O'Farrel, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Osborne, R. A. O. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4030–4034.
Pai, E. F., Sachsenheimer, W., Schirmer, R. H., & Schulz, G. E. (1977) *J. Mol. Biol. 114*, 37–45.
Sarup, J. C., & Fridland, A. (1987) *Biochemistry 26*, 590–597.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.
Verhoef, V., Sarup, J., & Fridland, A. (1981) *Cancer Res. 41*, 4478–4483.
Yamada, Y., Goto, H., & Ogasawara, N. (1985) *Int. J. Biochem. 17*, 425–428.

Purification and Physicochemical Characterization of a Human Placental Acid Phosphatase Possessing Phosphotyrosyl Protein Phosphatase Activity†

Abdul Waheed, Piotr M. Laidler,‡ Yu-Yuan P. Wo, and Robert L. Van Etten*
Chemistry Department, Purdue University, West Lafayette, Indiana 47907
Received May 20, 1987; Revised Manuscript Received February 18, 1988

ABSTRACT: A 17-kilodalton (kDa) human placental acid phosphatase was purified 21 400-fold to homogeneity. The enzyme has an isoelectric point of pH 7.2 and a specific activity of 106 μmol min^{-1} mg^{-1} using *p*-nitrophenyl phosphate as a substrate at pH 5 and 37 °C. This placental acid phosphatase showed activity toward phosphotyrosine and toward phosphotyrosyl proteins. The pH optima of the enzyme with phosphotyrosine and with phosphotyrosyl band 3 (from human red cells) were between pH 5 and 6 and pH 5 and 7, respectively. The K_m for phosphotyrosine was 1.6 mM at pH 5 and 37 °C. Phosphotyrosine phosphatase activity was not inhibited by tartrate or fluoride, but vanadate, molybdate, and zinc ions acted as strong inhibitors. Enzyme activity was also inhibited by DNA, but RNA was not inhibitory. It is a hydrophobic nonglycoprotein containing approximately 20% hydrophobic amino acids. The average hydrophobicity was calculated to be 903 cal/mol. The absorption coefficient at 280 nm, $E^{1\%}_{1cm}$, was determined to be 5.7. The optical ellipticity of the enzyme at 222 nm was −5200 deg cm^2 dmol^{-1}, which would correspond to a low helical content. Free sulfhydryl and histidine residues were necessary for the enzyme activity. The enzyme contained four reactive sulfhydryl groups. Chemical modification of the sulfhydryls with iodoacetate resulted in unfolding of the protein molecule as detected by fluorescence emission spectroscopy. Antisera against both the native and the denatured protein were able to immunoprecipitate the native enzyme. However, upon denaturation, the acid phosphatase lost about 70% of the antigenic determinants. Both antisera cross-reacted with a single 17-kDa polypeptide on immunoblotting.

Phosphorylation and dephosphorylation of proteins at serine residues represent one important mechanism for the regulation of enzymes in eukaryotes (Cohen, 1982; Ingebristen & Cohen, 1983). The phosphorylation of tyrosyl residues, which is thought to be more rare, has been suggested to be involved in cell proliferation and differentiation (Hunter & Cooper, 1985). In general, the phosphorylation state of any such protein, and therefore the physiological processes it controls, may be the result of a balance between competing protein kinase and protein phosphatase activities (Frank & Sartorelli, 1986). The physiological significance of tyrosine kinase in the regulation of cell proliferation and in transformation has been very well documented (Hunter & Sefton, 1980; Cooper et al., 1982). In contrast, very little is known about the role of phosphotyrosyl phosphatases. Recently, Klarlund described exciting experiments in which vanadate, an inhibitor of certain phosphatases, was able to transform NRK-1 cells (Klarlund, 1985). Transformation of the cells by vanadate was accompanied by an increase in the phosphotyrosyl content. This increase in the phosphotyrosyl content of the cellular proteins may be due to an increase in the tyrosine kinase level, but it could also result from an inhibition of phosphotyrosyl phosphatases by vanadate. There is evidence that pp60$^{v\text{-}src}$ kinase shows increased activity upon vanadate treatment (Brown & Gordon, 1984). At the same time, vanadate and related early transition-metal oxoanions possess broad potential as transition-state analogues of phospho transfer reactions in general (Van Etten et al., 1974), and of acid phosphatases in particular (Van Etten et al., 1974; Van Etten, 1982). More recently, vanadate has been shown to act as an inhibitor of phosphotyrosyl phosphatases (Swarup et al., 1984; Okada et al., 1986). Thus, elevated levels of phosphotyrosine in cellular proteins may be the result of decreased phosphatase activities as well as from increased tyrosine kinase activities.

Several acid phosphatases of differing molecular weights have been characterized in mammalian tissues (Heinrikson, 1969; Ostrowski et al., 1976; Lawrence & Van Etten, 1981), and some of them show phosphotyrosyl phosphatase activity (Okada et al., 1986; Li et al., 1984; Lin & Clinton, 1986; Lau et al., 1985; Chernoff & Li, 1985; Shriner & Brautigan, 1984; Apostol et al., 1985). High molecular weight (lysosomal) and low molecular weight acid phosphatases from human liver have been purified and characterized (Saini & Van Etten, 1978; Taga & Van Etten, 1982). It was observed that these enzymes efficiently hydrolyze phenyl phosphate, although their activity against phosphotyrosyl-containing proteins was not specifically tested. From earlier work (DiPietro & Zengerle, 1967; Rehkop & Van Etten, 1975), it was clear that human liver and placenta contain at least three different types of acid

†This research was supported by USDHHS Research Grant GM 27003 from the National Institute of General Medical Sciences.
‡Permanent address: Institute of Medical Biochemistry, Medical Academy, ul. Kopernika 7, 31-034 Krakow, Poland.

0006-2960/88/0427-4265$01.50/0

phosphatases and that a low molecular weight acid phosphatase [corresponding to peak III in the Sephadex G-200 chromatographic profile of Rehkop and Van Etten (1975)] constituted around half of the total tissue acid phosphatase activity (as measured using nitrophenyl phosphate as a substrate). Similar observations were reported for several bovine tissues (Heinrikson, 1969). A related low molecular weight acid phosphatase from bovine heart has been partially purified and has been shown to possess phosphotyrosyl phosphatase activity (Lin & Clinton, 1986). However, the relatively abundant low molecular weight acid phosphatase from human placenta still has not been purified and studied in detail.

Due to our interest in the chemical and kinetic characterization of acid phosphatases and in understanding their physiological functions, we have now purified the low molecular weight human placental acid phosphatase to homogeneity. The homogeneous enzyme was used to develop antisera against native and denatured protein molecules which could be used in studies of the biosynthesis of the protein. In the present paper, we describe in detail the isolation and physicochemical and enzymatic properties of a homogeneous low molecular weight (17K) acid phosphatase from human placenta. From the present results, it is evident that the 17-kilodalton (kDa) enzyme is a hydrophobic, basic protein that possesses substantial acid phosphatase and phosphotyrosyl phosphatase activity.

Experimental Procedures

Materials. Human placentas were obtained from a local hospital. L-Phosphotyrosine, -serine, and -threonine, *p*-nitrophenyl phosphate, nicotinamide adenine dinucleotide phosphate, diethyl pyrocarbonate, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), fluorescein mercuric acetate, RNA (bakers' yeast), and DNA (calf thymus) were from Sigma Chemical Co. Iodoacetate, which was recrystallized before use, was from MCB Co. SP-Sephadex and Sephadex G-75 were from Pharmacia. [γ-^{32}P]ATP was from Amersham. The cytoplasmic domain of human erythrocyte band 3 was a gift from Prof. P. S. Low, while calf thymus tyrosine-specific kinase (P^{40}) and angiotensin I were from Dr. Marrietta L. Harrison. Protein A bacterial adsorbant was from Miles Laboratories. Prestained and radioactive protein standards were purchased from Bethesda Research Laboratories.

Acid Phosphatase Activity. Human placenta acid phosphatase activity was assayed at 37 °C in a 100-μL reaction mixture containing 10 mM *p*-nitrophenyl phosphate in 100 mM sodium acetate, pH 5.0 (Taga & Van Etten, 1982). One unit of the enzyme is defined as the amount of enzyme required to produce 1 μmol of product per minute at 37 °C.

Phosphoamino Acid Phosphatase Activity. The rates of dephosphorylation of phosphoserine and phosphothreonine were measured by determining the production of inorganic phosphate (P_i). The concentration of P_i was determined by using acidic ammonium molybdate solution in the presence of Triton X-100 (Kyaw et al., 1985). All other experimental conditions were as described for phosphotyrosine phosphatase activity measurement.

Phosphotyrosine Phosphatase Activity. Tyrosine phosphate phosphatase activity was determined in a 100-μL reaction mixture containing 10 mM L-tyrosine phosphate in 100 mM sodium acetate, pH 5.0 at 37 °C. Reaction medium buffers were as follows: pH 3.5, 100 mM sodium formate; pH 4–5.5, 100 mM sodium acetate; pH 6 and 6.5, 100 mM sodium succinate; and pH 7–9, 100 mM sodium carbonate. L-Tyrosine produced during the reaction was determined by Lowry's method (Lowry et al., 1951) because phosphotyrosine does not react with Lowry's reagent. Inhibition of the tyrosine phosphate phosphatase activity by added chemicals was determined by first exposing the enzyme to the required concentration of the chemicals in 100 mM sodium acetate buffer, pH 5.0, for 5 min at room temperature. Then the reaction was initiated by addition of 10 mM L-tyrosine phosphate at 37 °C.

Michaelis Constants and Inhibition Constants. Kinetic parameters were measured at 37 °C in 0.05 M tris(hydroxymethyl)aminomethane (Tris)–acetate buffer at pH 5 and 7 using *p*-nitrophenyl phosphate as a substrate together with highly purified (>50%) enzyme that exhibited only one activity band on gel electrophoresis. On the basis of preliminary estimates, 10–12 substrate concentrations were used that spanned the concentration range $0.1K_m < K_m < 10K_m$. At least two inhibitor concentrations were used in measurements of K_I. Duplicate sets of data were obtained at each pH and inhibitor concentration. The best-fit inhibition mode and K_I values were obtained by direct computer fitting of the data using a Hewlett-Packard HP-85 computer together with the program ENZYME (Lutz & Rodbard, 1985).

Phosphotyrosyl Protein Phosphatase Activity. The phosphotyrosyl protein phosphatase activity was monitored by the release of [^{32}P]phosphate from [^{32}P]phosphotyrosyl-band 3, angiotensin, or kinase P^{40}. [^{32}P]Phosphotyrosyl proteins were prepared as described (Zioncheck et al., 1986). Band 3 (1 mg/mL) was 75% phosphorylated on tyrosine residues 8 and 22 (Low et al., 1987). Angiotensin (3 mM) was approximately 10% phosphorylated on the single tyrosine. Due to the nature of the autophosphorylation reaction of kinase P^{40}, only trace amounts of protein were labeled. [^{32}P]Phosphotyrosyl proteins (10 μL containing 13 000 cpm), 100 mM sodium acetate, pH 5.0, and homogeneous phosphatase were placed in a typical 20-μL reaction mixture. At pH 3 and 4, 100 mM sodium formate, at pH 6, 100 mM sodium succinate, and at pH 7 and 8, 100 mM Tris-HCl buffers were used. The reaction mixture was incubated at 37 °C for the required time. The reaction was quenched with 20 μL of 2×-concentrated Laemmli's solubilizer (Laemmli, 1970) by heating at 98 °C for 3 min. The sample was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography in order to monitor the release of [^{32}P]phosphate from the protein. The reaction was quantitated from densitometric tracings of the fluorogram.

DNA Binding. DNA–cellulose gel (200 μL in Eppendorf vials) was equilibrated with 10 mM sodium acetate, pH 5.0, or 10 mM Tris-HCl, pH 7.5, buffers. Homogeneous acid phosphatase (3.5 μg in 100 μL) was mixed with DNA–cellulose gel and 400 μL of the required buffer. The resin slurry was mixed and left on ice for 30 min with intermittent shaking. The unbound protein was recovered after centrifugation. The nonspecifically bound protein was further washed with an additional 0.5 mL of equilibrating buffer. The bound protein was eluted with 0.5 mL of equilibrating buffer containing different concentrations of sodium chloride (0.1–2 M). The enzyme activity was monitored in each fraction using phosphotyrosine as a substrate.

Electrophoresis and Isoelectric Focusing. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 12% acrylamide was performed according to Laemmli (1970). Prestained molecular weight standards contained insulin (3K), bovine trypsin inhibitor (6.2K), cytochrome *c* (12.3-K), trypsinogen (25.7K), and ovalbumin (43K). During fluorography of the gel, ^{14}C-methylated protein molecular weight standards containing lysozyme (14.3K), β-lactoglobulin (18.4K), α-chymotrypsinogen (25.7K), ovalbumin, bovine serum albumin

(68K), phosphorylase B (97.4K), and myosin H chain (200K) were used.

Isoelectric focusing was performed on precast gels from LKB according to the manufacturer's protocol. The protein bands were stained for enzyme activity using 1 mg/mL each of β-naphthyl phosphate and fast red TR salt in 100 mM sodium acetate, pH 5.0 at 37 °C.

Absorption Spectral Measurements. Absorption measurements and spectra were determined on an IBM 9420 UV–vis spectrophotometer. Optical density measurements at single wavelengths were also made on a Beckman Acta V spectrophotometer. All measurements were made at room temperature in 1-cm cuvettes.

Fluorescence Measurements. Fluorescence emission spectra and the relative fluorescence at a single wavelength were determined by using a Perkin-Elmer MPF-44A fluorescence spectrophotometer. The ratio mode of operation was used in all measurements in order to minimize the effect of variation in the xenon lamp intensity. The excitation and emission slits were kept constant at 6 nm.

Circular Dichroism Measurement. Circular dichroism of the enzyme was recorded at room temperature on a Cary 60 spectropolarimeter using a 1-cm light path cell. Mean residue ellipticities, $[\theta]$ (in degrees centimeter squared per decimole), were calculated from the measured ellipticities by the equation $[\theta] = (\theta/10)(M_0/lC)$ where θ is the measured ellipticity in degrees, l is the optical path length in centimeters, C is the protein concentration (grams per milliliter), and M_0 is the mean residue weight (molecular weight divided by the number of amino acid residues) which in this case is 112.

Amino Acid Analysis. Approximately 300 μg of pure enzyme was denatured, reduced, and alkylated for amino acid analysis. Lyophilized samples were dissolved in 0.5 mL of 6 N HCl, and the vacuum-sealed samples were hydrolyzed for 12, 24, and 48 h at 100 °C. The hydrolyzed samples were lyophilized and subjected to amino acid analysis using a Beckman Model 7300 analyzer. The number of amino acid residues were calculated by an integer fit method (Hoy et al., 1974). The number of residues were corrected for time-dependent destruction or release.

Preparation of Antisera and Quantitative Immunoprecipitation. The antigen injection schedule was similar to the antibody production protocol used previously with human urine arylsulfatase A (Laidler et al., 1985). Immunotitration of placental 17-kDa acid phosphatase was performed as described (Waheed et al., 1985) using 0.67 μg of homogeneous acid phosphatase.

Immunoblotting. Enzyme samples containing 5 μg of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in a 12% acrylamide gel. The polypeptides were transferred electrophoretically to a nitrocellulose strip (Burnette, 1981). After electrophoretic transfer, the nitrocellulose sheet was soaked overnight at room temperature in a blocking buffer (Johnson et al., 1984), and the acid phosphatase polypeptide was visualized by incubation with 100×-diluted rabbit anti-human placental 17-kDa acid phosphatase antiserum, followed by goat anti-rabbit IgG peroxidase (500×-diluted).

Determination of Free Sulfhydryl Groups. The number of free sulfhydryl residues in the native enzyme were determined by direct titration or by reaction with a 10-fold excess of DTNB and of fluorescein mercuric acetate (FMA) (Laidler et al., 1982).

Inactivation of the Enzyme by DTNB. In a typical inactivation experiment, enzyme (23.5 μM in 0.1 M iodoacetate pH 8.0) ws titrated with increasing concentrations of DTNB. An aliquot of the reaction mixture was removed at each new addition of DTNB for the enzyme assay. An identical experiment was performed in the presence of 10 mM phosphate. The percent residual activity of the enzyme was calculated from the initial activity and activities determined after each addition of DTNB.

Inactivation of the Enzyme by Iodoacetate. The placental acid phosphatase (23.5 μM) was incubated with 10 mM iodoacetate in 0.1 M Tris-HCl, pH 8.0, in the presence and absence of 10–20 mM phosphate. An aliquot of the reaction mixture was removed at different time intervals, and the enzyme activity was determined. The percent residual activity was calculated from the difference between control and iodoacetate-treated samples.

In order to determine the number of reactive sulfhydryls using iodoacetate, the enzyme was first treated with iodoacetate to different levels of residual activity. The iodoacetate-inactivated enzyme was then titrated with DTNB. The number of free sulfhydryl groups was calculated from the absorbance at 412 nm of the reaction mixture as indicated earlier. The number of sulfhydryls essential for enzyme activity were also determined. In a typical experiment, the enzyme samples (containing 50 μg of protein) were treated with iodoacetate in the presence and absence of phosphate. The reaction mixtures were desalted on a PD-10 column. The fractions containing protein were pooled, lyophilized, and subjected to amino acid analysis. The enzyme sample without any treatment was also used in amino acid analysis as a control. The amounts of (carboxymethyl)cysteine and cysteic acid were determined.

Modification of the Enzyme with Diethyl Pyrocarbonate. Acid phosphatase (10 μM) was incubated with 10–85 mM diethyl pyrocarbonate in 100 mM sodium acetate, pH 6.0, in the presence and absence of 10–20 mM phosphate. Aliquots of the reaction mixture were removed at required intervals, and enzyme activities were determined.

Purification of 17-kDa Acid Phosphatase. Several frozen human placentas were cut into small pieces after membranes, blood clots, and major veins and arteries were removed. Cleaned placenta pieces were frozen at −70 °C before use. Partially thawed placenta (1 kg) was homogenized in 2.5 L of cold 50 mM Tris-HCl, pH 7.5, buffer. The homogenate was stirred for at least 3 h at 4 °C and then centrifuged.

The proteins were precipitated from the supernatant (3.85 L) at pH 7–7.5 using ammonium sulfate (0.5 kg/L of supernatant). After standing at 4 °C for 4–6 h, the protein precipitate was recovered by centrifugation and solubilized in 1500 mL of cold deionized water. After centrifugation, the resulting protein solution (1.9 L) was subjected to ammonium sulfate precipitation using 374 g of ammonium sulfate/L of supernatant. The precipitate thus obtained was dissolved in 3 L of 10 mM sodium acetate, pH 4.5, buffer, and insoluble proteins were removed by centrifugation. The supernatant was applied to an SP-Sephadex column (5 × 30 cm) packed and equilibrated with 10 mM sodium acetate, pH 4.5, plus 50 mM ammonium sulfate buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA). After the protein sample was adsorbed on the column, the column was washed with 10 mM sodium acetate, pH 4.5, plus 100 mM ammonium sulfate until the absorbance at 280 nm dropped near zero. Bound proteins were eluted with 10 mM sodium acetate, pH 5.0, plus 500 mM sodium phosphate, pH 5.0. The fractions containing enzyme activity were pooled and concentrated on an ultrafiltration assembly using a YM5 membrane. The concentrated enzyme

Table I: Purification of the 17-kDa Human Placental Acid Phosphatase[a]

purification step	total protein (mg)	enzyme (units)	sp act. (units/mg)	yield (%)
tissue extract	105875	547	0.005	(100)
ammonium sulfate pptn	42350	392	0.009	72
SP-Sephadex C-50	45	280	6.2	51
Sephadex G-75 I	3.0	275	92	50
Sephadex G-75 II	2.2	235	107	43

[a] The purification started with 1.0 kg of human placenta. The enzyme was assayed using 10 mM *p*-nitrophenyl phosphate in 100 mM sodium acetate, pH 5.0, buffer at 37 °C.

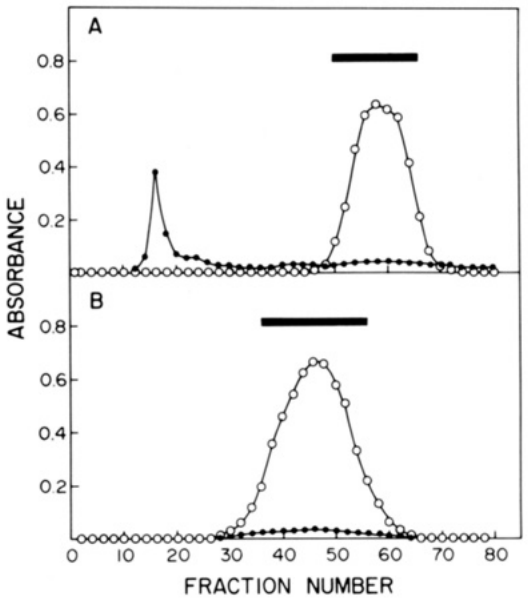

FIGURE 1: Elution profile of the acid phosphatase from a Sephadex G-75 column. (Panel A) The enzyme preparation after SP-Sephadex chromatography was applied on a 2.5 × 102 cm Sephadex G-75 column which was equilibrated with 25 mM sodium acetate, pH 5.0, buffer containing 30 mM phosphate, 1 mM EDTA, and 100 mM sodium chloride. The fractions were monitored at 280 nm for protein content (●) and at 405 nm for enzyme activity (○). (Panel B) The enzyme preparation obtained from the column of panel A was applied again on the Sephadex G-75 column. The fractions were monitored for protein content (●) and enzyme activity (○). Solid bars indicate the enzyme fractions pooled during purification.

sample was applied to a Sephadex G-75 column (2.5 × 102 cm) packed and equilibrated with a buffer containing 25 mM sodium acetate, pH 5.0, plus 30 mM sodium phosphate plus 1 mM EDTA and 100 mM sodium chloride. The enzyme sample was eluted in 4.2-mL fractions at a flow rate of 60 mL/h. Each fraction was monitored for protein at 280 nm and enzyme activity at 405 nm. Fractions containing activity were pooled and concentrated. The concentrated enzyme preparation was rechromatographed on the same column to check the chromatographic profile.

RESULTS

An acid phosphatase was purified to homogeneity from human placenta. The enzyme activity was determined during purification using *p*-nitrophenyl phosphate as a substrate at pH 5.0. The purification procedures are summarized in Table I. When a post-SP-Sephadex enzyme preparation was applied to the Sephadex G-75 column, two protein peaks were observed (Figure 1A). The first protein peak, which constituted more than 90% of the total protein and eluted with an apparent molecular weight of 68K, did not show acid phosphatase activity. The second peak, which constituted only 3–4% of the total protein, showed acid phosphatase activity. The enzyme

Table II: Relative Activity of the Homogeneous Human Placental Acid Phosphatase with Phosphoamino Acid Substrates

substrate	rel act.[a]	substrate	rel act.[a]
p-nitrophenyl phosphate	(100)	serine phosphatase	2
tyrosine phosphate	36	threonine phosphate	5

[a] Determined from the release of inorganic phosphate in the reaction mixture. The substrate concentration in each case was 10 mM.

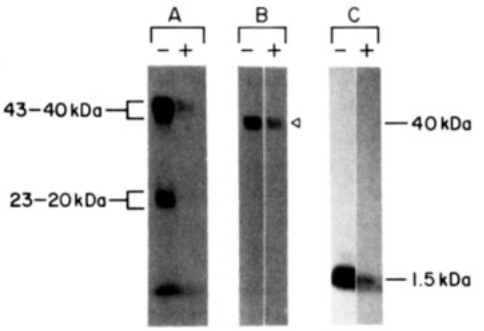

FIGURE 2: Phosphotyrosyl protein phosphatase activity of human placental 17-kDa acid phosphatase. Phosphorylated band 3 (A), tyrosine kinase p^{40} (B), or angiotensin I (C) containing 13 000 cpm of radioactive phosphate was incubated with (+) and without (–) acid phosphatase in 50 mM sodium acetate, pH 5.0, buffer at 37 °C for 60 min. The samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography. The apparent molecular weights of the polypeptides are marked. The arrow shows the position of the 40-kDa tyrosine kinase band.

preparation from the second peak was rechromatographed on the same column using a reduced fraction size. Homogeneous enzyme was eluted as a single symmetrical peak coincident with acid phosphatase activity (Figure 1B). The homogeneous enzyme had a specific activity of 106 units mg^{-1}, the yield was 43%, and the overall purification factor was 21 400. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the enzyme preparation showed a single polypeptide with an apparent molecular weight of 16.9K. Isoelectric focusing results indicate that the human placental acid phosphatase is a basic protein with an isoelectric point of pH 7.2. The homogeneous enzyme, 0.5 mg/mL, was found to be stable for 6 months when stored in 10 mM sodium acetate, pH 5.0, containing 10 mM phosphate and 10% (v/v) glycerol at 4 °C. The enzyme stability was very poor when phosphate and glycerol were not present. Enzyme stored near its isoelectric point or in high salt concentrations showed a loss of enzyme activity with time. Enzyme inactivation during storage was accompanied by protein aggregation. Addition of 5 mM EDTA and 1 mM dithiothreitol (DTT) helped to stabilize the enzyme activity.

The substrate specificity of the enzyme was checked using *p*-nitrophenyl phosphate and three phosphoamino acids. Results are shown in Table II. Because phosphotyrosine was nearly as good a substrate as *p*-nitrophenyl phosphate, we further characterized the 17-kDa acid phosphatase for its phosphotyrosyl phosphatase activity. The enzyme was able to cleave phosphate from tyrosine residues of human band 3, angiotensin I, and calf thymus tyrosine kinase (Figure 2). After band 3 and angiotensin I were phosphorylated using [γ-^{32}P]ATP in the presence of calf thymus tyrosine kinase (or autophosphorylated in the case of tyrosine kinase), excess ATP was removed by chromatography on a PD-10 column. The desalted band 3 reaction mixture showed two polypeptides with apparent molecular weights of 40–43K and 20–23K, respectively. These polypeptides were characterized as the intact cytoplasmic domain of band 3 (40–43K) and proteolysis fragments of band 3 (20–23K). [The presence of proteolysis fragments in band 3 preparations is not unusual (Low, 1986;

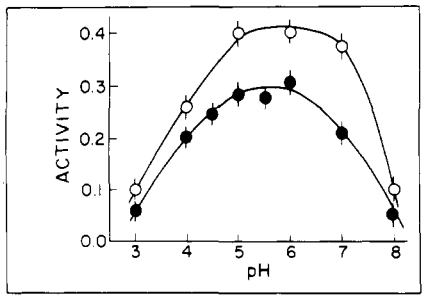

FIGURE 3: Effect of pH on the phosphotyrosine phosphatase activity of the acid phosphatase. Phosphatase activity was assayed with phosphotyrosine (●) and phosphotyrosyl band 3 (○) at various pH values obtained with 50 mM sodium malonate (pH 3.0), sodium acetate (pH 4–5.5), sodium succinate (pH 6.0), or sodium bicarbonate (pH 7 and 8). At pH 5.0, the K_m for phosphotyrosine was 1.6 mM.

Table III: Effect of Chemicals on the Phosphotyrosine Phosphatase Activity of the 17-kDa Acid Phosphatase

addition	concn	act. (%)
none[a]		(100)
serine phosphate	10 mM	100
threonine phosphate	10 mM	100
NADP	10 mM	100
sodium fluoride	0.5 mM	100
L-tartrate	1 mM	100
RNA	5 μg	100
DNA	5 μg	24
zinc chloride	1 mM	46
p-nitrophenyl phosphate	1 mM	60

[a] The enzyme control activity was established by using 10 mM tyrosine phosphate in 100 mM sodium acetate, pH 5.0, buffer. Thus, the substrate concentration was $6.3K_m$.

Kaul et al., 1983).] The desalted reaction mixtures of tyrosine kinase and angiotensin I showed an apparent molecular weight of 40K and 1.5K, respectively. Incubation of radioactive band 3, angiotensin I, or tyrosine kinase with the acid phosphatase for 60 min resulted in 80–90% loss of the radioactivity from these proteins. Prolonged incubation of the reaction mixture of angiotensin I resulted in a complete removal of the radioactivity from the polypeptide.

The pH optima were determined for the enzyme acting on phosphotyrosine and on phosphotyrosyl band 3 (Figure 3). With both phosphotyrosine and phosphotyrosyl band 3, the pH optimum was between pH 5 and 7 (Figure 3). At pH 5, the K_m for phosphotyrosine was determined to be 1.6 mM. The K_m values for *p*-nitrophenyl phosphate were 0.169 and 2.1 mM at pH 5 and 7, respectively. The K_m for phosphotyrosyl band 3 could not be determined with the limited amount of material available.

Further characterization of the enzyme was performed by using various possible effectors together with phosphotyrosine as a substrate. Serine phosphate, threonine phosphate, and nicotinamide adenine dinucleotide phosphate (10 mM) did not inhibit the enzyme activity, but 1 mM *p*-nitrophenyl phosphate inhibited the phosphotyrosine phosphatase activity by 40% (Table III). Sodium fluoride and L-tartrate were weak inhibitors compared to their effect on prostatic acid phosphatase, for example (Vescia & Chance, 1958). Zinc chloride (1 mM), another specific phosphatase inhibitor (Shriner & Brautigan, 1984), was able to inactivate the enzyme activity by 54%. Inhibition constants were measured at pH 5 and 7 (Table IV). Noteworthy is the potent inhibition by vanadate at pH 7.

When the enzyme was incubated with DNA from calf thymus, the phosphotyrosine phosphatase activity was inhibited by 76%, although RNA was completely ineffective as an inhibitor. Since calf thymus DNA was an inhibitor of the enzyme, the possibility of a DNA interaction with the acid

Table IV: Competitive Inhibition Constants of the 17-kDa Placental Acid Phosphatase[a]

inhibitor	inhibn constants, K_I (M), at pH 5.0	pH 7.0
fluoride	3.04×10^{-2}	7.9×10^{-3}
vanadate	1.26×10^{-5}	1.02×10^{-7}
molybdate	4.19×10^{-6}	1.48×10^{-5}
tungstate	5.55×10^{-5}	2.2×10^{-4}

[a] All of the inhibition data fitted (Lutz & Rodbard, 1985) the pattern expected for competitive inhibition.

Table V: Amino Acid Composition of the Enzyme

amino acid	residues/ molecule	amino acid	residues/ molecule
Asp	18	Leu	14
Thr	8	Tyr	4
Ser	11	Phe	5
Glu	18	His	1
Pro	8	Lys	4
Gly	14	Arg	6
Ala	13	Trp	3[a]
Cys	4	total no. of residues	151
Val	14	mol wt due to amino	16280
Met	1	acid residues	
Ile	5		

[a] Determined from the ratio of the fluorescence quantum yield of tryptophan over tyrosine.

phosphatase was explored. The human placenta 17-kDa acid phosphatase was found to bind to a DNA–cellulose matrix at pH 5.0 in 10 mM sodium acetate buffer. The majority of the bound enzyme was eluted with 200 mM sodium chloride in 10 mM sodium acetate, pH 5.0, buffer. However, the enzyme did not bind to the DNA–cellulose matrix at pH 7.5 in 10 mM Tris-HCl buffer.

The amino acid composition of the enzyme is shown in Table V. The enzyme molecule contains four cysteines and one residue each of histidine and methionine. About 20% of the amino acids were hydrophobic in nature. Basic amino acids constituted 7% of the total amino acid residues, whereas acidic amino acids constituted 24% of the total amino acids. However, because the protein actually exhibits a basic isoelectric point, most of these acidic amino acids are probably present as amide derivatives. The molecular weight of the polypeptide due to amino acid residues was calculated to be 16.3K, a value which was similar to that (16.9K) obtained by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The homogeneous enzyme molecule did not bind to concanavalin A–Sepharose, and no detectable reaction was observed with Schiff reagent.

The low molecular weight acid phosphatases from human (Taga & Van Etten, 1982) and bovine liver (Lawrence & Van Etten, 1981) and bovine brain (Chaimovich & Nome, 1970) contain sulfhydryl residue(s) at or near the active site. Results on the modification of histidine residues of the bovine liver enzyme using diethyl pyrocarbonate suggested that histidine might not be necessary for the enzyme activity (Lawrence & Van Etten, 1981). This was in contrast to results obtained with the human liver enzyme, where histidine modification resulted in a loss of enzyme activity (Taga & Van Etten, 1982). Therefore, we determined the number of free (reactive) sulfhydryls and explored the role of sulfhydryl and histidine residues in catalysis. Results of the titration of free sulfhydryls of the protein molecule are shown in Figure 4. The titration of the protein with DTNB gave a value of four reactive sulfhydryls (Figure 4A), suggesting that all four of the cysteine residues that are present in the enzyme are in fact free. Interestingly, when the enzyme was titrated with FMA, we found only three reactive sulfhydryls (Figure 4B). Similarly, when the enzyme was treated first with iodoacetate and then with

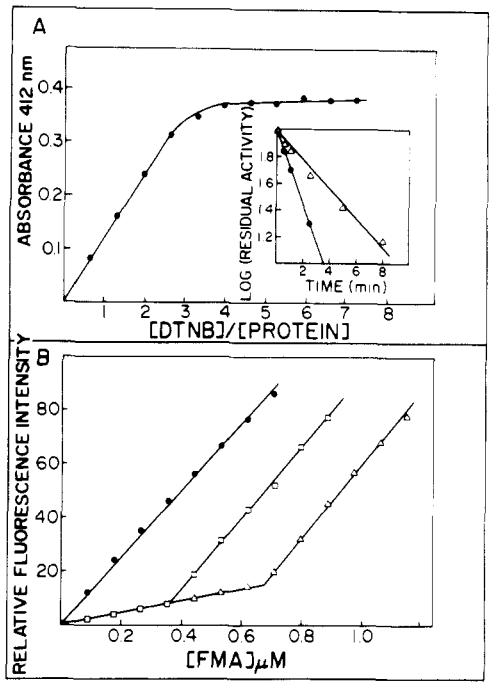

FIGURE 4: Titration of sulfhydryl groups of the acid phosphatase. (Panel A) Spectrophotometric titration of the enzyme with 5,5′-dithiobis(2-nitrobenzoate). The enzyme solution (7.5 μM) was titrated with 1.92 mM 5,5′-dithiobis(2-nitrobenzoate) in 0.1 M Tris-HCl, pH 7.5, buffer. The change in absorbance at 412 nm (●) was recorded against a blank from which enzyme was omitted. The inset shows the inactivation of the enzyme by 5,5′-dithiobis(2-nitrobenzoate). The enzyme (20 μM) was incubated with 83 mM 5,5′-dithiobis(2-nitrobenzoate) in 100 mM Tris-HCl, pH 8.0, buffer in the presence (△) and absence (●) of 20 mM phosphate at room temperature. The enzyme activity was determined at different times using *p*-nitrophenyl phosphate as a substrate. (Panel B) Fluorescence titration of the acid phosphatase with fluorescein mercuric acetate in 0.1 M Tris-HCl, pH 8.0, buffer. In the titration, different concentrations of the enzyme ranging from zero (●) to 0.22 mM (□) and 0.43 mM (△) were titrated with increasing concentrations of fluorescein mercuric acetate. The fluorescence intensity of the sample at 520 nm was recorded by exciting the sample at 495 nm.

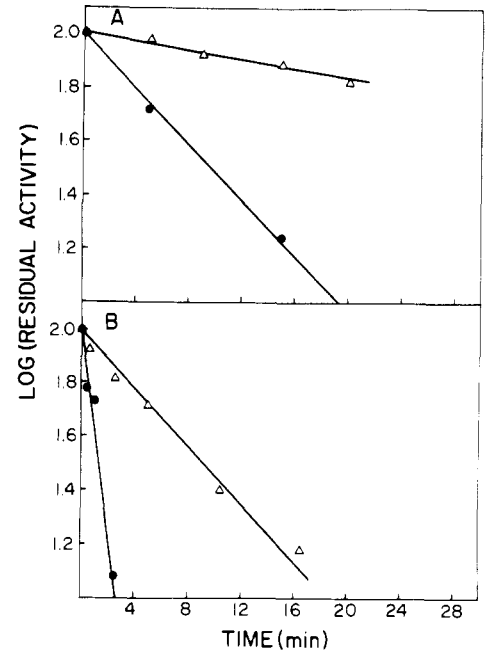

FIGURE 5: Inactivation of the acid phosphatase by iodoacetate and diethyl pyrocarbonate. (Panel A) Inactivation of the enzyme with iodoacetate was performed in 0.1 M Tris-HCl, pH 8.0, buffer. The enzyme (25.5 mM) was incubated with 10 mM iodoacetate in presence (△) and absence (●) of phosphate (20 mM). (Panel B) Inactivation of the enzyme with diethyl pyrocarbonate was carried out in 0.1 M sodium acetate, pH 6.2. The enzyme (10 mM) was incubated with 85 mM diethyl pyrocarbonate in the presence (△) and absence (●) of 20 mM phosphate.

DTNB, one sulfhydryl was found to be unreactive with iodoacetate (results not shown). Thus, the 17-kDa acid phosphatase contains four DTNB-reactive sulfhydryls, while only three sulfhydryls are reactive toward FMA and iodoacetate. During titration of the enzyme with DTNB or iodoacetate, the enzyme activity was reduced. Phosphate, a competitive inhibitor (Rehkop & Van Etten, 1975), was able to protect the enzyme against a loss in activity (Figure 4A and Figure 5A). Amino acid analysis of control enzyme and of enzyme treated with iodoacetate in the presence and absence of phosphate revealed the presence of cysteic acid and (carboxymethyl)cysteine (results not shown). In the presence of 10 mM phosphate, the alkylation of cysteine was decreased by about 34%. The decrease in alkylation was accompanied by a 22% increase in the amount of cysteic acid. From these results, we conclude that of three iodoacetate-reactive cysteine residues, one appears necessary for enzyme activity. The results of histidine modification with diethyl pyrocarbonate in the presence and absence of phosphate are shown in Figure 5B. From this result, it was clear that, like sulfhydryl residue(s), a histidine of the protein molecule is also necessary for the enzyme activity.

The absorption spectrum of the homogeneous acid phosphatase in 100 mM sodium acetate, pH 5.0, was characterized by a maximum near 275 nm and a shallow trough near 260 nm. The extinction coefficient of the acid phosphatase for 1% (w/v) protein at 280 nm was determined to be 5.7. Fluorescence emission spectra of the native and denatured enzyme molecule were recorded at an excitation wavelength of 282 nm. The fluorescence spectrum of the native enzyme showed a maximum near 350 nm that is characteristic of proteins that contain tryptophan. However, the fluorescence spectrum of the reduced and alkylated molecule was characterized by a shoulder near 310 nm corresponding to tyrosine emission and a maximum near 350 nm corresponding to tryptophan emission. There was a 50% loss of emission intensity upon alkylation of the enzyme. The circular dichroism spectrum of the native enzyme at pH 5 showed a minimum near 222 nm. The mean residue ellipticities below 218 nm were not calculated because the protein solution had a high absorption and consequently a relatively noisy spectrum resulted. The optical ellipticity of the enzyme near 222 nm was -5200 deg cm^2 dmol^{-1}. The latter value can be used to estimate a 17% helix content in the native molecule (Chen et al., 1974).

Antisera raised against both native and denatured enzyme were used to check the cross-reactivity with the native enzyme in quantitative immunoprecipitation experiments. Both antisera were able to immunoprecipitate the native enzyme molecule. However, the antiserum against denatured enzyme was able to precipitate only 30% of the native enzyme, suggesting that only a few of the antigenic determinants present in the denatured molecules are common to the native enzyme molecule or that the titer of the antiserum against denatured enzyme is lower. The two antisera each cross-react with a single polypeptide of 17 kDa in immunoblotting (results not shown).

Discussion

In human placenta, three different acid phosphatases with apparent molecular weights of >200K, 150K, and 35K have been characterized (DiPietro & Zengerle, 1967). However, that study did not report the presence of any acid phosphatase of molecular weight 14–23K. The present homogeneous en-

zyme preparation showed a single polypeptide chain of apparent molecular 16.9K on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Small acid phosphatases having apparent molecular weights of 14–23K have been purified from human liver (Taga & Van Etten, 1982), bovine liver (Heinrikson, 1969; Lawrence & Van Etten, 1981), heart (Chernoff & Li, 1985), and brain (Chaimovich & Nome, 1970), and rat brain (Okada et al., 1986). The pure 17-kDa acid phosphatase has an isoelectric point of 7.2, so that unlike the lysosomal and prostatic acid phosphatases (Lam et al., 1982; Taga et al., 1983), the placental 17-kDa acid phosphatase is a basic protein. The acid phosphatase that was purified to homogeneity from human placenta showed a specific activity of 106 units/mg of protein, using *p*-nitrophenyl phosphate as a substrate at pH 5 and 37 °C. The present purification scheme resulted in a high enzyme yield of 43%. The enzyme recovery may be due in part to an avoidance of the acid precipitation and several ion-exchange chromatography steps used in other purification schemes (Taga & Van Etten, 1982; Heinrikson, 1969; Lawrence & Van Etten, 1981). The enzyme preparation was very stable in 10 mM sodium acetate, pH 5.0, containing 10% glycerol, 5 mM EDTA, 10 mM phosphate, and 1 mM DTT.

Our results on the substrate specificity of the enzyme suggested that the placental acid phosphatase was able to cleave tyrosine phosphate relatively better than other naturally occurring phosphoamino acids (Table II). Therefore, we examined the phosphotyrosyl phosphatase activity of the placental acid phosphatase using three different well-characterized phosphotyrosyl proteins including human red cell band 3 (Low, 1986), angiotensin I, and calf thymus tyrosine kinase (Zioncheck et al., 1986). The homogeneous acid phosphatase catalyzed the hydrolysis of phosphate from all these phosphotyrosyl proteins. Several acid phosphatases from mammalian sources have been shown to contain phosphotyrosyl phosphatase activity (Okada et al., 1986; Li et al., 1984; Lin & Clinton, 1986; Lau et al., 1985; Chernoff & Li, 1985; Shriner & Brautigan, 1984; Apostol et al., 1985), but as yet we do not know the extent to which phosphotyrosyl phosphatases are different from the various acid and alkaline phosphatases which are also able to cleave phosphate from the phosphotyrosyl proteins (Leis & Kaplan, 1982; Swarup et al., 1982). More detailed structural comparisons of homogeneous phosphatases are necessary in order to provide complete answers to such questions.

We studied the enzymatic and physicochemical properties of the placental enzyme in order to compare the 17-kDa phosphotyrosine phosphatase with several other low molecular weight acid phosphatases. The pH optimum for hydrolysis of tyrosine phosphate and for the hydrolysis of phosphotyrosyl band 3 was pH 5–7 (Figure 3). The placental acid phosphatase possesses a high activity toward phosphotyrosyl band 3 at physiological pH. There are few reports where it has been shown that ostensible acid phosphatases exhibit neutral pH optima toward phosphotyrosyl proteins (Leis & Kaplan, 1982). As is the case with several low molecular weight acid phosphatases (Taga & Van Etten, 1982; Chernoff & Li, 1985), the placental phosphotyrosine phosphatase was significantly inhibited by fluoride or L-tartrate, although these ions are powerful inhibitors of the larger human prostatic (secretory) and human liver (lysosomal) acid phosphatases. The enzyme was strongly inhibited by vanadate, molybdate, and zinc (Tables III and IV). Such a property seems generally to be shared by phosphotyrosine phosphatases (Okada et al., 1986; Lau et al., 1985; Chernoff & Li, 1985; Shriner & Brautigan, 1984). An interesting feature of the present phosphatase was that the enzyme activity was inhibited by DNA and that the enzyme binds to a DNA–cellulose matrix at pH 5.0. The DNA-bound enzyme was eluted with 0.2 M sodium chloride. The enzyme did not bind to DNA at pH 7.5, suggesting that DNA binding to the protein molecule at pH 5.0 is due to electrostatic interactions. However, RNA did not bind to the protein at pH 5.0. At present, we do not know the reasons for this difference.

The results of sulfhydryl titration with DTNB suggested that the native enzyme molecule has four reactive sulfhydryl groups. However, only three sulfhydryls were reactive toward FMA and iodoacetate. The chemical modification of the sulfhydryls resulted in a loss of enzyme activity, but phosphate, a competitive inhibitor, was able to protect against a loss of enzyme activity. From these results, we conclude that one or more sulfhydryls are near the active site. Since the enzyme has several free sulfhydryls, we sought to determine the number of sulfhydryls actually required for enzyme activity. The results of amino acid analyses of control enzyme and enzyme treated with iodoacetate in the presence and absence of phosphate indicated that one sulfhydryl out of four is present at the active site and possibly necessary for the enzyme activity. Several low molecular weight acid phosphatases have been found to contain free sulfhydryls that are necessary for activity (Taga & Van Etten, 1982; Lawrence & Van Etten, 1981; Laidler et al., 1982; Chaimovich & Nome, 1970). The number of free, reactive sulfhydryl residues differs from previous results obtained with the bovine and human liver enzymes (Taga & Van Etten, 1982; Lawrence & Van Etten, 1981; Laidler et al., 1982), although this could be due to structural variations in the enzyme molecule. The results of chemical modification of the enzyme with diethyl pyrocarbonate in the presence and absence of phosphate suggest that histidine may also be required for enzyme activity. The role of histidine in other acid phosphatases has been carefully documented (Van Etten, 1982). From the present results, we conclude that in the 17-kDa human placental acid phosphatase, one sulfhydryl residue and one histidine are important for the enzyme activity.

Either the homogeneous enzyme is not a glycoprotein or else it possesses at most only a few sugar residues. It neither binds to concanavalin A–Sepharose nor is it stained with Schiff reagent, a glycoprotein staining reagent (Zacharius et al., 1969). The amino acid composition of the enzyme shows the presence of four cysteines and only one residue each of methionine and histidine. Another unique feature of the enzyme is that it contains 20% hydrophobic amino acids. Using the procedure of Nozaki and Tanford (1971), we were able to calculate the average hydrophobicity of the protein to be 903 cal/mol. From this result, we conclude that 17-kDa acid phosphatase is a hydrophobic protein. This observation also explains the reason for the aggregation of the protein molecule in solution at higher protein concentrations and in the presence of moderate or high salt concentrations. The presence of 10–20% glycerol in the protein solution protects the enzyme from aggregation. Protein aggregation leads to a loss of enzyme activity. Such an aggregation may be accompanied by a formation of intermolecular disulfide bonds between reactive thiol groups. However, the inactive aggregated enzyme can be fully reactivated by exposure to 4 M guanidine hydrochloride containing 10 mM DTT followed by dialysis against 10 mM sodium acetate, pH 5.0, buffer (B. Dayton and R. L. Van Etten, unpublished results).

The absorption spectrum of the enzyme in the ultraviolet region did not show any unusual features except for a very shallow trough near 260 nm. This could be due to a high content of free sulfhydryl groups because cysteine absorbs

strongly below 260 nm (Fasman, 1975). The fluorescence spectrum of the enzyme revealed a significant feature of this protein in that tyrosine fluorescence is completely quenched due to tryptophan in the native conformation. However, alkylation of the sulfhydryls with iodoacetate results in an elimination of the quenching of the tyrosine fluorescence emission by tryptophan and an overall decrease in emission intensity. From these results, we conclude that alkylation of all thiol groups of the enzyme induces an unfolding of the protein molecule. Similar features in the fluorescence emission spectra are observed when proteins are denatured by guanidine hydrochloride (Corbett et al., 1986). Circular dichroism measurements can be used to estimate that the acid phosphatase contains only about 17% helix.

Antisera against the native and the denatured enzyme molecule both cross-react with a single 17-kDa polypeptide in immunoblots. The antiserum against native enzyme cross-reacts with several other mammalian low molecular weight acid phosphatases (unpublished results). These results suggest that the enzyme is widely present and the structure is relatively conserved, at least in mammals.

In conclusion, we have purified and characterized a 17-kDa tartarate-resistant and vanadate- and zinc-sensitive acid phosphatase from human tissue. The homogeneous acid phosphatase is a hydrophobic, basic protein molecule. This placental acid phosphatase also possesses significant phosphotyrosine and phosphotyrosyl protein phosphatase activities. Elucidating the true physiological role of this enzyme will probably require extensive kinetic studies using numerous, perhaps so far uncharacterized, phosphotyrosyl protein substrates. Still, it already seems likely that information about the regulation, biosynthesis, and activity of enzymes such as this will be necessary for a full understanding of the processes such as transformation that may involve disturbances in the balance of phosphokinase and phosphohydrolase activities.

ACKNOWLEDGMENTS

We thank Harry Whatley and Prof. Michael Laskowski for amino acid analysis and Matthew McDonough for the circular dichroism spectrum.

Registry No. Acid phosphatase, 9001-77-8; phosphotyrosyl protein phosphatase, 79747-53-8; phosphotyrosine, 21820-51-9; *p*-nitrophenyl phosphate, 330-13-2; histidine, 71-00-1; fluoride, 16984-48-8; vanadate, 37353-31-4; molybdate, 11116-47-5; tungstate, 12737-86-9.

REFERENCES

Apostol, I., Kuciel, R., Wasylewska, E., & Ostrowski, W. (1985) *Acta Biochim. Pol. 32*, 187–197.

Brown, D. J., & Gordon, J. A. (1984) *J. Biol. Chem. 259*, 9580–9586.

Burnette, W. M. (1981) *Anal. Biochem. 112*, 195–203.

Chaimovich, H., & Nome, F. (1970) *Arch. Biochem. Biophys. 139*, 9–15.

Chen, Y., Yang, J. T., & Chan, H. H. (1974) *Biochemistry 13*, 3350–3359.

Chernoff, J., & Li, H.-C. (1985) *Arch. Biochem. Biophys. 240*, 135–145.

Cohen, P. (1982) *Nature (London) 296*, 613–620.

Cooper, J. A., Bowen-Pope, D. F., Raines, E., Ross, R., & Hunter, T. (1982) *Cell (Cambridge, Mass.) 31*, 263–273.

Corbett, R. J. T., Ahmad, F., & Roche, R. S. (1986) *Biochem. Cell Biol. 64*, 953–961.

DiPietro, D. L., & Zengerle, F. S. (1967) *J. Biol. Chem. 242*, 3391–3396.

Fasman, G. D. (1975) *Handbook of Biochemistry and Molecular Biology*, 3rd ed., Vol. I, pp 186–199, CRC, Cleveland, OH.

Frank, D. A., & Sartorelli, A. C. (1986) *Biochem. Biophys. Res. Commun. 140*, 440–447.

Heinrikson, R. L. (1969) *J. Biol. Chem. 244*, 299–307.

Hoy, T. G., Ferdinand, N., & Harrison, P. M. (1974) *Int. J. Pept. Protein Res. 6*, 121–140.

Hunter, T., & Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1311–1315.

Hunter, T., & Cooper, J. A. (1985) *Annu. Rev. Biochem. 54*, 897–930.

Ingebristen, T. S., & Cohen, P. (1983) *Science (Washington, D.C.) 221*, 331–338.

Johnson, D. A., Gautsch, J. W., Sportsman, J. R., & Elder, J. H. (1984) *Gene Anal. Tech. 1*, 3–8.

Kaul, R. K., Prasanna Murthy, S. N., Reddy, A. G., Steck, T. L., & Kohler, H. (1983) *J. Biol. Chem. 278*, 7981–7990.

Klarlund, J. K. (1985) *Cell (Cambridge, Mass.) 41*, 707–717.

Kyaw, A., Maung-U, K., & Toe, T. (1985) *Anal. Biochem. 145*, 230–234.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Laidler, P. M., Taga, E. M., & Van Etten, R. L. (1982) *Arch. Biochem. Biophys. 216*, 512–521.

Laidler, P. M., Waheed, A., & Van Etten, R. L. (1985) *Biochim. Biophys. Acta 827*, 73–83.

Lam, K. W., Li, C. Y., Yam, L. T., Smith, R. S., & Hacker, B. (1982) *Ann. N.Y. Acad. Sci. 390*, 1–15.

Lau, K.-H. W., Farley, J. R., & Baylink, D. J. (1985) *J. Biol. Chem. 260*, 4653–4660.

Lawrence, G. L., & Van Etten, R. L. (1981) *Arch. Biochem. Biophys. 206*, 122–131.

Leis, J. F., & Kaplan, N. O. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6507–6511.

Li, H.-C., Chernoff, J., Chen, L. B., & Kirschonbaum, A. (1984) *Eur. J. Biochem. 138*, 45–51.

Lin, M.-F., & Clinton, G. M. (1986) *Biochem. J. 235*, 351–357.

Low, P. S. (1986) *Biochim. Biophys. Acta 864*, 145–167.

Low, P. S., Allen, D. P., Zioncheck, T. F., Chari, P., Willardson, B. M., Geahlen, R. L., & Harrison, M. L. (1987) *J. Biol. Chem. 262*, 4592–4596.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lutz, R. A., & Rodbard, D. (1985) *Clin. Chem. (Winston-Salem, N.C.) 31*, 656.

Nozaki, Y., & Tanford, C. (1971) *J. Biol. Chem. 246*, 2211–2217.

Okada, M., Owada, K., & Nakagawa, H. (1986) *Biochem. J. 239*, 155–162.

Ostrowski, W., Bhargawa, A. K., Dziember, E., Gizler, M., Gryszkiewicz, J., & Bernard, E. A. (1976) *Biochim. Biophys. Acta 453*, 262.

Rehkop, D. M., & Van Etten, R. L. (1975) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 1775–1782.

Saini, M. S., & Van Etten, R. L. (1978) *Arch. Biochem. Biophys. 191*, 613–624.

Shriner, C. L., & Brautigan, D. L. (1984) *J. Biol. Chem. 259*, 11383–11390.

Swarup, G., Speg, K. V., Jr., Cohen, S., & Garbers, D. L. (1982) *J. Biol. Chem. 257*, 7298–7301.

Swarup, G., Cohen, S., & Garbers, D. L. (1984) *Biochem. Biophys. Res. Commun. 107*, 1104–1109.

Taga, E. M., & Van Etten, R. L. (1982) *Arch. Biochem. Biophys. 214*, 505–515.

Taga, E. M., Moore, D. L., & Van Etten, R. L. (1983)

Prostate (*N.Y.*) *4*, 141–150.
Van Etten, R. L. (1982) *Ann. N.Y. Acad. Sci. 390*, 27–51.
Van Etten, R. L., Waymack, P. P., & Rehkop, D. M. (1974) *J. Am. Chem. Soc. 96*, 6782–6785.
Vescia, A., & Chance, E. K. (1958) *Biochim. Biophys. Acta 30*, 446–447.
Waheed, A., Van Etten, R. L., Gieselmann, V., & Von Figura, K. (1985) *Biochem. Genet. 23*, 309–319.
Zacharius, R. M., Zell, T. E., Morrison, J. H., & Woodlock, J. J. (1969) *Anal. Biochem. 30*, 148–152.
Zioncheck, T. F., Harrison, M. L., & Geahlen, R. L. (1986) *J. Biol. Chem. 261*, 15637–15643.

Catecholate Complexes of Ferric Soybean Lipoxygenase 1†

Mark J. Nelson

Central Research and Development Department, E. I. du Pont de Nemours & Company, Inc., Wilmington, Delaware 19898

Received November 2, 1987; Revised Manuscript Received January 14, 1988

ABSTRACT: Lipoxygenases are non-heme iron enzymes that catalyze the peroxidation of unsaturated fatty acids containing 1,4-dienes to yield 1,3-diene-5-hydroperoxides. The mechanism is thought to include a ferrous–bis(allyl) radical complex as an intermediate. The complexes formed between ferric soybean lipoxygenase 1 and a series of 4-substituted catechols have been used to study the iron environment. At pH 7, substituted catechols with sufficiently electron-withdrawing substituents (e.g., 3,4-dihydroxybenzonitrile, 3,4-dihydroxybenzaldehyde) form stable complexes that are best viewed as Fe^{3+}–catechol^{2-} on the basis of the positions and number of charge-transfer bands in their visible spectra. Catechols with less electron-withdrawing substituents (e.g., catechol, 2,3-dihydroxynaphthalene) efficiently reduce the iron. More of the substituted catechols are competent to reduce the iron at pH 8, and at pH 9 all of the catechols tested reduce ferric lipoxygenase. Estimates of the pH-dependent oxidation potentials of the catechols suggest the reduction potential of the ferric ion in lipoxygenase is approximately $+0.6 \pm 0.1$ V (relative to the normal hydrogen electrode) from pH 7 to 9. These catechols are slow-binding inhibitors of lipoxygenase at pH 7, with final values of K_i between 6 and 100 μM. The magnitude of K_i does not correlate with the electron-withdrawing character of the substituents; presumably, other effects such as charge and polarity are more important.

Mononuclear non-heme iron-containing dioxygenases catalyze a variety of reactions, among which are the oxygenation of benzene and benzoic acid to yield dihydrocatechols, the oxygenation of catechols to open the aromatic ring, the α-keto acid dependent hydroxylation of, e.g., proline residues in collagen, and the peroxidation of unsaturated fatty acids to produce fatty acid hydroperoxides (Que, 1980). It is fascinating to consider that such a broad range of reactions is catalyzed in each case specifically by a reagent that superficially appears to be identical: protein-bound iron.

Lipoxygenases represent a distinct class of non-heme iron dioxygenases (Vliegenthart & Veldink, 1982; Veldink & Vliegenthart, 1984). These enzymes catalyze the reaction of oxygen with unsaturated fatty acids containing 1,4-*cis*,*cis*-diene units to yield 1,3-*cis*,*trans*-diene-5-hydroperoxides. Soybean lipoxygenase is of particular interest as an example of a metallooxygenase for which there is yet no evidence for oxygen activation at the metal (Feiters et al., 1985). To date, the best evidence suggests a mechanism involving activation of the diene by Fe^{3+} in the enzyme active site, with an intermediate bis(allyl) radical (or analogous iron-coordinated) species reacting directly with dioxygen (DeGroot et al., 1975). By comparison, the intradiol catechol dioxygenases utilize a grossly similar mechanism in which the catechol is activated toward attack by dioxygen by coordination to Fe^{3+} (Que et al., 1987), while the extradiol dioxygenases apparently activate both substrates at the metal ion (Arciero & Lipscomb, 1986).

Despite the differences in the reactions catalyzed, the Fe^{2+} in soybean lipoxygenase 1 (as isolated) shares many common features with the Fe^{2+} in protocatechuate 4,5-dioxygenase, especially as revealed in studies of their nitric oxide complexes by EPR (Nelson, 1987). However, one critical distinction in their metal sites does involve the coordination of substrates: In the extradiol dioxygenase, substrate and NO may be coordinated to the iron simultaneously, while ferrous lipoxygenase binds substrate to the exclusion of NO. To the extent that nitric oxide is an analogue of dioxygen, this implies that substrate and dioxygen are not bound simultaneously to the iron in lipoxygenase during the course of the reaction. Also, the iron in the catechol dioxygenases has phenolate ligands supplied by tyrosine residues of the protein (Que et al., 1980; Que & Epstein, 1981), but tyrosine coordination of the Fe^{3+} in lipoxygenase is ruled out by the absence of long-wavelength charge-transfer bands in the visible spectrum. The differences in reactivity between these enzymes presumably arise in large part in the differences in their iron coordination environment. Until recently, however, the precise nature of the ligand field of the iron in lipoxygenase has not been appreciated.

The positions of charge-transfer bands of Fe^{3+}–cat^{2-} complexes[1] of non-heme iron enzymes may be used to approximate the identity of the iron ligands provided by the protein (Cox et al., 1988). While this technique was applied to soybean lipoxygenase 1, the reactions between this enzyme and nine

† This is Contribution No. 4580 from the Central Research and Development Department, Du Pont.

[1] Abbreviations: catH_2, neutral catechol; catH^-, catechol monoanion; cat^{2-}, catechol dianion; sqH, neutral semiquinone; sq$^-$, semiquinone monoanion; salen, *N*,*N*′-ethylenebis(salicylideneaminato).

0006-2960/88/0427-4273$01.50/0