Kinetics and Optical Spectroscopic Studies on the Purple Acid Phosphatase from Beef Spleen†

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ABSTRACT: A new purification scheme has been developed for the purple acid phosphatase from beef spleen; typical yields are 8 mg of homogeneous enzyme per kg of spleen in only five steps. Kinetics studies have shown that the enzyme is strongly inhibited by fluoride, phosphate, and [*p*-(acetylamino)-benzyl]phosphonate, a nonhydrolyzable substrate analogue; the last two of these show simple competitive inhibition. In contrast, cyanide, azide, tartrate, and *p*-nitrophenol show no inhibition at concentrations up to 10 mM. Molecular weight estimations by gel electrophoresis and gel permeation chromatography give a value of 40 000 for the native enzyme, which is shown to consist of two subunits of apparent molecular weight 24 000 and 15 000. Careful metal analyses indicate the presence of 2.1 ± 0.1 iron atoms per enzyme molecule, and less than 0.1 copper, zinc, nickel, or manganese atom per enzyme. The purple enzyme (λ_{max} 550 nm) is reversibly converted to a pink, active form (λ_{max} 505 nm) upon treatment with mild reducing agents (dithioerythritol or ascorbate). Addition of competitive inhibitors to the pink form causes rapid reversion to the purple form. Electron paramagnetic resonance spectroscopy at several temperatures showed only weak g = 4.3 signals (<0.1 spin/molecule) for the native, reduced, and inhibited forms of the enzyme.

Enzymes that catalyze the hydrolysis of phosphate esters play a vital role in the regulation of physiological levels of inorganic phosphate and phosphorylated metabolites and, in some cases, in regulating the activity of certain phosphorylated enzymes. The zinc-containing alkaline phosphatases have been studied in great detail (Chlebowski & Coleman, 1976; Spiro, 1973; Reid & Wilson, 1971), as have the acid phosphatases that contain no known prosthetic group (Hollander, 1971). In contrast, only isolated reports have appeared on a series of relatively poorly characterized purple phosphatases from diverse sources: bovine spleen (Glomset & Porath, 1960; Campbell & Zerner, 1973; Campbell et al., 1978); porcine uterine fluid (Campbell et al., 1978; Schlosnagle et al., 1974, 1976); red kidney bean (Nochumson et al., 1974); sweet potato (Uehara et al., 1971, 1974a,b); soybean (Fujimoto et al., 1977a,b); spinach leaves (Fujimoto et al., 1977c); cultured rice plant cells (Igaue et al., 1976); *Neurospora crassa* (Jacobs et al., 1971); and *Micrococcus sodonensis* (Glew & Heath, 1971). All but the last of these are basic glycoproteins with acid phosphatase activity; the *Micrococcus* enzyme is atypical in that it is an acidic glycoprotein, is an alkaline phosphatase, and contains approximately eight Ca^{2+} ions per molecule (Glew & Heath, 1971). All are characterized by an intense ($\epsilon \sim 2500$ M^{-1} cm^{-1}) absorption band at 510–550 nm and a shoulder at 310–320 nm. Only recently has the presence of tightly bound iron been demonstrated for the enzymes from beef spleen (Campbell & Zerner, 1973; Campbell et al., 1978), porcine uterine fluid (Schlosnagle et al., 1974; Campbell et al., 1978; Keough et al., 1980), and kidney bean (Nochumson et al., 1974), while manganese is claimed to be present in the enzymes from sweet potato (Uehara et al., 1974a; Sugiura et al., 1980), soybean (Fujimoto et al., 1977a,b), spinach leaves (Fujimoto et al., 1977c), and rice cells (Igaue et al., 1976).

There are thus two major questions about these enzymes that are not yet satisfactorily answered: (1) What is the nature of the unusual chromophore? Although both the identity and the oxidation state of the metal cofactor in all of these enzymes remain to be established, coordination by tyrosyl phenolate groups is suggested by resonance Raman spectra of the porcine (Gaber et al., 1979) and sweet potato (Sugiura et al., 1980) enzymes. (2) Is the metal chromophore directly involved in catalysis? Roberts has recently ascribed to the purple phosphatase from porcine uterine fluids a role in iron transport, rather than in phosphate metabolism (Buhi et al., 1979). This obviously cannot be the case for the *Neurospora* and *Micrococcus* enzymes, both of which are extracellular and secreted into the growth medium in response to phosphate depletion.

Of the two mammalian enzymes in this class, which appear to be similar to one another in both metal content and overall properties (Campbell et al., 1978), that from spleen tissue is by far the more poorly characterized, due to its relatively low abundance in bovine spleen and to the lack of a convenient, high-yield purification scheme. Because of our continuing interest in metalloproteins of the reticuloendothelial system, and because of the implications of the possible involvement of iron in an enzyme catalyzing a nonredox reaction,[1] we have chosen to examine the purple acid phosphatase from beef spleen in more detail. In this paper, we report a significantly improved purification of the enzyme, additional physical characterization data, including the results of careful metal analyses, kinetics and visible spectral data on the interaction of the enzyme with inhibitors, the existence of and interconversion of purple (inactive) and pink (active) forms of the enzyme, and the absence of significant electron paramagnetic resonance signals typical of high-spin ferric iron.

Experimental Procedures

Extraction and Purification of the Enzyme. The purple acid phosphatase was extracted from beef spleen strips (spleens were obtained from the local abattoir) suspended in 0.25 M KCl (2 mL/g of spleen) at 4 °C and homogenized in a Waring

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[1] Only three other examples are known: two involve iron–sulfur prosthetic groups [aconitase (Kurtz et al., 1979) and glutamine phosphoribosylpyrophosphate amidotransferase (Averill et al., 1980)], and the other, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase from *E. coli*, shows significant similarities in both optical spectrum and amino acid sequence to hemerythrin (McCandliss & Herrmann, 1978; Herrmann et al., 1980).

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blender at medium speed for 1 min and high speed for 2 min. The homogenate was adjusted to pH 3.5 with 6 M HCl and stirred at room temperature for 3 h, followed by centrifugation at 9000*g* for 10 min at 20 °C. The clarified supernate was filtered through glass wool to remove small waxy particles, and the resulting filtrate was made 0.1 M in ascorbic acid and adjusted to pH 5.5 with 12% NaOH. The solution was then treated with 1 g of cellulose phosphate (P-11, Whatman) per 2000 units of enzyme. The P-11 was filtered, washed with 2 × 100 mL of H_2O, resuspended in 200 mL of 2.0 M KCl, and stirred for 2 h. The P-11 was filtered and washed with 50 mL of 2.0 M KCl, resulting in a pale yellow-green filtrate. At this point, slightly cloudy solutions were centrifuged at 9000*g* for 20 min at 20 °C for clarification, to avoid plugging the chromatography column in the succeeding step. (All subsequent operations were carried out at 5–10 °C in a cold box.)

The clear filtrate was diluted to 0.15 M KCl and loaded onto a 2.5 × 20 cm carboxymethylcellulose (CM-52, Whatman) column at 6.0 mL/min. The column (with an intense purple band) was washed with 100 mL of 0.2 M KCl and 0.05 M sodium acetate, pH 5.0, and eluted with a 0.15–1.0 M KCl gradient in 0.05 M sodium acetate buffer, pH 5.0. The KCl concentration was monitored by measuring the conductivity of the samples and comparing the results to a set of KCl/buffer standards. The fractions with $A_{280}/A_{550} < 40$ were pooled, concentrated to 5 mL by ultrafiltration (Amicon 8MC, PM-10 membrane), loaded onto a Sephadex G-75 column (1.5 × 85 cm), and eluted with 0.2 M KCl in 0.05 M sodium acetate buffer, pH 5.0. The purple fractions with $A_{280}/A_{550} < 25$ were combined and used in the subsequent step. The dilute purple solution from the G-75 column (typically 50 mL) was pumped at 1 mL/min through a 1.5 × 3 cm bed of hydroxylapatite (Bio-Rad Bio-Gel HTP) previously equilibrated with the G-75 elution buffer. A greenish yellow band slowly appeared, usually occluding ~50% of the hydroxylapatite. Residual purple acid phosphatase was removed by washing the column with 10 mL of 0.75 M KCl and 0.05 M acetate, pH 5.0. The resulting solution was typically concentrated as before to ~8 mL and normally had an A_{280}/A_{550} of 15–17.

Molecular Weight Determination. Molecular weight data were obtained by gel permeation chromatography using Sephadex G-75, with bovine albumin, egg albumin, α-chymotrypsinogen, and β-lactoglobulin (all from Sigma) as standards. Polyacrylamide gel electrophoresis (both native and $NaDodSO_4$)[2] was done on a Bio-Rad Model 220 electrophoresis cell with Coomassie blue development of protein bands (O'Farrell, 1975). For $NaDodSO_4$ gels, the same molecular weight standards were used as in the G-75 experiments, with the addition of lysozyme (Sigma). To determine whether or not proteases were affecting the observed molecular weight, we ran a sample isolated in the presence of 1 mM phenylmethanesulfonyl fluoride (Fahrney & Gold, 1963) on the G-75 column.

Spectral Characterization. Spectral data for protein estimation (A_{280}) and for determination of enzyme activity were obtained on a Beckman DU equipped with a Gilford Model 252-D accessory. Visible and UV spectra and some kinetics data were obtained on a Cary 219 spectrophotometer. The EPR spectra were run on a Varian E-4 spectrometer equipped with an Oxford liquid helium cryostat. All optical spectra of reduced enzyme were run in the presence of excess reductant; reduction was accomplished with either 0.4 M ascorbate or 50 mM DTE. These levels are similar to the 0.1 M β-mercaptoethanol used by other workers (Keough et al., 1980; Schlosnagle et al., 1974); lower levels of reductant (0.01 M ascorbate or 2.5 mM DTE) were not effective in converting the purple to the pink form.

Table I: Purification of Purple Acid Phosphatase from Beef Spleen

step	yield (units)	% recovery	sp act. (units/mg)
acid extraction (1000 g of spleen)	20 000	100	0.24
cellulose phosphate (CP-11) batch adsorption	17 000	85	32
carboxymethylcellulose (CM-52) column	13 600	68	480
Sephadex G-75 column	11 600	58	1110
hydroxylapatite column	10 000	50	1240

Analytical Methods. The iron content of the purified purple acid phosphatase was determined in two ways. A chemical method (Van de Bogart & Beinert, 1967) using formation of a ferrous ion–bathophenanthroline complex absorbing at 535 nm was routinely used. In addition, iron, copper, manganese, molybdenum, mangnesium, and zinc were determined by using inductively coupled plasma emission spectroscopy on a Jarrell-Ash Model 955 plasma atomcomp using the G-75 and hydroxylapatite elution buffer as a blank solution. Protein was determined with the Lowry method (Lowry et al., 1951).

Phosphatase activity was determined by using *p*-nitrophenyl phosphate (Campbell et al. 1978) with 0.1 M Mes buffer at pH 6.00. All assays were done in duplicate (triplicate for kinetics and limiting specific activity experiments). Fresh spleens were assayed by excising ~1 g from the thickest portion of the spleen, followed by grinding in 10 mL of 0.25 M KCl in a 15-mL tissue grinder. After adjustment to pH 3.5 with 6 M HCl, the samples were centrifuged for 5 min in a bench-top Waco separator and assayed for phosphatase activity. Typically, spleens with less than 20 units/g were discarded.

Results

Purification. The improved purification of purple acid phosphatase from beef spleen described under Experimental Procedures results in 50% recovery of enzyme purified to homogeneity in five steps. Yield and recovery data are given in Table I. The combined use of a preassay to screen individual spleens for activity and improved acid extraction conditions increases the initial amount of activity obtained. Spleens ranged from 1 unit/g to as high as 100 units/g with an average of 22 units/g for 131 spleens. Figure 1 shows the elution profile of the purple phosphatase from the carboxymethylcellulose column, using the KCl gradient shown. Absorption at 280, 550, and 430 nm is shown, as well as the phosphatase activity profile. The late-eluting 430-nm peak is the green heme peroxidase reported elsewhere (Davis & Averill, 1981), which copurifies with the purple phosphatase prior to CM-52 chromatography. Figure 2 shows the results of Sephadex G-75 column chromatography; absorption at both 280 and 550 nm is plotted. The small 550-nm peak at 105-mL elution volume is caused by the green heme peroxidase impurity, which has a significant absorbance at 550 nm.

Following the G-75 step, hydroxylapatite chromatography was successfully used to purify the enzyme, but with unac-

[2] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; EPR, electron paramagnetic resonance; DTE, dithioerythritol; PABP, [*p*-(aminoacetyl)benzyl]phosphonate; PNPP, *p*-nitrophenyl phosphate; PNP, *p*-nitrophenol; P_i, inorganic phosphate; PMSF, phenylmethanesulfonyl fluoride; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

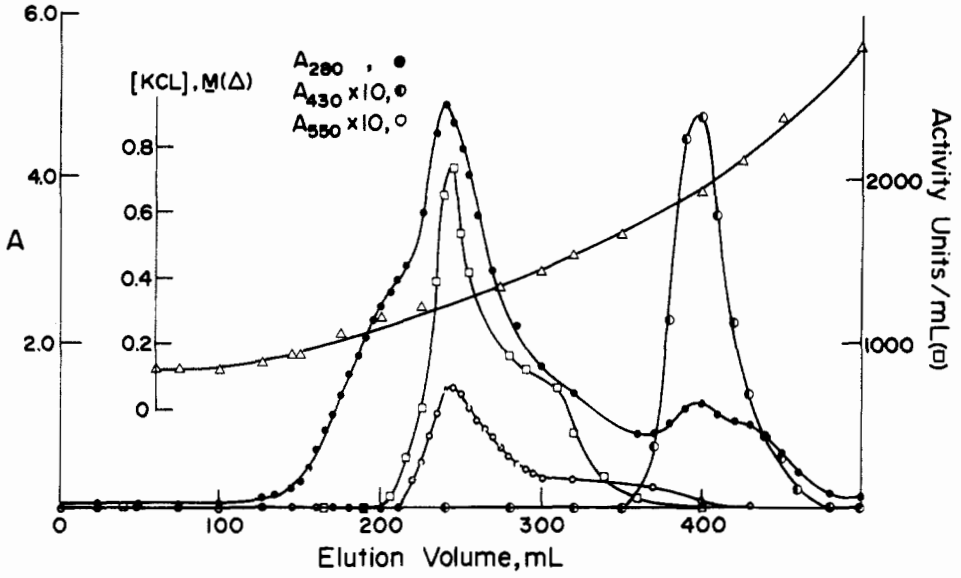

FIGURE 1: Carboxymethylcellulose (CM-52) ion-exchange chromatography of the beef spleen purple acid phosphatase. Elution by KCl gradient (△) with absorption at 280 (●), 430 (◐), and 550 nm (○) and phosphatase activity (□) shown.

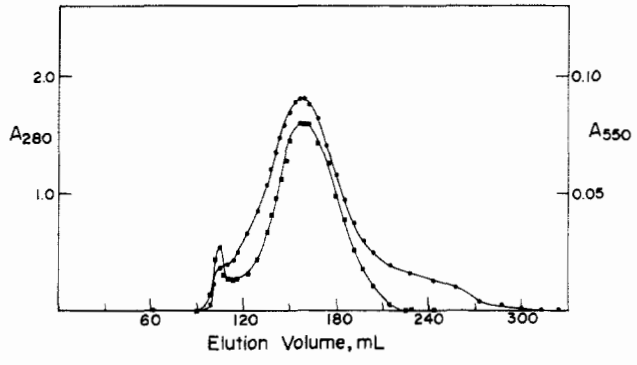

FIGURE 2: Chromatography of the purple fractions from CM-52 on Sephadex G-75 with absorption at 550 (■) and 280 nm (●).

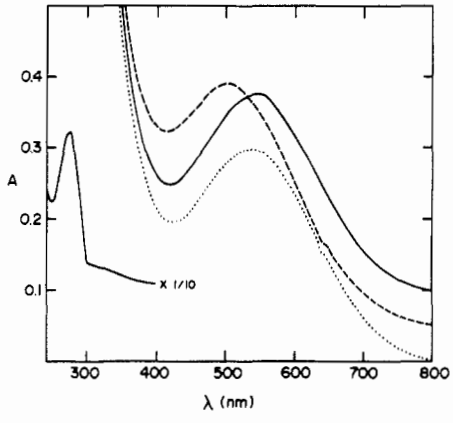

FIGURE 3: Visible absorption spectra of the purple acid phosphatase. Shown are the native enzyme (—) and the reduced enzyme with 10 mM F^- (---) and 10 mM phosphate (···). Note that the spectra of the fluoride-treated enzyme and the native enzyme are offset from that of the phosphate-treated enzyme by +0.05 *A* and +0.10 *A*, respectively.

ceptable loss of activity. Accordingly, a small bed of hydroxylapatite was used as a "polishing" column, which successfully removed remaining impurities with an 86% recovery of phosphatase activity.

Metal Content and Protein Assay. The iron content of the purified enzyme was determined on duplicate samples and gave values of 2.2 per 40000 molecular weight for the chemical method and 2.0 per mole for the plasma emission method. Magnesium was undetected at a detection limit of 0.4 mol per mol of enzyme, while copper, manganese, molybdenum, and zinc were all undetected at a detection limit of 0.1 mol per mol of enzyme. Protein assay by the Lowry method, with bovine serum albumin as standard, consistently gave values 1.6 times the value obtained by using the previously reported value of ϵ_{550} (Campbell et al., 1978). This is probably due to the difference in tyrosine content of the bovine serum albumin standard and the purple phosphatase; the ϵ_{550} determined gravimetrically by Campbell et al. provides a better estimation of protein content.

Visible Spectra of Native and Reduced Enzyme in the Presence of Inhibitors. The first visible spectrum of the beef spleen purple phosphatase was published in 1960 (Glomset & Porath, 1960), but to our knowledge, the beef enzyme has not previously been shown to undergo reversible reduction to a pink form, as has been found for the porcine enzyme (Schlosnagle et al., 1974). Figure 3 shows the optical spectrum of the native enzyme (λ_{max} 550 nm) and the spectra of the enzyme reduced with DTE in the presence of phosphate or fluoride (λ_{max} 545 and 505 nm, respectively). The spectra in Figure 4 show that

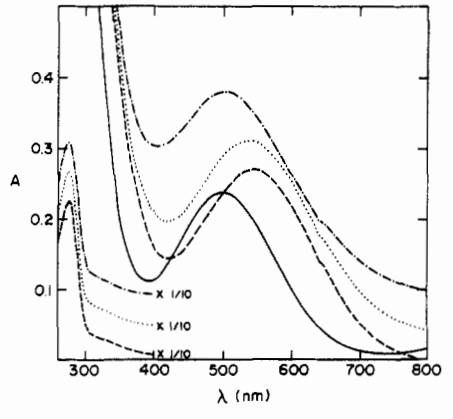

FIGURE 4: Visible absorption spectra of the purple acid phosphatase. Shown are the time course of dithioerythritol reduction at (---) 0, (···) 10, and (-·-·-) 30 min and the reduction with ferrous ion and ascorbate at $t = 0$ (—). Note that the spectra obtained at 10 and 30 min are offset from the other two by +0.05 *A* and +0.10 *A*, respectively.

conversion from the purple to the pink form is relatively slow when DTE without ferrous ion is used as a reductant. Similar slow conversion is observed with ascorbate in the absence of ferrous ion. The spectrum of the pink form produced by

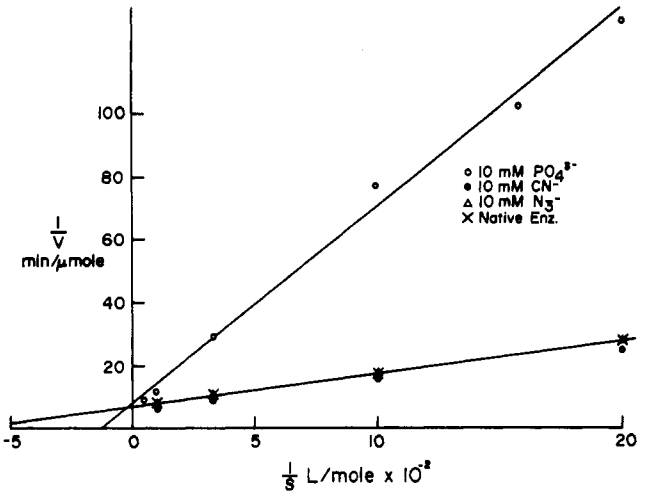

FIGURE 5: Lineweaver–Burk plots of kinetics data with *p*-nitrophenyl phosphate as substrate. Shown are native enzyme (×) alone and with 10 mM phosphate (○), cyanide (●), and azide (△).

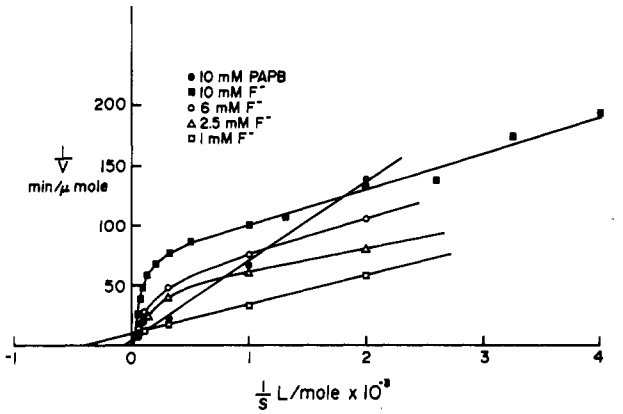

FIGURE 6: Lineweaver–Burk plots of kinetics data with *p*-nitrophenyl phosphate as substrate. Shown are fluoride inhibition at 10 (■), 6 (○), 2.5 (△), and 1 mM (□) and PABP inhibition at 10 mM (●).

reduction of the native form with 0.4 M ascorbate and 0.08 M Fe^{2+} is also shown in Figure 4; reduction under these conditions is complete within 1 min.

Molecular Weight Data. Two methods were used to determine the molecular weight of the beef spleen purple phosphatase. Gel permeation chromatography on Sephadex G-75 gave a molecular weight of ~40 000 for the native form. $NaDodSO_4$ gel electrophoresis was used to obtain information on the subunit molecular weights. The purple acid phosphatase consists of two nonidentical subunits of molecular weight ~24 000 and ~15 000. Gel electrophoresis of native enzyme, followed by staining and scanning with a Gilford gel scanner, showed the purity of the enzyme to be greater than 95% (data not shown).

EPR Spectra. EPR spectra at various temperatures (4.2–300 K) were obtained in an attempt to provide further information on the iron environment in the enzyme, but only insignificant signals (<0.1 spin/40 000 M_r) were observed. Both oxidized and DTE-reduced enzyme with and without inhibitors were studied. Typical conditions of spectroscopy were the following: gain, 10 000; power, 20 mW; microwave frequency, 9.088 GHz; modulation amplitude, 5 G; time constant, 1.0 s; modulation frequency, 100 kHz; enzyme concentration, 15 mg/mL (0.75 mM Fe).

Kinetics Data. The data in Figure 5 indicate that this purple acid phosphatase obeys Michaelis–Menten kinetics for *p*-nitrophenyl phosphate as substrate. No inhibition with 10 mM cyanide or azide was observed; the cyanide data are corrected for the pH shift (ca. 0.3 pH unit) caused by high concentrations of cyanide. Values of K_m and V_{max} obtained for *p*-nitrophenyl phosphate are 2.0 mM and 580 s^{-1}, respectively. Phosphate is a potent competitive inhibitor with K_i = 0.13 mM. Data for *p*-nitrophenol (not shown) showed no detectable product inhibition even at 5 mM.

The data in Figure 6 show that [*p*-(aminoacetyl)benzyl]-phosphonate (PABP), a substratelike molecule with a carbon–phosphorus linkage instead of the normal oxygen–phosphorus bond in the substrate, is also a very good competitive inhibitor, with a value of K_i of 0.13 mM. The remaining data in Figure 6 show that fluoride exhibits complex inhibition behavior, shifting from apparently competitive inhibition at low fluoride (~1 mM) concentrations (apparent K_i ~2 mM) to more complex inhibition at higher (2.5 mM) concentrations. Table II summarizes the data on inhibitors of the purple acid phosphatase from bovine spleen.

Table II: Inhibition of Purple Acid Phosphatase by Selected Compounds and Inorganic Ions

inhibitor[a]	concn	substrate concn (mM)	K_i (mM)	% inhibition
P_i	10 mM	1.0	0.13	74
PABP	10 mM	1.0	0.13	75
fluoride	10 mM	1.0		82
ascorbate/Fe^{2+}	10 mM/20 μM	10		500 (activation)

[a] No inhibition by 10 mM cyanide or azide, 5 mM *p*-nitrophenol, or 40 mM DL-tartrate at 1 mM substrate was observed.

Discussion

The improved purification for spleen purple acid phosphatase has a number of advantages over previous methodology. Most important, of course, is the improved yield (8 mg of homogeneous enzyme per kg of spleen) over that of the previous procedure (<1 mg/kg) (Campbell et al., 1978). This remarkable improvement is not due to a single step but derives from three separate features. First, using 0.25 M KCl (Sundararajan & Sarma, 1954) and pH 3.5 (Singer & Fruton, 1957), thereby combining conditions used by previous researchers, increases the yield at the initial acid extraction step by as much as 3-fold. In addition, these conditions provide faster extraction (3 h), although standing for up to 20 h does not affect the yield. By preassaying spleens and using only those with average or above average enzyme content, it is possible to obtain another 2–3-fold increase in extraction stage yield, due principally to the large variation in enzyme content from spleen to spleen. Finally, the use of cellulose phosphate as a batch adsorbent, replacing the more conventional ammonium sulfate precipitation, not only is more convenient but also increases yield slightly in the second step. A batch extraction method using carboxymethylcellulose has been alluded to previously (Campbell et al., 1978), but to our knowledge has not yet been published. The 130-fold purification on cellulose phosphate is surprisingly good for such a crude method; it is not clear whether this is due simply to the highly basic nature of the protein or to a form of affinity adsorption occurring via the phosphate groups. The final three purification steps are relatively conventional chromatographic procedures giving the expected extent of purification.

The molecular weight of the beef spleen acid phosphatase has been shown by this study to be ~40 000. The protein is composed of two nonidentical subunits of molecular weight ~24 000 and ~15 000. No significant change in these values is observed when 1 mM PMSF (a serine protease inhibitor)

is maintained throughout the preparation, suggesting that degradation of the protein during purification is not occurring. This thesis is also supported by the unusual stability of the enzyme observed during purification (pH 3.5, 24 °C, up to 20 h), as well as its resistance to damage during repeated freeze–thaw cycles. The data obtained in this study also substantiate previous findings (Campbell et al., 1978), where essentially identical values for A_{280}/A_{550} (15.1), molecular weight of holoenzyme (38 000), and subunit molecular weights (15 000 and 26 000) were reported.

The iron content reported previously (Campbell et al., 1978) (two irons per 39 000 molecular weight) is also in good agreement with those values reported here (2.2 and 2.0). Unfortunately, literature values for the iron content of the similar porcine enzyme indicate some confusion. One group (Schlosnagle et al., 1974) reports one iron per 32 000 molecular weight, while another (Campbell et al., 1978; Keough et al., 1980) consistently finds two irons per 36 000–38 000 molecular weight, similar to our results for the beef spleen enzyme. As has been pointed out previously, however (Campbell et al., 1978), the discrepancies in data obtained for the porcine enzyme by the two groups are due to the use of different methods for assaying protein concentration and to differences in assumed molecular weights. Correction for the former and substitution of a more reasonable value for $\bar{V}$ in the calculation of molecular weight via sedimentation equilibrium (Schlosnagle et al., 1974) bring the values of both the molecular weight and iron content found by the two groups into excellent agreement, at ~40 000 and 2 Fe/molecule, respectively. Adding further confusion is the report (Antanaitis et al., 1980) of a novel EPR signal centered at $g = 1.74$ for the porcine enzyme, while the previously reported EPR spectra of the beef enzyme indicated either no detectable signal even at maximum sensitivity (Glomset & Porath, 1960) or a weak signal at $g \sim 4.3$ (Schlosnagle et al., 1976). In our study, we see less than 0.1 spin per 40 000 molecular weight at $g = 4.3$ compared to a conalbumin standard and no detectable $g = 1.74$ signal. Quite likely, the small signal seen at $g = 4.3$ for our sample is due to the slight excess of iron present over the stoichiometry of 2 per molecule (i.e., 5–10% "adventitious" iron). These results suggest that the iron is either ferrous or a spin-coupled ferric dimer; in view of the low-energy features in the visible spectrum (presumably ligand to metal charge transfer), the latter seems more likely.

A recent report on a manganese-containing purple acid phosphatase from sweet potato (Sugiura et al., 1980) claimed that one Mn(III) ion per 110 000 molecular weight enzyme was present; no indication was given either of the method of manganese analysis or of any attempt to analyze for other metals (i.e., Fe). Manganese is known to be abundant in plants and likely to appear as nonspecifically bound metal in plant proteins. Evidence for manganese binding from resonance Raman spectroscopy was also presented; however, the data are remarkably similar to those observed by others (Gaber et al., 1979) for the porcine purple acid phosphatase and attributed to the presence of iron. Comparison is made to a manganese superoxide dismutase from *Escherichia coli* (Fee et al., 1976), which has a chromophore with ϵ of 400 M^{-1} cm^{-1} and λ_{max} of 473 nm (vs. ϵ of 2460 M^{-1} cm^{-1} and λ_{max} 515 nm for the sweet potato enzyme). It is unfortunate that a comparison to either the beef spleen or the porcine enzyme (Campbell et al., 1978) was not made, since the ϵ of 2000 M^{-1} cm^{-1} and λ_{max} of 500–550 nm (depending on the oxidation state) are remarkably close to those of the sweet potato enzyme. Certainly, these similarities cannot be ignored, and further evidence of the presence of manganese and *absence* of iron is required to clarify this situation.

The visible and ultraviolet (where appropriate) spectra of the purple acid phosphatase shown in Figures 3 and 4 indicate that the spleen enzyme undergoes reversible conversion to a "pink" form when exposed to mild reducing agents. Dithionite, however, causes bleaching and complete loss of both the purple color and activity. Only in the presence of ferrous ion does conversion to the pink form occur rapidly. Without ferrous ion, total conversion requires at least 1 h at room temperature with either ascorbate or DTE. Of special interest is the nearly immediate (<1 min) reversion to the purple form upon addition of 10 mM phosphate, even in the presence of excess reductant; similar behavior is observed for PABP. In this case, however, conversion to the purple form is not complete as the absorption maximum is 535 nm rather than 550 nm. Such a reversal is not observed with fluoride at 5–10 mM. These results agree with the postulation (Schlosnagle et al., 1976) that a protein conformation change caused by reduction of protein disulfide bonds(s) and resulting in a change of environment at the iron sites, rather than direct reduction of Fe(III) to Fe(II), is the cause of the shift in λ_{max} from 550 to ~500 nm. The shift back to the purple form in the presence of inhibitors such as phosphate, coupled with the evidence that both the porcine (Schlosnagle et al., 1974) and beef (this study; Campbell et al., 1978) enzymes are active in the pink form but much less so in the purple form, supports such a conformational change, but direct evidence is lacking. Experiments designed to examine this point further are in progress.

The results described above show that phosphate and PABP, a substrate analogue, are competitive inhibitors of the enzyme. The lack of inhibition by *p*-nitrophenol coupled with inhibition by phosphate suggests that the hydrolysis reaction proceeds via sequential release of *p*-nitrophenol and phosphate:

$$E + PNPP \rightleftharpoons E\cdot PNPP \rightleftharpoons E\cdot P_i\cdot PNP \overset{PNP}{\rightleftharpoons} E\cdot P_i \rightleftharpoons E + P_i$$

The substrate analogue PABP is an inhibitor because the normally hydrolyzed P–O bond is replaced by a C–P bond. The increased bulk of this molecule over that of phosphate may be the reason the enzyme–PABP complex shows an absorption maximum (535 nm) intermediate between the pink and purple forms. The observed peak does not appear to be broader than that for either native or phosphate-inhibited enzyme, suggesting that the majority of the enzyme molecules are locked into a conformation between the pink and purple forms. Further studies will be required to substantiate this.

The lack of inhibition by cyanide or azide, normally strong Fe(II) or Fe(III) ligands, suggests either that the irons are in a kinetically nonlabile complex or that addition of such ligands to the iron does not affect the catalytic properties of the enzyme. The absence of any change in the optical spectrum of the iron chromophore argues that these anions do not bind to the metal. The anomalous fluoride inhibition observed implies some type of mixed competitive/noncompetitive kinetics; however, the absence of any change in the optical spectrum implies that the inhibition is, at least in part, due to effects at a site remote from the iron centers.

Others (Keough et al., 1980) have questioned the use of the term "uteroferrin" (Gaber et al., 1979) for the porcine enzyme. It is difficult to assign a role as an iron transport protein to the spleen purple acid phosphatase when it is one of the most potent phosphatases known. Further, the widespread occurrence of apparently similar purple phosphatases in animals, plants, bacteria, and fungi, where in some cases an iron

transport function is most unlikely, suggests an alternate function utilizing the phosphatase activity. The absence of phosphatase activity in transferrin and conalbumin (J. C. Davis and B. A. Averill, unpublished experiments) also implies an enzymatic rather than an iron transport role for the purple acid phosphatases.

Clearly, determining the function of the spleen enzyme and the role of iron therein requires much additional work. EPR and resonance Raman spectroscopic investigations with both iron and other metals in either of the iron sites (Keough et al., 1980) are currently under investigation in our laboratories, as are Mössbauer spectroscopic investigations of ^{57}Fe-enriched enzyme. In addition, immunochemical studies and examination of the enzyme from human spleen are under way in cooperation with the Michigan State University Medical Center.

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