Derivatives of the Purple Phosphatase from Red Kidney Bean: Replacement of Zinc with other Divalent Metal Ions

JENNIFER L. BECK, MARK J. McARTHUR, JOHN DE JERSEY and BURT ZERNER*
Department of Biochemistry, University of Queensland, St Lucia, Qld. 4067, Australia
(Received February 5, 1988)

Abstract

Apoenzyme, containing ⩽0.1 zinc atoms and ⩽0.2 Fe atoms per subunit and with ⩽3% of the phosphatase activity, has been prepared from native red kidney bean purple phosphatase. Treatment of this apoenzyme with Fe^{3+} or Zn^{2+} separately gave very little recovery of activity, whereas treatment with both Fe^{3+} and Zn^{2+} resulted in complete restoration of activity, indicating that both metal ions are essential. Zn–Fe enzyme with close to one iron and one zinc atom per subunit has been reconstituted by this procedure. Essentially full reactivation was also achieved by addition of Fe^{3+} together with Fe^{2+} or Co^{2+} to the apoenzyme; Fe^{3+} and Cd^{2+} gave 27% restoration of activity, whereas Fe^{3+} with Mn^{2+}, Cu^{2+}, Ni^{2+} or Hg^{2+} gave little or no increase in activity. Kinetic parameters for the hydrolysis of *p*-nitrophenyl phosphate and ATP by the Fe–Fe derivative are reported.

Introduction

Red kidney bean phosphatase is a recently defined, purple, iron-containing phosphatase. It consists of two subunits of ~60 kDa, each containing one zinc and one iron atom [1]. The enzyme is an efficient ATPase and exhibits a broad visible absorption spectrum centred around 560 nm (ϵ_{560} per iron = 3360 M^{-1} cm^{-1}) [1]. The most extensively studied of this group of related proteins is pig allantoic fluid acid phosphatase. This enzyme and beef spleen acid phosphatase contain two iron atoms per molecule of ~40 kDa and exist in two redox states [2]. The oxidized Fe(III)–Fe(III) form of both these enzymes is catalytically inactive and has a visible absorption maximum at 550 nm. The reduced Fe(II)–Fe(III) form, however, is catalytically active with λ_{max} = 510 nm [3, 4]. Sodium dithionite rapidly bleaches the colour of the purple phosphatases, concomitant with the reduction of the ferric iron. We have previously reported the preparation of enzymatically active Zn–Fe, Cu–Fe, and Hg–Fe derivatives of pig allantoic fluid acid phosphatase [4, 5]; an active Zn–Fe derivative of the beef enzyme has also been prepared [6].

We now report results on the preparation of metal-free apoenzyme from native red kidney bean enzyme, and its reactivation by combinations of Fe^{3+} with any of several divalent metal ions including Zn^{2+}, Co^{2+} and Cd^{2+}. These results confirm that both Fe^{3+} and a divalent metal ion are required for phosphatase activity.

Experimental

Materials

p-Nitrophenyl phosphate (disodium salt hexahydrate) was obtained from Calbiochem, sodium dithionite from Merck, and Sephacryl S-300 and Concanavalin A-Sepharose 4B from Pharmacia. ATP (disodium salt from equine muscle, Grade I) was obtained from Sigma, and 2-mercaptoethanol from Eastman. Metal ion salts and buffer components were analytical grade reagents. Metal ion standards for atomic absorption spectroscopy (British Drug Houses) were made up in 10% (v/v) nitric acid.

Enzyme Preparation and Assay

Red kidney bean phosphatase was purified by a method based on that developed by Nochumson and co-workers [1, 7] *viz.* salt extraction, ethanol fractionation, ammonium sulfate precipitation, low salt dialysis, gel filtration, and affinity chromatography on Con A-Sepharose. In more recent preparations, however, the low salt dialysis step has been omitted owing to its poor reproducibility. In the modified procedure, enzyme recovered by ammonium sulfate precipitation is loaded directly onto a Con A-Sepharose column before the final gel filtration step. Enzyme purified by this procedure had the same spectral characteristics and phosphatase activity as enzyme prepared by the published procedure [1]. Enzyme samples were assayed at 25 °C

*Author to whom correspondence should be addressed.

0020-1693/88/$3.50

with *p*-nitrophenyl phosphate as substrate using the previously described assay II [2] in 0.1 M acetate buffer (pH 4.9), or at 30 °C with ATP as substrate in 0.2 M succinate buffer, 0.25 M in NaCl (pH 6.5) [1]. One unit (U) is defined as the amount of enzyme activity which hydrolyses 1 μmol of substrate per minute. Specific activity is expressed in U ml^{-1} A_{280}^{-1}. Enzyme subunit concentrations were calculated from ultraviolet spectra using a value of M_r of 60 000 and an $A^{1\%}_{1\,cm}$ at 280 nm of 20.3 [1]. Metal ion analyses were carried out by atomic absorption spectroscopy using a Varian AA-6 spectrometer. The metal ion content of enzyme samples is given as moles of metal ion per 60 000 g of protein, as indicated by the ratio $[M^{n+}]/[E]$.

Preparation of Apoenzyme

Native enzyme (10–40 μM) was dialysed at 4 °C against 0.1 M acetate buffer (pH 4.5), 0.5 M in NaCl, 2 mM in pyridine-2, 6-dicarboxylate. Sodium dithionite was then added to dialysed enzyme at 25 °C to an initial concentration of 10 mM. After 3 h, excess reagents were removed by gel filtration on a Sephadex G-25 column equilibrated at room temperature with 0.1 M acetate buffer (pH 4.9), 0.5 M in NaCl. During the 3-h period, the solution became orange owing to the formation of a complex between iron and the chelating agent; presumably zinc is also bound to the pyridine-2,6-dicarboxylate.

Reactivation of Apoenzyme with Added Metal Ions

Ten reaction mixtures were set up at 25 °C in which the concentration of apoenzyme subunits was 5.14 μM: the first was a control with no added metal ions, while the remaining nine contained a four-fold molar excess of Fe^{3+} (as ferric chloride) over enzyme subunits. After ~12 h, a twenty-fold excess of Zn^{2+} (as zinc sulfate), Co^{2+} (as cobaltous chloride), Cu^{2+} (as cupric sulfate), Mn^{2+} (as manganous chloride), Ni^{2+} (as nickel chloride), Cd^{2+} (as cadmium acetate) and Fe^{2+} (as ammonium ferrous sulfate) was added to seven of the tubes containing Fe^{3+}. An eighth tube contained only Fe^{3+}, while Hg^{2+} (as mercuric chloride) was added in two-fold excess to the remaining Fe^{3+}-treated apoenzyme. The specific activities of the various reaction mixtures towards *p*-nitrophenyl phosphate were determined at various times.

In a similar experiment designed to investigate the effect of the order of addition of the metal ions, the divalent metal ions Mn^{2+}, Co^{2+}, Cu^{2+} and Fe^{2+} (±0.12 M 2-mercaptoethanol) were added to apoenzyme (3.76 μM) in 200-fold molar excess, and Zn^{2+} was added in 590-fold molar excess. After 18 h, Fe^{3+} was added to the reaction mixtures in ~9-fold molar excess. In the reverse experiment, apoenzyme (3.76 μM) was first treated with Fe^{3+} for 18 h, followed by the addition of the divalent metal ions at the concentrations given above.

Samples of reactivated enzyme in 0.1 M acetate buffer (pH 4.9), 0.5 M in NaCl, were treated with 10.9 mM H_2O_2 at 25 °C. Aliquots were assayed at various times after addition of H_2O_2.

Reconstitution of Zn–Fe Enzyme from Apoenzyme

Apoenzyme (7.5 μM; $[Fe]/[E] = 0.23$; $[Zn]/[E] = 0.12$) was treated with a four-fold molar excess of ferric ion for 2 h prior to the addition of a 20-fold molar excess of zinc. After a further 65 h, excess metal ions were removed by dialysis of 1 ml of enzyme against 1 litre of 0.1 M acetate buffer (pH 4.9, 0.5 M in NaCl, 10 mM in EDTA) for 2 h, followed by dialysis against buffer minus EDTA (2 × 1 l; 2 h between changes; 4 °C).

Preparation of Fe–Fe Red Kidney Bean Phosphatase

Apoenzyme (~10 μM in 0.1 M acetate buffer, pH 4.9, 0.5 M in NaCl) was treated with a 200-fold molar excess of Fe^{2+} and 0.14 M 2-mercaptoethanol at 25 °C. After 48 h, excess reagents were removed by gel filtration on a Sephadex G-25 column equilibrated at room temperature with 0.1 M acetate buffer (pH 4.9, 0.5 M in NaCl).

Results

Preparation of Apoenzyme

Using the method described above, apoenzyme with ≤0.1 atoms of iron and zinc per subunit, and a specific activity of ≤5 U ml^{-1} A_{280}^{-1} (*cf.* 210 U ml^{-1} A_{280}^{-1} for native enzyme) was prepared in ~60% yield. If higher concentrations of native enzyme were used (>40 μM), losses of up to 60% of the protein occurred during the preparation, mostly as a result of precipitation. Further, some iron (up to 0.23 iron atoms per subunit) remained bound to the enzyme.

Reactivation of Apoenzyme with Added Metal Ions

Figure 1 shows the result of an experiment in which apoenzyme was treated first with Fe^{3+} and then with various divalent metal ions. When the divalent metal ions Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+} and Fe^{2+} were added first, only the apoenzyme samples treated with Fe^{2+} gained substantial activity: 160 U ml^{-1} A_{280}^{-1} and 330 U ml^{-1} A_{280}^{-1} after 49 h in the absence or presence of 2-mercaptoethanol. Addition of Zn^{2+} alone to apoenzyme resulted in an increase in specific activity from ≤1 to 36 U ml^{-1} A_{280}^{-1}. Since this sample of apoenzyme contained 0.21 atoms of residual iron per subunit, the addition of Zn^{2+} to form Zn–Fe enzyme would be expected to produce an enzyme sample with ~21% of the specific activity of the native enzyme (210 U ml^{-1}

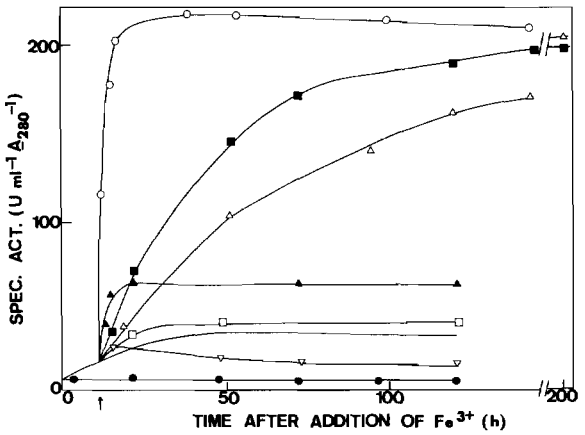

Fig. 1. Apoenzyme (5.14 μM; [Fe]/[E] = 0.22; [Zn]/[E] = 0); specific activity ≤1 U ml^{-1} A_{280}^{-1}) was treated with a 4-fold molar excess of Fe^{3+} in 0.1 M acetate buffer (pH 4.9), 0.5 M in NaCl, at 25 °C. After ~12 h, divalent metal ions were added as outlined in the text (indicated by an arrow), and aliquots were taken at various times for assay (assay II). Control, no additions (●); Fe^{3+} only, Fe^{3+}/Hg^{2+} and Fe^{3+}/Ni^{2+} followed essentially the same time course for activation (——); Fe^{3+}/Cu^{2+} (▽); Fe^{3+}/Mn^{2+} (□); Fe^{3+}/Cd^{2+} (▲); Fe^{3+}/Co^{2+} (△); Fe^{3+}/Fe^{2+} (■); Fe^{3+}/Zn^{2+} (○).

TABLE I. Effect of H_2O_2 on Samples of Metal Ion-activated Apo-red Kidney Bean Phosphatase[a]

Sample[b]	Original specific activity (U ml^{-1} A_{280}^{-1})	Specific activity after H_2O_2 treatment (2 min) (U ml^{-1} A_{280}^{-1})	Specific activity after 2 h (U ml^{-1} A_{280}^{-1})
Apoenzyme	3	3	4
Fe^{3+} only	37	38	37
$Fe^{3+} + Fe^{2+}$	193	21	27
$Fe^{3+} + Zn^{2+}$	223	231	224
$Fe^{3+} + Co^{2+}$	205	166	44
$Fe^{3+} + Cu^{2+}$	12	11	7
$Fe^{3+} + Mn^{2+}$	41	28	37
$Fe^{3+} + Ni^{2+}$	36	25	41
$Fe^{3+} + Cd^{2+}$	76	75	76
$Fe^{3+} + Hg^{2+}$	36	35	36

[a]Samples were treated with 10.9 mM H_2O_2 at 25 °C in 0.1 M acetate buffer (pH 4.9), 0.5 M in NaCl, and aliquots were taken for assay (assay II). [b]Samples are those from Fig. 1.

A_{280}^{-1}). The observed specific activity of 36 U ml^{-1} A_{280}^{-1} is consistent with this interpretation. The order of addition of Fe^{3+} and the divalent metal ions Fe^{2+}, Mn^{2+} and Co^{2+} made no difference to the reactivation rates or final specific activities. However, when Zn^{2+} in large molar excess was added to apoenzyme prior to Fe^{3+}, reactivation was much slower than that observed when Fe^{3+} was added first, but the same final specific activity was reached.

Reconstitution of Zn–Fe Enzyme from Apoenzyme

Enzyme prepared by treatment of apoenzyme with Fe^{3+} and Zn^{2+} followed by dialysis contained 1.18 iron atoms and 1.18 zinc atoms per subunit and had a specific activity of 150 U ml^{-1} A_{280}^{-1} with *p*-nitrophenyl phosphate as substrate.

Preparation of Fe–Fe Red Kidney Bean Phosphatase

Three samples of the Fe–Fe derivative of red kidney bean phosphatase, prepared as described in Experimental, had the following properties (mean ± S.D.): [Fe]/[E] = 1.62 ± 0.15; [Zn]/[E] = 0.11 ± 0.09; specific activity = 268 ± 55 U ml^{-1} A_{280}^{-1}; specific activity after H_2O_2 treatment for 2 min = 29 ± 7 U ml^{-1} A_{280}^{-1}; $A_{280}/A_{max(vis)}$ = 33.5 ± 2.5; $\lambda_{max(vis)}$ = 560 nm).

Reactions of Enzyme Derivatives with H_2O_2

The effect of H_2O_2 on the specific activities of the reactivated samples from Fig. 1 is shown in Table I. The activities of apoenzyme samples treated with Fe^{3+} and Zn^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+}, Mn^{2+} or Hg^{2+} were unaffected by H_2O_2. The Fe^{3+}/Fe^{2+} sample lost 89% of its activity after 2 min with 10.9 mM H_2O_2 with no additional loss on longer treatment. The Fe^{3+}/Co^{2+} sample lost 19% of its activity in 2 min, and 79% in 2 h. Reactivation of a H_2O_2-inactivated sample of the Fe–Fe derivative (which retained 13% of its original activity) was attempted by assay in 0.1 M MES buffer, pH 6.0, with added ferrous ion and ascorbate (assay I of ref. 2). H_2O_2-treated enzyme, pretreated for 5 min in the assay mixture containing Fe^{2+} and ascorbate before addition of substrate, gave an initial rate which was 67% of the control value, whereas the same sample showed only 13% of the control activity measured in assay II (in the absence of reducing agents). Therefore, inactivation by H_2O_2 was largely reversed by mild reducing agents.

Kinetic Properties of Fe–Fe Red Kidney Bean Phosphatase

Values of K_m and k_{cat} for the hydrolysis of *p*-nitrophenyl phosphate, and the specific activity for ATP hydrolysis by Fe–Fe red kidney bean phosphatase were determined using a sample with the following properties: [Fe]/[E] = 1.70; [Zn]/[E] = 0.16; specific activity = 331 U ml^{-1} A_{280}^{-1}; specific activity after H_2O_2 treatment = 34 U ml^{-1} A_{280}^{-1}. For *p*-nitrophenyl phosphate hydrolysis in 0.1 M acetate buffer, pH 4.9, K_m = 0.126 ± 0.021 mM and k_{cat} = 438 ± 8 s^{-1} at 25 °C. At substrate concentrations higher than 2.1 mM, substrate inhibition was observed. ATP hydrolysis under standard

assay conditions ($[S]_0 = 3$ mM; pH 6.5; 30 °C) corresponded to a specific activity of 490 U ml^{-1} A_{280}^{-1}.

Discussion

The procedure developed here for the preparation of apoenzyme incorporates: (i) the use of low pH (pH 4.5) to protonate or partially protonate metal ion ligands; (ii) the use of sodium dithionite to reduce ferric ion, since earlier studies have shown the weaker binding of ferrous ion to the purple phosphatases [5]; (iii) the use of a metal ion chelator to remove residual metal ions, particularly zinc; in early experiments where pyridine-2,6-dicarboxylate was not used, apoenzyme contained no iron but significant levels of zinc; and (iv) the use of a high salt concentration to increase the solubility of the apoenzyme. In preliminary experiments where apoenzyme was prepared using buffers without added salt, essentially all of the enzyme was lost by precipitation. Apoenzyme prepared as recommended had low residual Fe and Zn and activity, and thus was suitable for reactivation and reconstitution experiments.

The time courses for reactivation shown in Fig. 1 clearly demonstrate that both Fe^{3+} and a divalent metal ion are required to reconstitute active red kidney bean phosphatase. Addition of Fe^{3+} alone, or divalent metal ions (other than Fe^{2+}) alone, caused only small increases in activity, consistent with the generation of active enzyme molecules incorporating residual ions in the apoenzyme and the complementary metal ion added. Addition of Fe^{3+} to apoenzyme containing no detectable zinc also caused some activation (Fig. 1), suggesting that trace amounts of adventitious metal ions might have been present in reactivation mixtures.

With the exception of Cu^{2+}, Ni^{2+}, and Hg^{2+}, each of the divalent metal ions tested caused some degree of activation of Fe^{3+}-treated apoenzyme, in the order $Zn^{2+} > Fe^{2+} > Co^{2+} > Cd^{2+} > Mn^{2+}$ (Fig. 1). Zn^{2+} increased the specific activity to 210 U ml^{-1} A_{280}^{-1} (Fig. 1), whereas the maximum specific activity previously obtained in five preparations of native red kidney bean enzyme was 178 ± 5 U ml^{-1} A_{280}^{-1} [1]. The source of this variation is under investigation, and appears to be due to a slight deficiency of zinc in some samples of the purified enzyme. Zn^{2+} and Cd^{2+} each activated Fe^{3+}-treated apoenzyme rapidly: the final specific activity was achieved within 1 h of the addition of the divalent metal ions (Fig. 1). Mn^{2+}, Fe^{2+} and Co^{2+} caused much slower activation. For the Fe^{3+}/Zn^{2+} combination, the order of addition of the divalent metal ion and ferric ion affected the rate of reactivation after the second metal ion was added. When Zn^{2+} was added first, the rate of activation when Fe^{3+} was added was slower than the rate observed when Zn^{2+} was added to Fe^{3+}-treated enzyme. A possible explanation for this behaviour is that Zn^{2+} can bind to the Fe^{3+} site although not with the same affinity as Fe^{3+}: when Fe^{3+} is then added, it competes with the Zn^{2+}, eventually replacing it. More detailed studies of the kinetics of reconstitution are necessary to comment on this and other possibilities. The order of addition of Fe^{3+} and Mn^{2+} or Co^{2+} had little, if any, effect on the rates of reactivation or the final specific activities of the Fe^{3+}/Mn^{2+}-treated and Fe^{3+}/Co^{2+}-treated apoenzymes. The observations made above indicate that one of the metal ion binding sites of red kidney bean phosphatase favours ferric ion (or possibly other metal ions of similar size and ligand requirements) and that the other favours divalent metal ions.

Zn–Fe enzyme prepared by dialysis of Fe^{3+}/Zn^{2+}-treated apoenzyme bound slight excesses of Zn^{2+} and Fe^{3+} (1.18 of each ion/subunit). Short additional dialysis against another change of EDTA or gel filtration on a column of Sephadex G-25 may have removed the small excess of iron and zinc bound to the enzyme. However, the potential usefulness of such steps must be balanced against the possible loss of divalent metal ion from its binding site. The low activity (150 U ml^{-1} A_{280}^{-1}) of the reconstituted Zn–Fe enzyme (compared with the specific activity of 210 U ml^{-1} A_{280}^{-1} reached after reactivation with Fe^{3+} and Zn^{2+}; Fig. 1) may reflect either irreversible inactivation of the enzyme during handling (which affected catalysis, but not the metal ion binding capacity), or inhibition of the enzyme by non-specific binding of excess iron and zinc. Studies are currently under way to prepare and isolate active Zn–Fe, Co–Fe, Cd–Fe and Mn–Fe derivatives from apo-red kidney bean phosphatase.

The Fe(II)–Fe(III) derivative of red kidney bean phosphatase may be prepared by treatment of apoenzyme with Fe^{2+} and 2-mercaptoethanol, a procedure analogous to that used for reconstitution of pig allantoic fluid acid phosphatase [5]. Reconstituted enzyme samples contained close to two iron atoms per subunit ($[Fe]/[E] = 1.62 \pm 0.15$) and very little zinc ($[Zn]/[E] = 0.11 \pm 0.09$). The specific activity (268 ± 55 U ml^{-1} A_{280}^{-1}) was somewhat greater than that observed for the native Zn–Fe enzyme (178 ± 5 U ml^{-1} A_{280}^{-1}, ref. 1; or 210 U ml^{-1} A_{280}^{-1}, Fig. 1). The variability observed in metal ion content and specific activity of these reconstituted samples probably reflects some degree of irreversible inactivation during the preparation of apoenzyme. The presence of a ferric ion in the normal Fe^{3+} binding site is shown by the close agreement between the $A_{280}/A_{max(vis)}$ ratio of the reconstituted Fe–Fe enzyme (33.5 ± 2.5) and that of the native Zn–Fe enzyme (35–38, ref. 1).

The visible absorption is almost certainly due to Fe^{3+}–tyrosinate bonds [1]. The presence of Fe^{2+} in the second site is shown by the susceptibility of the Fe–Fe enzyme to oxidation by H_2O_2 (see below).

Neither native Zn–Fe red kidney bean phosphatase nor the Zn–Fe derivative of pig allantoic fluid enzyme [4] is affected by treatment with 10 mM H_2O_2. As expected, Fe^{3+}/Zn^{2+}-treated apoenzyme was also resistant to oxidation (Table I). In contrast, Fe–Fe enzyme was largely inactivated by short treatment with 10.9 mM H_2O_2. Using a value for the specific activity of Zn–Fe enzyme of 210 U ml^{-1} A_{280}^{-1}, the residual specific activity after H_2O_2 treatment (29 ± 7 U ml^{-1} A_{280}^{-1}) was in reasonable agreement with that expected from the residual zinc content ([Zn]/[E] = 0.11 ± 0.09). The rapid loss of activity seen on oxidation of Fe–Fe red kidney bean phosphatase indicates that at least one ferrous ion is required for phosphatase activity. Furthermore, the reversibility of this inactivation as shown by assay in the presence of reducing agents indicates that the decrease in activity is not due to loss of iron from the enzyme. Similar behaviour observed for pig allantoic fluid acid phosphatase corresponds to the interconversion of the Fe(II)–Fe(III) and Fe(III)–Fe(III) forms of the binuclear metal ion binding site. The slower loss of activity observed for Fe^{3+}/Co^{2+}-treated apoenzyme on treatment with H_2O_2 suggests that oxidation causes conversion to an inactive Co(III)–Fe(III) enzyme. Similar behaviour has been observed in other systems: Co(II)–carbonic anhydrase can be oxidized by H_2O_2 to form an inactive Co(III)–enzyme [10], and *m*-chloroperbenzoic acid oxidizes Co(II)–carboxypeptidase A to inactive Co(III)–enzyme [11].

The kinetic studies carried out in this work show that the specific ATPase activity of Fe–Fe red kidney bean phosphatase (490 U ml^{-1} A_{280}^{-1}) is approximately half of that of native enzyme (1068 U ml^{-1} A_{280}^{-1}, ref. 1). The value of k_{cat} for the hydrolysis of *p*-nitrophenyl phosphate by Fe–Fe enzyme at pH 4.9 (438 s^{-1}) is comparable with the value of 417 s^{-1} obtained for pig allantoic fluid enzyme (J. de Jersey and B. Zerner, unpublished results). The Michaelis constant for the Fe–Fe red kidney bean enzyme (0.126 mM) is ~10 times lower than the value for the pig enzyme (1.32 mM). Corresponding data for native red kidney bean enzyme have not yet been obtained; at pH 6.5, the K_m is 36 mM [1]. Studies aimed at determining and comparing the catalytic mechanisms of Zn–Fe and Fe–Fe forms of the pig allantoic fluid and red kidney bean enzymes are proceeding.

The reactivation studies reported above demonstrate that red kidney bean phosphatase contains essential zinc and iron, making it the first known example of such a metalloenzyme. Calcineurin, a phosphoprotein phosphatase from bovine brain, contains stoichiometric amounts of these two metal ions [12], but experiments analogous to those described in this work involving removal and replacement of the metal ions with loss and regeneration of catalytic activity have not been reported.

Purple phosphatases from plant sources other than red kidney bean have been reported to contain significant amounts of manganese [13–16]. Sweet potato enzyme is the most extensively studied, and has been reported to contain one or two manganese atoms per molecule of 110 kDa, although the presence of iron and zinc was not rigorously excluded [13, 17]. Recently, Hefler and Averill [18] purified a purple phosphatase from sweet potato with a specific activity much higher than any previously reported. Their enzyme contained two atoms of iron per molecule but only small amounts of manganese and zinc.

It seems possible that red kidney bean phosphatase binds its metal ions in a binuclear unit such as is present in the beef spleen and pig allantoic fluid enzymes. The presence of two antiferromagnetically coupled iron atoms in a binuclear unit in the pig enzyme was confirmed using a combination of Mössbauer and ESR studies and magnetic susceptibility measurements [3, 19]. Such studies are being pursued using metal ion derivatives of red kidney bean phosphatase and the native Zn–Fe enzyme.

Acknowledgements

We thank Mr Andrew Summors for competent technical assistance. This research was supported in part by the Australian Research Grants Scheme. One of us (J.L.B.) acknowledges the receipt of a Commonwealth Postgraduate Award.

References

1 J. L. Beck, L. A. McConachie, A. C. Summors, W. N. Arnold, J. de Jersey and B. Zerner, *Biochim. Biophys. Acta, 869*, 61 (1986).
2 H. D. Campbell, D. A. Dionysius, D. T. Keough, B. E. Wilson, J. de Jersey and B. Zerner, *Biochem. Biophys. Res. Commun., 82*, 615 (1978).
3 P. G. Debrunner, M. P. Hendrich, J. de Jersey, D. T. Keough, J. T. Sage and B. Zerner, *Biochim. Biophys. Acta, 745*, 103 (1983).
4 J. L. Beck, D. T. Keough, J. de Jersey and B. Zerner, *Biochim. Biophys. Acta, 791*, 357 (1984).
5 D. T. Keough, D. A. Dionysius, J. de Jersey and B. Zerner, *Biochem. Biophys. Res. Commun., 94*, 600 (1980).
6 J. C. Davis and B. A. Averill, *Proc. Natl. Acad. Sci. U.S.A., 79*, 4623 (1982).

7 S. Nochumson, J. J. O'Rangers and N. F. Dimitrov, *Fed. Proc., 33,* 1378 (1974).
8 B. C. Antanaitis and P. Aisen, *J. Biol. Chem., 257,* 1855 (1982).
9 R. B. Lauffer, B. C. Antanaitis, P. Aisen and L. Que, Jr., *J. Biol. Chem., 258,* 14212 (1983).
10 H. Shinar and G. Navon, *Biochim. Biophys. Acta, 334,* 471 (1974).
11 H. E. Van Wart and B. L. Vallee, *Biochemistry, 17,* 3385 (1978).
12 M. M. King and C. Y. Huang, *J. Biol. Chem., 259,* 8847 (1984).
13 Y. Sugiura, H. Kawabe, H. Tanaka, S. Fujimoto and A. Ohara, *J. Biol. Chem., 256,* 10664 (1981).
14 S. Fujimoto, T. Nakagawa and A. Ohara, *Agric. Biol. Chem., 41,* 599 (1977).
15 S. Fujimoto, T. Nakagawa, S. Ishimitsu and A. Ohara, *Chem. Pharm. Bull., 25,* 1459 (1977).
16 I. Igaue, H. Watabe, K. Takahashi, M. Takekoshi and A. Morota, *Agric. Biol. Chem., 40,* 823 (1976).
17 S. Fujimoto, A. Ohara and K. Uehara, *Agric. Biol. Chem., 44,* 1659 (1980).
18 S. K. Hefler and B. A. Averill, *Biochem. Biophys. Res. Commun., 146,* 1173 (1987).
19 E. Sinn, C. J. O'Connor, J. de Jersey and B. Zerner, *Inorg. Chim. Acta, 78,* L13 (1983).

Note Added in Proof

The EPR spectrum of the Fe–Fe derivative of this enzyme has been reported and compared to that of the pig allantoic fluid enzyme [20].

20 J. F. Beck, J. de Jersey, B. Zerner, M. P. Hendrich and P. G. Debrunner, *J. Am. Chem. Soc.,* 1988, in press.