12]); and (c) Fe(III)Co(II)–KBPAP in 100 mM Bistris buffer, containing 0.1 M NaCl, in 15% (v/v) glycerol/water solution, pH 6. Each protein concentration was 1 mM. The temperature was 30 °C. Shaded signals correspond to exchangeable protons in $^2\text{H}_2\text{O}$.

Table 1 ^1H NMR parameters (400 MHz) for the Fe(III)Fe(II) and Fe(III)Co(II) derivatives of KBPAP at 30 °C

Peak	δ (p.p.m.)	$\Delta\nu_{1/2}$ (Hz)	Integration
Fe(III)Fe(II)KBPAP			
a	88.4	2200	1
a' (ex)	88.4	2200	1
b, b'	65	—	2
c (ex)	39.8	1600	1
d, d', d'', d'''	25.8	2800	4
e	12.6	360	1
Fe(III)Co(II)KBPAP			
A (ex)	105	—	1–2
B	98.6	3300	3–4
C	62	—	2
D	46.2	600	1
E	39.3	1400	2
F	29.5	1400	1
G	20.6	1300	1

Table 2 Proposed assignments for the ¹H NMR hyperfine-shifted resonances of di-iron PAPs

The Uf and BSPAP peak, δ and assignment columns are taken from [13]. Abbreviation used: n.o., not observed.

KBPAP Peak	δ (p.p.m.)	Assignment	Peak	δ (p.p.m.)		Assignment
				Uf	BSPAP	
<i>a</i>	88.4	C β H-Tyr-167	<i>a</i>	86.6	69.5	C β H-Tyr-Fe(III)
<i>a'</i> (ex)	88.4	N δ H-His-325	<i>b</i> (ex)	88.4	83.2	N δ H-His-Fe(III)
<i>b</i>	65	C δ H-Tyr-167	<i>c</i>	70	69.5	C δ H-Tyr-Fe(III)
<i>b'</i>	65	C δ H-Tyr-167	<i>d</i>	62.4	69.5	C δ H-Tyr-Fe(III)
<i>c</i> (ex)	39.8	NeH-His-323	<i>e</i> (ex)	44	39.9	NeH-His-Fe(II)
<i>d</i>	25.8	C β H-His-325	<i>i</i>	23.4	24	C β H-His-Fe(III)
<i>d'</i>	25.8	C δ H-His-323	<i>g</i>	43.5	39.9	C δ H-His-Fe(II)
<i>d''</i>	25.8	?	<i>f</i>	43.5	39.9	?
<i>d'''</i>	25.8	?	<i>h</i>	29.5	30.0	?
<i>e</i>	12.6	C β H-Tyr-167	<i>j</i>	15.1	n.o.	C β H-Tyr-Fe(III)

be a clue to achieve a tentative assignment of the hyperfine-shifted signals of Fe(III)Fe(II)-KBPAP, whose chemical shifts, along with those previously reported for the reduced mammalian enzymes [13,14], are listed in Table 2.

The chemical shifts of the two pairs of signals *a*, *e* and *b*, *b'* are almost coincident with those of the resonances assigned to the C β H and C δ H protons, respectively, of the tyrosine bound to Fe(III) in Uf [13–15]. This striking similarity suggests that the same assignments also apply for the present di-iron derivative of KBPAP. Therefore, on the basis of the X-ray structure [10], peaks *a* and *e* and peaks *b* and *b'* can be assigned to the C β H and C δ H protons, respectively, of Tyr-167. The remarkable broadening of *b* and *b'* as compared with the corresponding peaks in Uf (*c* and *d*), which are well resolved [13], may be tentatively attributed, besides to the effects of Curie relaxation, to an intermediate exchange of the two tyrosine C δ H protons on the NMR time scale due to ring flipping [13,15]. This cannot be verified by decreasing the temperature, because of the dramatic increase in efficiency of Curie relaxation and the consequent general broadening of the spectral features. The strong similarity of the shift of the C β H protons in Fe(III)Fe(II)-KBPAP and Uf, which differ from those of BSPAP (one of which falls in the diamagnetic region), indicates that the conformation of the tyrosine ligand in the former two enzymes is the same, given that the shift of the two C β H protons is controlled by the torsion angle involving H β -C β -C γ (π orbital on C γ) [13,14], and hence is sensitive to the conformation of the β -CH₂ group relative to the aromatic ring. As a consequence, the difference in the visible-band maximum between the reduced Fe(III)Fe(II)-KBPAP and Uf (565 versus 515 nm) [4] is not to be ascribed to different conformations of the Fe(III)-binding tyrosine, but to other factors related to the co-ordination sphere.

The chemical shift of signal *a'* (solvent exchangeable) and that of the four-proton peak *d-d'''* closely reproduce those of the resonances assigned to an N δ H and a C β H proton, respectively, of the Fe(III)-co-ordinating histidine in the mammalian enzymes [13,14]. Hence peak *a'* and one of the components of the above composite peak, e.g. *d*, probably arise from the above protons of His-325, thereby confirming the N ϵ -co-ordination proposed for the Fe(III)-bound histidine in mammalian PAPs [13,14]. No upfield-shifted signals analogous to that observed for Uf at -68 p.p.m., tentatively assigned to the C ϵ H proton(s) of the Fe(III)-binding tyrosine [14], were detected.

The exchangeable peak *c* at 39.8 p.p.m. falls in almost the same position of that assigned to the NeH proton of a Fe(II)-bound histidine in reduced mammalian enzymes and model complexes [13,14]. The dipolarly connected C δ H proton of the same histidine, which was found at 43.5 p.p.m. in Uf and at 39.9 p.p.m. in BSPAP [13,14], possibly contributes to the four-proton peak at 25.8 p.p.m. in the present derivative. Therefore peak *c* and, e.g., *d'*, can be assigned to the NeH and C δ H protons of His-323 respectively, although the latter peak shows a remarkably lower frequency than that in the mammalian enzymes [13,14]. The one-proton resonances at about 40 and 30 p.p.m. in reduced Uf and BSPAP (peaks *f* and *h*), which turn out not to correspond to dipolarly connected protons [13], were tentatively assigned to C β H protons of the bridging carboxylate residue and of that bound to the bipovalent metal [13]. These two peaks probably correspond to the remaining two protons at 25.8 p.p.m., i.e. *d''* and *d'''*, in the present derivative, which can thus arise from the β -CH₂ group of the bridging Asp-164 and of the Fe(II)-binding Asn-201.

No exchangeable peak corresponding to the N δ H proton of the second Fe(II)-bound histidine (His-286) is detected in the downfield region of the ¹H NMR spectrum of Fe(III)Fe(II)-KBPAP. Since the X-ray structure of the native enzyme indicates that this residue is, at least partially, accessible to the solvent [10,11], it is possible that its N δ H proton is in fast exchange with bulk solvent, as previously reported for the a similar proton of one of the Zn-binding histidines in Co-substituted carboxypeptidase A [16]. The same argument may apply to mammalian PAPs; hence at least one of the above two unassigned signals may also arise from the second Fe(II)-binding histidine.

Insertion of Co(II) in the bipovalent metal site of KBPAP, which yields a catalytically active species [5], does not facilitate signal assignment. Despite the faster electronic relaxation rate of Co(II) as compared with Fe(II) [17], the spectrum of Fe(III)Co(II)-KBPAP still contains broad resonances with sub-millisecond *T*₁ values (Figure 2 and Table 1). In contrast, the homologous derivative of Uf showed remarkably increased *T*₁ values that allowed partial signal assignment through two-dimensional techniques [12]. Comparison of the spectral changes determined by Co(II) substitution of the bipovalent iron in KBPAP and Uf shows that in both derivatives the exchangeable peaks, assigned to the N δ H and NeH proton of the histidine binding to the tripositive and bipovalent metal respectively [12,13], undergo a frequency increase. In addition, the peaks attributed to the Fe(III)-binding tyrosine in di-iron KBPAP, namely *a* and *b*, *b'*, would share the small changes in chemical shift observed for the homologous peaks in Uf (*a*, *c* and *d*) [12,13], provided we assume that they correspond to peaks B and C, respectively, in the Co(II) derivative. Finally, in both Co(II) derivatives the 20–40 p.p.m. region appears more resolved as compared with the di-iron species. Attempts to obtain one-dimensional nuclear Overhauser enhancement and two-dimensional NOESY spectra at a lower field are currently underway in our laboratory.

In conclusion, the present spectral comparison, although forcibly based on qualitative grounds due to the impossibility of achieving an independent signal assignment for both derivatives of the plant enzyme, indicates that signal assignment obtained for Uf and BSPAP [12–15] is fully consistent with the X-ray structure of KBPAP, and that the co-ordination environments of the metal sites of the mammalian and plant enzymes are very similar, especially that of the tripositive metal centre. Some spectral differences appear to arise mainly from the M(II) site, probably due to a different spatial arrangement of the ligands (possibly also determined in part by metal substitution), which

sequence-alignment studies indicate to be conserved [9]. Structural changes at the bipositive centre would influence the electronic and redox properties of the di-iron core, possibly accounting for the different electronic band maxima and oxidation rates by dioxygen shown by Fe(III)Fe(II)-KBPAP and Uf [4]. These results strongly support the hypothesis that, despite the little sequence identity, mammalian and plant PAPs are derived from an old ancestor enzyme whose dimetal active centre has been conserved during evolution [9].

Financial support was from the Deutsche Forschungsgemeinschaft under Wi 1124/26-4. NMR measurements were carried out at the Centro Intedipartimentale Grandi Strumenti of the University of Modena.

REFERENCES

- Doi, K., Antanaitis, B. L. and Aisen, P. (1988) *Struct. Bonding* **70**, 1–26
- Que, Jr., L. and True, A. E. (1990) *Prog. Inorg. Chem.* **38**, 97–200
- Vincent, J. B., Olivier-Lilley, G. L. and Averill, B. A. (1990) *Chem. Rev.* **90**, 1447–1467
- Suerbaum, H., Körner, M., Witzel, H., Althaus, E., Mosel, B.-D. and Müller-Warmuth, W. (1993) *Eur. J. Biochem.* **214**, 313–321
- Beck, J. L., McArthur, M. J., De Jersey, J. and Zerner, B. (1988) *Inorg. Chim. Acta* **153**, 39–44
- Beck, J. L., Keough, D. T., De Jersey, J. and Zerner, B. (1984) *Biochim. Biophys. Acta* **791**, 357–363
- Beck, J. L., De Jersey, J., Zerner, B., Hendrich, M. P. and Debrunner, P. G. (1988) *J. Am. Chem. Soc.* **110**, 3317–3318
- Klabunde, T., Stahl, B., Suerbaum, H., Hahner, S., Karas, M., Hillenkamp, F., Krebs, B. and Witzel, H. (1994) *Eur. J. Biochem.* **226**, 369–375
- Klabunde, T., Sträter, N., Witzel, H. and Krebs, B. (1995) *FEBS Lett.* **367**, 56–60
- Sträter, N., Klabunde, T., Tucker, P., Witzel, H. and Krebs, B. (1995) *Science* **268**, 1489–1492
- Klabunde, T., Sträter, N., Frölich, R., Witzel, H. and Krebs, B. (1996) *J. Mol. Biol.* **259**, 737–748
- Holz, R. C., Que, Jr., L. and Ming, L.-J. (1992) *J. Am. Chem. Soc.* **114**, 4434–4436
- Wang, Z., Ming, L.-J., Que, Jr., L., Vincent, J. B., Crowder, M. W. and Averill, B. A. (1992) *Biochemistry* **31**, 5263–5268
- Scarrow, R. C., Pyrz, J. W. and Que, Jr., L. (1990) *J. Am. Chem. Soc.* **112**, 657–665
- Lauffer, R. B., Antanaitis, B. C., Aisen, P. and Que, Jr., L. (1983) *J. Biol. Chem.* **258**, 14212–14218
- Bertini, I., Turano, P. and Vila, A. J. (1993) *Chem. Rev.* **93**, 2833–2934
- Bertini, I. and Luchinat, C. (1986) *NMR of Paramagnetic Molecules in Biological Systems*, Benjamin & Cummings, Menlo Park, CA
- Inubushi, T. and Becker, E. D. (1983) *J. Magn. Reson.* **51**, 128–133