Vol. 94, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1980 Pages 600-605

IRON-CONTAINING ACID PHOSPHATASES: CHARACTERIZATION OF THE METAL-ION BINDING SITE OF THE ENZYME FROM PIG ALLANTOIC FLUID

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Received April 10,1980

Summary: All of the iron can be removed from the violet acid phosphatase of pig allantoic fluid by treatment with sodium dithionite at pH 4.9. Of the two moles of iron present per mole of enzyme (40,000 g), half is lost rapidly, and the remainder much more slowly. Removal of half of the iron causes complete loss of acid phosphatase activity. Conditions have been defined for the isolation and complete reconstitution [by Fe(II) and β-mercaptoethanol] of two apoenzymes, designated "iron-free" and "one-iron" apoenzymes. Zn^{2+} ions restore most of the acid phosphatase activity to the one-iron apoenzyme but not to the iron-free enzyme. No metal ions other than Fe(II) and Fe(III) restore significant activity to the iron-free apoenzyme, but Zn(II) and Ni(II) bind tightly to it.

Two iron-containing violet acid phosphatases, from beef spleen and pig allantoic fluid, have been purified to homogeneity (1-3). The enzymes have very similar physical properties, with molecular weights of 35-40,000, equivalent weights for iron of ~20,000, and strongly basic isoelectric points (4). Both enzymes exist in a violet oxidized form which is catalytically inactive (λ_{max} ~550 nm; ϵ = 2,000 in terms of iron), and a pink reduced form (λ_{max} ~510 nm) which is active. Several other acid phosphatases have been found which have some properties in common with the beef spleen and pig allantoic fluid enzymes, suggesting that the violet iron-containing acid phosphatases are widespread (4). Recently, the acid phosphatase of *Saccharomyces rouxii* has been shown to be activated by Fe(III) in the assay (5). In contrast, violet acid phosphatases from soy bean and other plant sources are reported to contain manganese rather than iron (6). In this communication, we describe experiments aimed at characterization of the metal ion binding site(s) of pig allantoic fluid acid phosphatase.

Experimental Section

All chemicals used were of the best grade commercially available, and all solutions were made with distilled deionized water. Spectral measurements and enzyme assays were carried out at 25°C using a Cary 17 recording spectrophotometer. Metal ion estimations were done by atomic absorption, using a Varian AA-6 spectrometer, except where otherwise indicated.

Enzyme was prepared as described previously (4). Three batches were used with specific activities and A_{280}/A_{530} ratios as follows: batch 1, 5.6 (mkat/L) /A_{280}, 14.9; batch 2, 6.0 (mkat/L) /A_{280}, 15.8; batch 3, 5.0 (mkat/L) /A_{280}, 17.0. The concentrations of enzyme and apoenzyme solutions were determined from the absorbance at 280 nm, using $A^{1\%}_{1cm}$ = 14.2, and a molecular weight of 40,000 (4). The acid phosphatase activity was determined in 0.1 M acetate buffer, pH 4.90, at 25°C, with *p*-nitrophenyl phosphate (5 mM) as substrate (4), the release of *p*-nitrophenol being measured at 390 nm ($\Delta\epsilon$ = 343). Except in reconstitution experiments, the enzyme was routinely activated prior to assay by equilibration at 25°C with 0.1 M β-mercaptoethanol and 1 mM ferrous ion. Under these conditions, maximum activity is reached after ~2 h, and is sustained for at least 72 h. Treatment of the isolated

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enzyme with β-mercaptoethanol in the absence of Fe(II) leads to an initial increase in activity, followed by a gradual decrease to zero, which is due to a loss of iron from the reduced enzyme. The maximum activity reached in the absence of added Fe(II) is considerably less than that reached in its presence.

Results

Roberts and his coworkers (7) found that treatment of the enzyme with sodium dithionite causes rapid bleaching and loss of activity, with concomitant loss of iron. We treated the enzyme with dithionite at pH 4.9 in the presence of 1,10-phenanthroline to monitor the removal of iron from the protein: the complex of Fe(II) with 1,10-phenanthroline absorbs maximally at 510 nm with ϵ = 11,100 (8). The progress curve is shown in Figure 1. Approximately half of the iron is removed rapidly, the remainder being removed over a period of several hours. Activity measurements on aliquots of this reaction mixture showed that virtually all of the activity was lost when *half* of the iron had been removed.

The progress curve suggested that two apoenzymes could be prepared: a "one-iron" apoenzyme by short term treatment with dithionite, and an "iron-free" apoenzyme by longer term treatment. This expectation has been realized. Treatment of enzyme (90 μM) with dithionite (10 mM) in 0.1 M acetate buffer, pH 4.90, at 25°C for 2 min, followed by chromatography on Sephadex G-25 yielded enzyme containing 0.81 Fe atoms per 40,000 daltons (determined by the bathophenanthroline disulfonate method, Ref. 1), with a specific activity of 0.2 (mkat/L) $/A_{280}$ (~3% of the maximum specific activity). Figure 2 shows the effect of Fe(II) in the presence and absence of β-mercaptoethanol, and of Zn^{2+} on the acid phosphatase activity of the one-iron apoenzyme. Complete recovery of

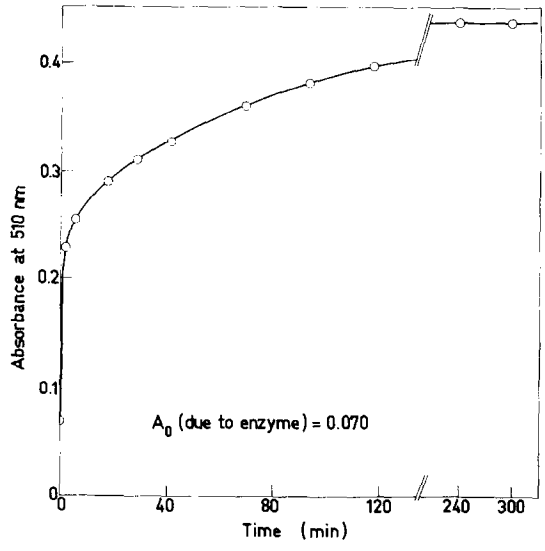

Figure 1. Progress curve for the formation of the Fe(II) - 1,10-phenanthroline complex, measured at 510 nm, on the addition of sodium dithionite (5.07 mM) to pig allantoic fluid acid phosphatase (22.4 μM) in 0.1 M acetate buffer, pH 4.90; 25°C; [1,10-phenanthroline] = 10.9 mM. The final absorbance of 0.44 represents an iron concentration of 40 μM as the 1,10-phenanthroline complex.

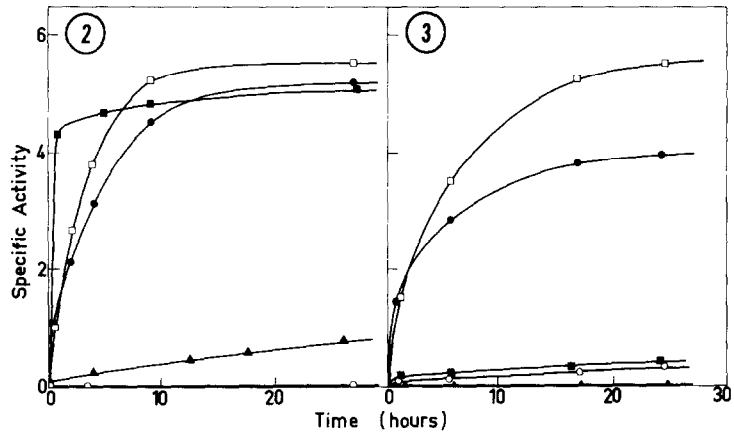

Figure 2. Time dependence of the reactivation of the one-iron apoenzyme of pig allantoic fluid acid phosphatase in 0.1 M acetate buffer, pH 4.90: 25°C; $[apoenzyme]_0$ = 7.4 μM; [metal ion] = 1.4 mM in each case; [β-mercaptoethanol] = 144 mM, when present; Fe^{2+} + β-mercaptoethanol (□); Fe^{2+} (●); Zn^{2+} (■); β-mercaptoethanol control (○); water control (▲).

Figure 3. Effect of metal ions on the activity of the iron-free apoenzyme of pig allantoic fluid acid phosphatase; reaction mixtures contained apoenzyme [≤ 0.02 Fe atoms per 40,000 daltons; specific activity ≤0.001 (mkat/L) $/A_{280}$; 0.84 μM], and metal ion (1.0 mM) in 0.1 M acetate buffer, pH 4.90, 25°C; [β-mercaptoethanol] = 100 mM when present; Fe^{2+} + β-mercaptoethanol (□); Fe^{2+} (●); Fe^{3+} (○); Zn^{2+} (■); control, with or without β-mercaptoethanol (▲).

activity is produced by Fe(II) and β-mercaptoethanol, while Zn^{2+} gives rapid restoration of most of the original activity. Cu^{2+} and several other metal ions including Ni^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Co^{2+}, and Al^{3+}, under similar conditions, have little effect on the slow recovery of activity observed in the water control, which may be due to a redistribution of the residual Fe atoms to form some enzyme molecules with a full complement of iron. Inclusion of EDTA (0.1 mM) in the control prevents this slow increase in activity. The iron content and residual specific activity of the one-iron apoenzyme, prepared as described above, are somewhat variable. This may be due in part to differences in the time of exposure to dithionite, but also to a failure of the procedure to give complete discrimination between the two types of bound iron represented by the two phases of the progress curve (Figure 1).

The inclusion of 2 M or 3 M guanidinium chloride in the reaction mixture was found to accelerate complete removal of the iron. Apoenzyme prepared by treatment with dithionite (10 mM) in 0.1 M acetate buffer, pH 4.9, 3 M in guanidinium chloride, for 1 h at 25°C, followed by chromatography on Sephadex G-25 was essentially iron-free and had zero acid phosphatase activity. Figure 3 shows the effect of Fe(II) with and without β-mercaptoethanol, Fe(III), and Zn^{2+} on this apoenzyme.

Equilibration of the apoenzyme with a high concentration of Fe(III) does not produce any increase in activity (Figure 3). However, when only a small excess of Fe(III) is added ([Fe(III)] = 62 μM; [apoenzyme] = 14 μM) significant return of activity is observed [specific activity = 1.0 (mkat/L) $/A_{280}$]. This compares with a specific

TABLE I

Properties of Metalloproteins Obtained from the Iron-Free Apoenzyme of Pig Allantoic Fluid Acid Phosphatase[a]

Metal Ion	[β-Mercaptoethanol] (mM)	Specific Activity [(mkat/L) /A_{280}]	Metal Ion Content (moles/40,000 g)
Fe(II),[b] 14.3 mM	130	4.9	2.0
Fe(II),[b] 14.3 mM	–	5.2	2.3
Zn(II),[c] 15.0 mM	140	0.12	2.0
Zn(II),[c] 15.2 mM	–	0.31	0.31
Zn(II),[c] 36 μM	140	0.14	0.77
Zn(II),[c] 36 μM	–	0.23	0.09
Ni(II),[d] 15.2 mM	130	0.01	1.40
Ni(II),[d] 15.2 mM	–	0.03	0.40
Ni(II),[d] 36 μM	137	0.01	1.23
Ni(II),[d] 36 μM	–	0.02	0.21

[a]Iron-free apoenzyme was equilibrated with metal ion in 0.1 M acetate buffer, pH 4.90, at 25°C. The mixture was then chromatographed on Sephadex G-25, and the specific activity and metal ion content of the void volume protein peak determined. [b]Equilibration for 30 h; $[apoenzyme]_o$ = 15.4 μM. [c]Equilibration for 45 h; $[apoenzyme]_o$ = 12.7 μM. [d]Equilibration for 45 h; $[apoenzyme]_o$ = 11.3 μM.

activity of 1.8 (mkat/L) /A_{280} obtained when the apoenzyme is equilibrated with Fe(II) under identical conditions ([Fe(II)] = 62 μM; [apoenzyme] = 14 μM). In contrast to these results, Schlosnagle *et al.* (7) reported that Fe^{3+} was much better than Fe^{2+} in the reactivation of apoenzyme ([apoenzyme] = 10 μM; 4-fold excess of metal ion). Reconstitution experiments with Fe(III) are complicated by polymerization which prevents removal of excess iron by gel filtration, and by inactivation of the enzyme by Fe(III), due at least in part to oxidation. The former problem can in principle be solved using Fe(III) complexes with citrate and nitrilotriacetate, as has been done with transferrin (9). Equilibration of iron-free apoenyzme (11 μM) with 1.8 mM Fe(III) and either citrate (9 mM) or nitrilotriacetate (4.9 mM) failed to give significant reactivation.

Apart from Fe(II) and Fe(III), none of the other metal ions tested with the iron-free apoenzyme (Zn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Al^{3+}) gave any significant restoration of activity.

A series of reconstitution experiments with iron-free apoenzyme was carried out to check for binding of metal ions to the protein. After equilibration of apoenzyme with a particular metal ion, excess metal ion was removed by gel filtration, and the metal ion content and specific activity of the resulting enzyme determined. Table 1 lists the results obtained.

The presence of two iron atoms per molecule raised the possibility of a bridging sulfur as in many of the iron-sulfur proteins. However, no inorganic sulfide could be detected by the standard method (10).

Discussion

Pig allantoic fluid acid phosphatase resembles the blood iron transport protein transferrin both in its visible spectrum and in having two metal ion binding sites per molecule. The resonance Raman spectra of the oxidized (violet) form of the acid phosphatase, transferrin, and the Fe(III) complex of enterobactin are very similar, strongly suggesting the existence of an Fe(III) – phenolate bond in both proteins (11). This result shows that the visible spectrum arises, *at least in part*, from an Fe(III)-tyrosine bond. As with acid phosphatase, dithionite accelerates the release of iron from iron-transferrin-anion complexes (12). Further, in transferrin, binding constants for the two iron atoms differ by a large factor (13). The time course of iron removal from acid phosphatase (Figure 1) is consistent with a similar presence of strong and weak iron binding sites.

Conditions have been established for preparation of the iron-free apoenzyme and a somewhat less well-defined one-iron apoenzyme. In both cases, treatment of the inactive apoenzyme with Fe(II) and β-mercaptoethanol leads to complete restoration of the acid phosphatase activity, with reconstituted enzyme containing 2 ± 0.2 iron atoms/40,000 daltons. Experimental results on the role of the metal ions in catalysis may be summarized as follows: (i) the one-iron apoenzyme is virtually devoid of phosphatase activity; (ii) the reactivation of the one-iron apoenzyme with Zn^{2+} suggests that the one-iron, one-zinc protein is about as active as the native two-iron protein; (iii) the iron-free apoenzyme will bind Zn(II) and Ni(II) (Table I); and (iv) no metal ions other than Fe(II) and Fe(III) can reactivate the iron-free apoenzyme. Further experiments are required to explain these results in detail and to delineate the role of the iron atoms more clearly.

The oxidation state of the iron atoms in the pink and violet forms of pig allantoic fluid acid phosphatase remains unclear. The electron paramagnetic resonance (7) and resonance Raman (11) experiments of Roberts and his coworkers indicate that at least some of the iron is present as high spin Fe(III) in the violet form, and also possibly in the pink form (7). However, neither technique gives a quantitative estimate of the iron being measured [*i.e.* Fe(III)]. The experiments described here clearly indicate that ferrous iron is more effective than ferric iron in reactivation of the apoenzymes. However, ferric iron is formed by air oxidation of ferrous iron, and it remains possible that the active enzyme contains at least some Fe(III). When the iron-free apoenzyme is equilibrated with Zn^{2+} or Ni^{2+}, the amount of bound metal ion is increased markedly by the presence of β-mercaptoethanol (Table I). The simplest explanation of this observation is that a group or groups in the enzyme (possibly a sulfhydryl group) must be maintained in the reduced form for metal ion binding to occur, and to this end, the presence of titratable sulfhydryl groups in different forms of the enzyme is currently under investigation. The possibility of intermediate

oxidation states with one iron present as Fe(II) and the other as Fe(III) also exists, and may account for the reactivation experiments.

Roberts and his coworkers (14) have suggested that pig allantoic fluid acid phosphatase should be termed "uteroferrin" to emphasize its relationship with transferrin and its putative function in iron transport from mother to fetus. While a role in iron transport seems an eminently reasonable one for this protein and perhaps also for the closely similar beef spleen acid phosphatase, it is difficult to discount the acid phosphatase activity. The pig allantoic fluid enzyme is an efficient acid phosphatase: k_{cat} for the hydrolysis of *p*-nitrophenyl phosphate (pH 4.9; 25°C) is 462 sec^{-1} (15), while k_{cat} for human prostatic acid phosphatase catalysis of *p*-nitrophenyl phosphate hydrolysis is ~250 sec^{-1} (16) under similar conditions.

Acknowledgements

This work was supported in part by a grant from the Australian Research Grants Committee. The invaluable assistance of Mr Bruce Wilson and Mr John McVey in the provision of pregnant sows and the collection of allantoic fluid, and the expert technical assistance of Mr Allen Tripp are gratefully acknowledged.

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