Purification and Properties of Pyruvate Dehydrogenase Phosphatase from Bovine Heart and Kidney†

W. Martin Teague,‡ Flora H. Pettit, Tyzz-Liang Wu, Steven R. Silberman, and Lester J. Reed*

ABSTRACT: Pyruvate dehydrogenase phosphatase was purified to apparent homogeneity from bovine heart and kidney mitochondria. The phosphatase has a sedimentation coefficient ($s_{20,w}$) of about 7.4 S and a molecular weight (M_r) of about 150 000 as determined by sedimentation equilibrium and by gel-permeation chromatography. The phosphatase consists of two subunits with molecular weights of about 97 000 and 50 000 as estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Phosphatase activity resides in the M_r 50 000 subunit, which is sensitive to proteolysis. The phosphatase contains approximately 1 mol of flavin adenine dinucleotide (FAD) per mol of protein of M_r 150 000. FAD is apparently associated with the M_r 97 000 subunit. The function of this subunit remains to be established. The phosphatase binds 1 mol of Ca^{2+} per mol of enzyme of M_r 150 000 at pH 7.0, with a dissociation constant (K_d) of about 35 μM as determined by flow dialysis. Use of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate (EGTA) at pH 7.6 in conjunction with flow dialysis gave a K_d value for Ca^{2+} of about 8 μM. In the presence of both the phosphatase and the dihydrolipoyl transacetylase (E_2) core of the pyruvate dehydrogenase complex, two equivalent and apparently noninteracting Ca^{2+}-binding sites were detected per unit of M_r 150 000, with a K_d value of about 24 μM in the absence and about 5 μM in the presence of EGTA. In the presence of 0.2 M KCl, which inhibits phosphatase activity about 95%, the phosphatase exhibited only one Ca^{2+}-binding site, even in the presence of E_2. The phosphatase apparently possesses an "intrinsic" Ca^{2+}-binding site, and a second Ca^{2+}-binding site is produced in the presence of E_2. The second site is apparently altered by increasing the ionic strength. It is proposed that the second site may be at the interface between the phosphatase and E_2, with Ca^{2+} acting as a bridging ligand for specific attachment of the phosphatase to E_2.

In eukaryotic cells, the pyruvate dehydrogenase complex is located in mitochondria, within the inner membrane–matrix compartment. The mammalian pyruvate dehydrogenase complex consists of three major components: pyruvate dehydrogenase (E_1),[1] dihydrolipoyl transacetylase (E_2), and dihydrolipoyl dehydrogenase (E_3) [for a review, see Reed (1981)]. The complex is organized about a 60-subunit core, consisting of E_2, to which multiple copies of E_1 and E_3 are bound by noncovalent bonds. The mammalian complex also contains small amounts of two regulatory enzymes, a kinase and a phosphatase, that modulate the activity of E_1 by phosphorylation (inactivation) and dephosphorylation (activation), respectively. Pyruvate dehydrogenase kinase is tightly bound to E_2 and copurifies with the complex, whereas pyruvate dehydrogenase phosphatase is loosely associated with the complex. The phosphatase is Mg^{2+} dependent and Ca^{2+} stimulated (Denton et al., 1972; Siess & Wieland, 1972; Hucho et al., 1972). In the presence of Ca^{2+}, the phosphatase binds to E_2 (Pettit et al., 1972).

In this paper, we report the purification to apparent homogeneity and some properties of pyruvate dehydrogenase phosphatase from bovine heart and kidney mitochondria.

Experimental Procedures

Materials. Poly(ethylene glycol) (M_r 6000) was obtained from J. T. Baker, sucrose (enzyme grade) was from Schwarz/Mann, Chelex-100 was from Bio-Rad Laboratories, hexokinase was from Boehringer, protamine sulfate was from Elanco Products, sodium ribonucleate and $^{45}Ca^{2+}$ were from ICN Pharmaceuticals, FAD, CNBr-activated Sepharose 4B, and sodium pyruvate (type II) were from Sigma, and [γ-^{32}P]ATP was from Amersham-Searle. D-Amino acid oxidase was furnished by Dr. Daniel Ziegler. The apooxidase was prepared as described by Burton (1955). Protein phosphatase inhibitor 1 and inhibitor 2 and porcine brain calmodulin were generous gifts from Dr. Philip Cohen and Dr. Edmond Fischer. All other reagents and materials were of the purest grade available commercially.

Pyruvate Dehydrogenase Phosphatase Assay. Assay of phosphatase activity is based on measurement of the initial rate of reactivation of phosphorylated, inactive pyruvate dehydrogenase complex from bovine kidney or heart (Linn et al., 1972). The reaction mixture contained 80 μg of phosphorylated complex, 10 mM Mops, pH 7.0, 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2 mM dithiothreitol, and 0.1–0.4 unit of phosphatase in a volume of 0.09 mL. Phosphatase was added last, the mixture was incubated at 30 °C for 1 min, and the reaction was started by addition of 0.01 mL of 0.1 M $MgCl_2$. After 2 min, a 0.02-mL aliquot was withdrawn for assay of pyruvate dehydrogenase complex activity as described (Stepp et al., 1981). One unit of phosphatase activity is defined as the amount of enzyme that reactivates 1 unit of pyruvate dehydrogenase complex per min at 30 °C. Activity of the complex is expressed as micromoles of NADH formed per minute. Protein was determined routinely by the biuret (Gornall et al., 1949) or Lowry (Lowry et al., 1951) method with crystalline serum albumin as the standard.

In certain experiments, phosphatase activity was determined by measuring the initial rate of release of ^{32}P-labeled phos-

† From the Clayton Foundation Biochemical Institute and the Department of Chemistry, The University of Texas at Austin, Austin, Texas 78712. *Received May 24, 1982.* Supported in part by Grant GM06590 from the National Institutes of Health, U.S. Public Health Service.

‡ Present address: CPC International, Inc., Argo, IL 60501.

[1] Abbreviations: E_1, pyruvate dehydrogenase; E_2, dihydrolipoyl transacetylase; E_3, dihydrolipoyl dehydrogenase; M_r, molecular weight; FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; Mops, 3-(*N*-morpholino)propanesulfonate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonate); $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; CoA, coenzyme A.

0006-2960/82/0421-5585$01.25/0

phoryl groups from enzyme complex that had been phosphorylated with [γ-^{32}P]ATP. The reaction mixture contained 78 μg of ^{32}P-labeled enzyme complex, 20 mM imidazole buffer, pH 7.0, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 mM dithiothreitol, 120 μg of bovine serum albumin, and an appropriate amount of phosphatase in a total volume of 50 μL. Phosphatase was added last, the mixture was incubated at 30 °C for 1 min, and the reaction was started by addition of 5 μL of 0.1 M $MgCl_2$. After 1.5 min, 0.25 mL of 10% trichloroacetic acid and 0.1 mL of serum albumin (25 mg/mL) were added. The mixture was centrifuged for 10 min at 13000g. A 0.2-mL sample was withdrawn, added to scintillation fluid, and counted.

Preparation of Phosphorylated Pyruvate Dehydrogenase Complex. A solution containing 1 mg of highly purified complex from bovine kidney (Linn et al., 1972), 10 mM Mops, pH 7.0, 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2 mM dithiothreitol, and 0.02 mM ATP in a total volume of 1.0 mL was incubated for 15 min at 30 °C. A 20-μL aliquot of the incubation mixture was assayed for pyruvate dehydrogenase complex activity, which should be less than 5% of the activity of the untreated complex. One micromole of glucose and 1 μg of hexokinase were added to scavenge the ATP. After 1 min at 30 °C, the preparation was placed in an ice bath.

In certain experiments, pyruvate dehydrogenase complex containing ^{32}P-labeled phosphoryl groups was used as substrate for the phosphatase. A solution containing about 16 mg of highly purified enzyme complex, 20 mM potassium phosphate, pH 7.0, 1 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, 20 mM NaF, and 0.7 mm [γ-^{32}P]ATP (200 000 cpm/nmol) was incubated for 15 min at 30 °C. NaF was included to inhibit a trace amount of pyruvate dehydrogenase phosphatase. The mixture was applied to a 1.5 × 15 cm column of Sephadex G-25 (fine) that had been equilibrated with 20 mM imidazole, pH 7.0, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM dithiothreitol at 4 °C. The complex was eluted with the same buffer. Incorporation of ^{32}P-labeled phosphoryl groups was 2.3–2.8 mol/mol of pyruvate dehydrogenase.

Coupling of Dihydrolipoyl Transacetylase to Sepharose 4B. A mixture of 15 g of CNBr-activated Sepharose 4B and 100 mL of 0.2 M $NaHCO_3$ was adjusted to pH 9.2 with NaOH. Eighty milligrams of highly purified dihydrolipoyl transacetylase from bovine heart (Linn et al., 1972; Pettit et al., 1982b) was added, and the mixture was shaken gently for 20 h. The gel was washed on a sintered glass funnel with 0.2 M $NaHCO_3$ and then with deionized water. The gel was shaken with a solution of 250 mg of bovine serum albumin in 100 mL of 0.2 M $NaHCO_3$ for 4 h and then washed well with 0.2 M $NaHCO_3$ and deionized water.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed as described by Ornstein (1964) and Davis (1964) in Tris–glycine buffer, pH 8.9. NaDodSO$_4$–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970) in 8.5% acrylamide gels and in slab gels (12.5% acrylamide). Subunit molecular weights were estimated by the procedure of Weber & Osborn (1969).

Sedimentation equilibrium analysis by the meniscus depletion technique (Yphantis, 1964) was used to determine the molecular weight of pyruvate dehydrogenase phosphatase. The interference photographs were measured with an automatic scanner (DeRosier et al., 1972), and the data were evaluated by a computer program (Munk & Halbrook, 1976).

Calcium Binding to Phosphatase. Buffers were passed through a Chelex-100 column (2.6 × 30 cm) to remove Ca^{2+}. Total calcium was measured with a Perkin-Elmer 306 atomic absorption spectrophotometer equipped with a flameless HGA70 graphite atomizer. Protein was determined by refractometry in the analytical ultracentrifuge (Babul & Stellwagen, 1969). Correction factors of 0.74 for the phosphatase and 1.09 for E_2 were applied to the results obtained by the Lowry method (Lowry et al., 1951).

The phosphatase and E_2 were dialyzed separately at 4 °C against three changes of Ca^{2+}-free buffer and then dialyzed overnight against 25 mL of buffer containing a small amount of $^{45}Ca^{2+}$ (720 mCi/nmol). Radioactivity in the sample and the dialyzate was measured, and total calcium in the dialyzate was determined by atomic absorption. The Ca^{2+} concentration in the sample was calculated from these data. The final Ca^{2+} concentration was about 2 μM.

Ca^{2+} binding was measured at 25 °C by the flow dialysis method of Colowick & Womack (1969) by using the apparatus described by Feldmann (1978). A 0.2-mL sample of protein solution containing about 400 000 cpm of $^{45}Ca^{2+}$ was placed in the upper chamber. The lower chamber was perfused with Ca^{2+}-free buffer at a flow rate of 1.9 mL/min with a LKB 2120 Varioperpex II pump. Fractions (8 drops) of the perfusate were collected directly in scintillation vials. Aliquots of nonradioactive $^{40}Ca^{2+}$ were added to the sample at every sixth fraction. The last addition was 30-fold excess of $^{40}Ca^{2+}$ over the concentration of Ca^{2+} binding sites. Ca^{2+} binding to the phosphatase was also measured by using EGTA in conjunction with flow dialysis as described by Haiech et al. (1980). The experiments were performed on $^{45}Ca^{2+}$-loaded protein to which aliquots of EGTA were added sequentially. Fischer Spectrapor dialysis tubing was boiled for 2 h in 1 mM EDTA (three changes) and then for 2 h in deionized water (three changes).

Results

Purification of Enzyme. (A) Preparation and Washing of Mitochondria. Unless specified otherwise, all operations were performed at 4 °C. About 20 lb of bovine heart was collected and chilled immediately after slaughter. After 24 h, the hearts were trimmed to remove fat and connective tissue, and the heart tissue was passed through an electric meat grinder. The ground meat was suspended in one-third volume of 0.25 M sucrose containing 10 mM potassium phosphate (pH 7.6) and 0.1 mM EDTA (sucrose–phosphate–EDTA). The pH was adjusted to 7.6 with NaOH, and the suspension (12 L) was stored overnight. The pH was adjusted to 6.8, and the suspension was passed through a continuous-flow homogenizer (Ziegler & Pettit, 1966) operated at a speed of about 2000 rpm. The pH was readjusted to 6.8, and the homogenate was centrifuged in 1-L bottles at 2000g for 10 min. For recovery of trapped mitochondria, the pellets were resuspended in an equal volume of the sucrose–phosphate–EDTA solution and processed like the original suspension. The supernatant fluids were combined and strained through eight layers of cheesecloth. The mitochondrial fraction was collected by centrifugation at 23000g (maximal centrifugal force) for 17 min in the type 15 rotor of a Beckman Model L3-40 centrifuge. The mitochondrial paste was resuspended in 4 L of deionized water with the aid of the continuous flow homogenizer. The pH was adjusted to 6.8, and the suspension was centrifuged at 22000g for 20 min. The mitochrondrial paste was washed twice with 4-L portions of 0.02 M potassium phosphate, pH 6.5; the paste was collected by centrifugation for 10 min. The yield of washed mitochondrial paste was 500–800 g (wet weight). It was resuspended with a minimal volume (about 150 mL) of 0.02 M potassium phosphate, pH 6.5. Portions (500 mL) of the suspension were shell-frozen in 4-L flasks in a −70 °C (solid CO_2–2-propanol) cooling bath. The frozen preparation was

stored at −20 °C for as long as 1 month.

(*B*) *Mitochondrial Extract.* Four batches of frozen mitochondria (from about 80 lb of heart) were thawed under running tap water. Ten milliliters of 5 M NaCl was added for every liter of suspension, and the pH was adjusted to 6.4. The suspension was passed through a Manton-Gaulin laboratory homogenizer operated at 4000 psi. The pH was adjusted to 6.2, and the mixture was centrifuged at 30000*g* for 30 min. The pellets (membrane fraction) contained 40–60% of the total phosphatase activity and were extracted as described below. The supernatant fluid was decanted carefully and warmed to 23 °C, 0.01 volume of 1 M $MgCl_2$ was added, and the mixture was stirred for 20 min to convert any phosphorylated, inactive pyruvate dehydrogenase complex to the dephosphorylated, active form.

(*C*) *Poly(ethylene glycol) Precipitation.* To the mitochondrial extract was added dropwise, with stirring, 0.1 volume of 50% (w/w) poly(ethylene glycol). The suspension was stirred for 10 min at 23 °C, and then the precipitate was collected by centrifugation for 10 min. This precipitate contained both the pyruvate dehydrogenase complex and the α-ketoglutarate dehydrogenase complex as well as pyruvate dehydrogenase phosphatase. It was resuspended, by means of a large glass homogenizer equipped with a motor-driven Teflon pestle, in 300 mL of cold 0.05 M Mops, pH 7.0, containing 1 mM $MgCl_2$, 2 mM dithiothreitol, and 0.02 mM thiamin pyrophosphate. The suspension was stored overnight in a refrigerator.

(*D*) *First Ultracentrifugation.* The suspension from the previous step was centrifuged at 40000*g* for 20 min; the precipitate was discarded. To the clear, amber supernatant fluid was added 0.01 volume of 0.2 M EGTA, pH 7.0. After 20 min, the solution was centrifuged at 105000*g* for 3.5 h in a Beckman type 30 rotor. The supernatant fluid (fraction 1) contained the phosphatase. The amber pellet contained both the pyruvate and α-ketoglutarate dehydrogenase complexes, which were purified further as described elsewhere (Pettit & Reed, 1982).

(*E*) *Extraction of Mitochondrial Membrane Fraction.* The membrane fraction was suspended in 2 L of 0.1 M Mops, pH 7.0, at 23 °C. The suspension was stirred for 1.5 h and then centrifuged at 30000*g* for 30 min at 20 °C; the pellets were discarded.

(*F*) *First Protamine Precipitation.* The supernatant fluid from the previous step (fraction 2) was cooled to 4 °C, and 0.011 volume of 2% protamine sulfate was added dropwise with stirring. After 15 min, the precipitate was collected by centrifugation at 30000*g* for 15 min; the supernatant fluid was discarded. The protamine precipitate was resuspended, with the aid of a glass–Teflon homogenizer, in 300 mL of buffer A [0.02 M Mops, pH 7.0, 5 mM $MgCl_2$ 10% (v/v) glycerol and 0.5 mM dithiothreitol] containing 0.16% yeast sodium ribonucleate. The suspension was stirred overnight, and then 0.01 volume of 0.2 M EGTA was added. The mixture was warmed at 23 °C for 20 min and then centrifuged at 30000*g* for 15 min at 20 °C. The clear, yellow supernatant fluid (fraction 3) was cooled to 4 °C; the pellets were discarded.

(*G*) *Second Protamine Precipitation.* Fraction 3 was combined with fraction 1. To this solution (fraction 4) was added dropwise, with stirring, 0.01 volume of 2% protamine sulfate. After 15 min, the precipitate was collected by centrifugation for 15 min. The protamine precipitate was extracted exactly as described in the previous step (fraction 5).

(*H*) *Second Ultracentrifugation.* Fraction 5 was centrifuged at 105000*g* for 3.5 h; the pellets were discarded.

Table I: Purification of Pyruvate Dehydrogenase Phosphatase from Bovine Heart[a]

fraction	volume (mL)	protein (mg)	sp act.[b]	recovery (%)
(1) first ultracentrifugation	260	923	8.5	
(2) membrane extract	1830	6240	2.1	
(3) first protamine precipitation	283	1190	9.5	
(4) combined fractions 1 and 3	543	2113	9.0	100
(5) second protamine precipitation	292	742	22.6	78
(6) second ultracentrifugation	280	403	36.3	69
(7) affinity chromatography	1.8	5.8	1655[c]	45

[a] From about 80 lb of heart. [b] Units of pyruvate dehydrogenase complex reactivated per minute per milligram of protein. [c] This specific activity corresponds to the release of about 2000 nmol of $^{32}P_i$ min^{-1} (mg of enzyme)$^{-1}$ from ^{32}P-labeled pyruvate dehydrogenase complex.

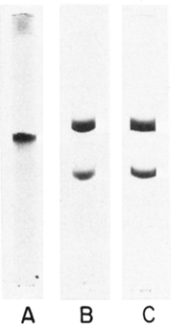

FIGURE 1: Polyacrylamide gel electrophoresis of highly purified pyruvate dehydrogenase phosphatase in the absence (A) and presence (B and C) of $NaDodSO_4$. A preparation of phosphatase from bovine heart was used in (A) and (B), and a preparation of phosphatase from bovine kidney was used in (C). Electrophoresis was performed as described by Davis (1964) with Tris–glycine buffer (A) and by Weber & Osborn (1969) in the presence of 0.1% $NaDodSO_4$ (B and C).

(*I*) *Affinity Chromatography.* To the supernatant fluid from the previous step (fraction 6) was added 0.05 volume of 0.1 M $CaCl_2$. The solution was applied slowly to a column (1.5 × 26 cm) of dihydrolipoyl transacetylase (E_2)–Sepharose 4B that had been equilibrated with buffer A containing 2 mM $CaCl_2$. The column was washed with 150 mL of this equilibration buffer. The phosphatase was eluted with buffer A containing 2 mM EGTA and 0.02% sodium azide. The active fractions were combined and concentrated by vacuum dialysis against buffer A in a Bio-Molecular Dynamics concentrator (fraction 7). A summary of the purification is presented in Table I.

Properties of Enzyme. (*A*) *Homogeneity.* Highly purified pyruvate dehydrogenase phosphatase, from both heart and kidney mitochondria, gave one band on polyacrylamide gel electrophoresis with Tris–glycine buffer in the absence of sodium dodecyl sulfate (Figure 1A). Some phosphatase preparations with equal specific activity gave a broad diffuse band. This phenomenon may be due to a tendency of the phosphatase to undergo aggregation. The sedimentation velocity pattern of highly purified phosphatase showed (Figure 2) a major component with a sedimentation coefficient ($s_{20,w}$) of about 7.4 S.

(*B*) *Molecular Weight.* Highly purified pyruvate dehydrogenase phosphatase, from both heart and kidney, gave two bands on polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (Figure 1B,C). The estimated M_r's of the two subunits are 97 000 and 50 000. No band corresponding in molecular weight to calmodulin was detected.

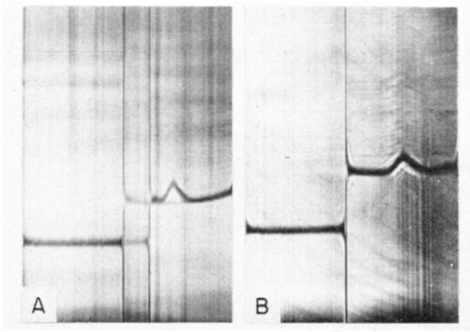

FIGURE 2: Sedimentation velocity patterns obtained with highly purified preparations of bovine kidney and heart pyruvate dehydrogenase phosphatase. (A) Kidney phosphatase (4.9 mg/mL) in 0.02 M potassium phosphate buffer, pH 7.0, and 0.5 mM dithiothreitol, after 16 min at 5 °C and 60 000 rpm. (B) Heart phosphatase (4.3 mg/mL) in 0.02 M Mops, pH 7.0, 0.5 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM EGTA after 28 min at 20 °C and 60 000 rpm.

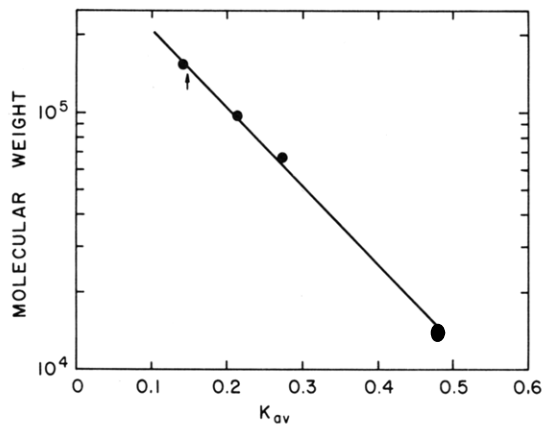

FIGURE 3: Determination of apparent molecular weight of pyruvate dehydrogenase phosphatase by gel-permeation chromatography on Sephadex G-150. Bovine kidney or heart pyruvate dehydrogenase phosphatase and protein standards in 0.5 mL were chromatographed on a 1.5 × 90 cm column of Sephadex G-150 that had been equilibrated with 20 mM phosphate buffer, pH 7.0, 0.1 M NaCl, 0.5 mM dithiothreitol, and 0.5 mM EGTA. The standard proteins and their molecular weights were as follows: mammalian pyruvate dehydrogenase, 154 000; hexokinase, 96 000; bovine serum albumin, 67 000; cytochrome *c*, 13 500. The arrow indicates the K_{av} of the phosphatase. K_{av} is calculated as $(V_e - V_0)/(V_t - V_0)$ where V_0, V_t, and V_e are void volume, total column volume, and peak elution volume, respectively.

The M_r of the intact phosphatase was estimated by gel-permeation chromatography on a calibrated Sephadex G-150 column (Figure 3). The M_r of the phosphatase was found to be about 150 000.

The M_r of the phosphatase was also determined by sedimentation equilibrium analysis. The sample contained 0.7 mg of protein/mL of 20 mM potassium phosphate buffer and 0.5 mM dithiothreitol, pH 7.0. The equilibrium run was conducted at 5 °C with a rotor speed of 12 000 rpm. The partial specific volume was taken to be 0.73 mL/g. Analysis of the data by a computer program (Munk & Halbrook, 1976) indicated heterogeneity of the sample, with a minimum weight-average molecular weight of about 140 000 and a maximum value of about 165 000. The best average value was about 150 000.

(*C*) *Specificity*. Pyruvate dehydrogenase phosphatase is inactive toward *p*-nitrophenyl phosphate. The enzyme exhibited only slight activity toward ^{32}P-labeled rabbit skeletal muscle phosphorylase *a*, i.e., about 10% of the activity observed with phosphorylated pyruvate dehydrogenase complex as substrate. Pyruvate dehydrogenase phosphatase was not inhibited by protein phosphatase inhibitor 1 or inhibitor 2 (Ingebritsen et al., 1980). Phosphatase activity was also not affected by the addition of highly purified calmodulin from porcine brain.

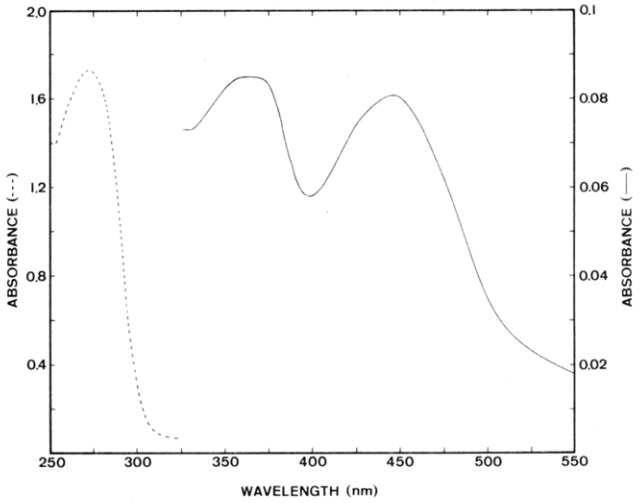

FIGURE 4: Absorption spectrum of pyruvate dehydrogenase phosphatase from bovine heart mitochondria. The solution contained 0.92 mg of phosphatase per mL of 0.02 M Mops (pH 7.0), 0.5 mM dithiothreitol, 5 mM $MgCl_2$, and 10% glycerol. The spectrum was taken with an Amico DW-2 UV/VIS spectrophotometer.

(*D*) *Identification of Chromophore*. Highly purified preparations of pyruvate dehydrogenase phosphatase exhibit a pale yellow color. The color was not removed by extensive dialysis, by gel filtration on Sephadex G-25, or by treatment with acid-washed charcoal. The absorption spectrum (Figure 4) showed maxima at 447 and 365 nm, suggesting the presence of a flavin. The chromophore was released from the phosphatase by heating for 60 s at 100 °C. Thin-layer chromatography of the extract, together with authentic samples of FAD and FMN, on cellulose plates with butanol–acetic–water (4:4:3) and 5% disodium hydrogen phosphate indicated that the chromophore was FAD (data not shown). This finding was confirmed by use of D-amino acid apooxidase with authentic FAD as the standard, in conjunction with a Clark oxygen electrode. The amount of FAD found in heat-denatured samples of highly purified pyruvate dehydrogenase phosphatase from both heart and kidney mitochondria was approximately 1 mol/mol of phosphatase of M_r 150 000.

The purified phosphatase showed no activity in a lipoamide dehydrogenase assay (Linn, 1971). The absorption spectrum in the visible region was not affected by addition of dihydrolipoamide, NADH, NADPH, glucose, glucose 6-phosphate, succinyl-CoA, butyryl-CoA, octanoyl-CoA, or palmitoyl-CoA.

The purified phosphatase contained less than 0.1 mol/mol of protein of iron, cobalt, or manganese as determined by atomic absorption measurements.

(*E*) *Limited Tryptic Digestion*. Pyruvate dehydrogenase phosphatase was subjected to limited tryptic digestion at pH 7.0 and 4 °C. Samples were removed at selected time intervals, and proteolysis was stopped by addition of excess trypsin inhibitor. Aliquots were analyzed for enzymatic activity and were examined by $NaDodSO_4$–polyacrylamide gel electrophoresis. The gel patterns (Figure 5) indicate that the small subunit, but not the large subunit, is sensitive to proteolysis under these conditions. Proteolysis of the small subunit was accompanied by loss of phosphatase activity (Figure 6).

(*F*) *Separation of Subunits*. Separation of the two phosphatase subunits with retention of enzymatic activity was

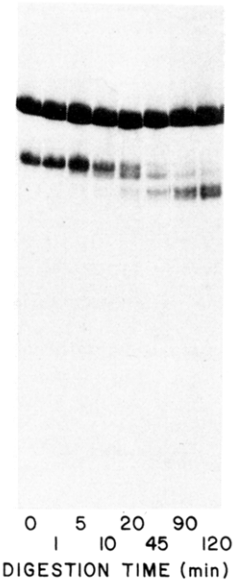

FIGURE 5: Time course of limited tryptic digestion of pyruvate dehydrogenase phosphatase. Bovine heart phosphatase (640 μg) was incubated with trypsin (6.4 μg) in 20 mM Mops, pH 7.0, 0.5 mM dithiothreitol, and 1 mM $MgCl_2$ in an ice bath. Trypsin was omitted from the control. Aliquots (15 μL) were taken at the indicated time intervals, proteolysis was stopped by addition of 2.5 μg of soybean trypsin inhibitor, and 32-μg samples were subjected to NaDod-SO_4–polyacrylamide gel electrophoresis with 12.5% acrylamide.

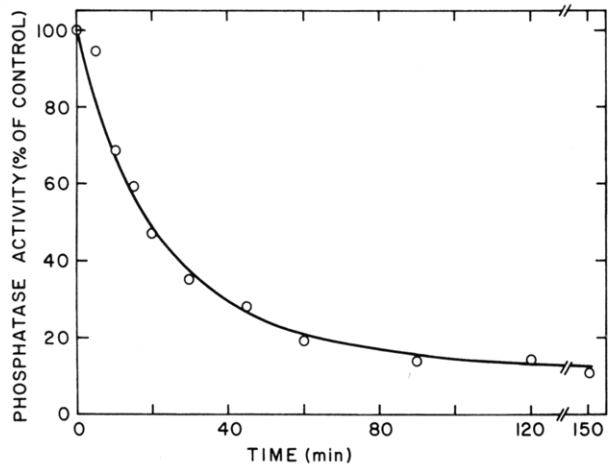

FIGURE 6: Loss of enzymatic activity accompanying limited tryptic digestion of pyruvate dehydrogenase phosphatase. Samples were the same as in Figure 5.

accomplished by treatment with 1 M KSCN followed by chromatography on Sephadex G-100 (Figure 7). FAD was released by this treatment and eluted from the column after the two protein peaks. Essentially all of the phosphatase activity was associated with peak II. Examination of fractions comprising the two peaks by NaDodSO_4 gel electrophoresis showed that peak I consisted mainly of the large subunit, whereas peak II consisted mainly of the small subunit (data not shown). Recovery of phosphatase activity in peak II varied between 45 and 90%. Phosphatase activity of peak II showed an absolute dependence on Mg^{2+} and was markedly stimulated by Ca^{2+}.

When a sample of pyruvate dehydrogenase phosphatase that had been incubated with trypsin for 3 h in an ice bath, to digest and inactivate the M_r 50 000 subunit (see Figures 5 and 6), was subjected to chromatography on Sephadex G-100, FAD was found to be associated with the M_r 97 000 subunit in peak I (data not shown). Little if any M_r 50 000 subunit was detected in peak I by NaDodSO_4 gel electrophoresis.

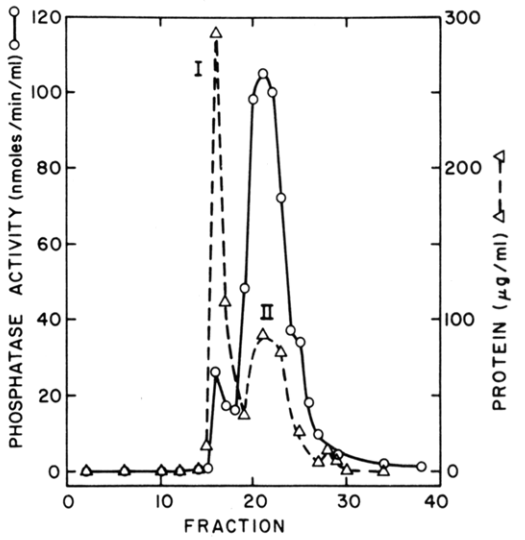

FIGURE 7: Separation of phosphatase subunits on Sephadex G-100. To a solution of 1.2 mg of heart phosphatase in 0.5 mL of buffer A was added 48.6 mg of KSCN. After 20 min at 30 °C, the solution was chromatographed on a column (1.5 × 60 cm) of Sephadex G-100 equilibrated and developed with 20 mM potassium phosphate, 0.1 mM EDTA, 2 mM dithiothreitol, and 20% glycerol, pH 7.0, at 5 °C. One-milliliter fractions were collected and analyzed for phosphatase activity and protein (Bradford, 1976). The void volume was 12 mL. The chromophore eluted in fractions 40–45.

(*G*) *Calcium Binding to Phosphatase.* Because the activity of the phosphatase is inhibited by increasing ionic strength (Siess & Wieland, 1972; Hucho et al., 1972), preliminary experiments were carried out to select an appropriate buffer and ionic strength for the Ca^{2+}-binding experiments. The effect of ionic strength on phosphatase activity appeared to be nonspecific. At $I = 0.15$, phosphatase activity was inhibited about 80%, and at $I = 0.25$, the phosphatase was essentially inactive. Among the buffers tested, Pipes proved satisfactory. At 50 mM Pipes and 0.5 mM dithiothreitol, pH 7.0, the phosphatase retained 85% of the control activity. This concentration of Pipes was sufficient to suppress nonspecific binding of Ca^{2+} to the plastic walls of the flow dialysis apparatus.

The binding isotherms for Ca^{2+} of pyruvate dehydrogenase phosphatase, dihydrolipoyl transacetylase (E_2), and a mixture of the two proteins are shown in Figure 8. E_2 alone exhibited weak, apparently nonsaturable binding of Ca^{2+}. Two or three moles of Ca^{2+} was bound per mol of E_2 of M_r 3.1×10^6 at Ca^{2+} concentrations up to 300 μM. The phosphatase, on the other hand, exhibited saturable binding of Ca^{2+}. A mixture of the two proteins bound substantially more Ca^{2+} than the individual proteins. A Scatchard plot (Scatchard, 1949) of the data (Figure 9A) showed that the free (i.e., uncomplexed) phosphatase bound one Ca^{2+} per molecule of M_r 150 000, with a dissociation constant (K_d) of about 35 μM. When both the phosphatase and E_2 were present, two equivalent and independent Ca^{2+}-binding sites were detected per unit of M_r 150 000, with a K_d value of about 24 μM. In all experiments involving a mixture of phosphatase and E_2, a molar ratio was chosen to ensure an excess of binding sites for the phosphatase on E_2. These observations indicate that the phosphatase possesses an intrinsic Ca^{2+}-binding site and that in the presence of both the phosphatase and E_2 a second high affinity Ca^{2+}-binding site is produced.

Effects of Mg^{2+} and salt concentration on Ca^{2+} binding to the phosphatase are summarized in Table II. At 1.5 mM, Mg^{2+} did not affect the stoichiometry of Ca^{2+} binding but increased the K_d for Ca^{2+} about 1.6-fold. In the presence of

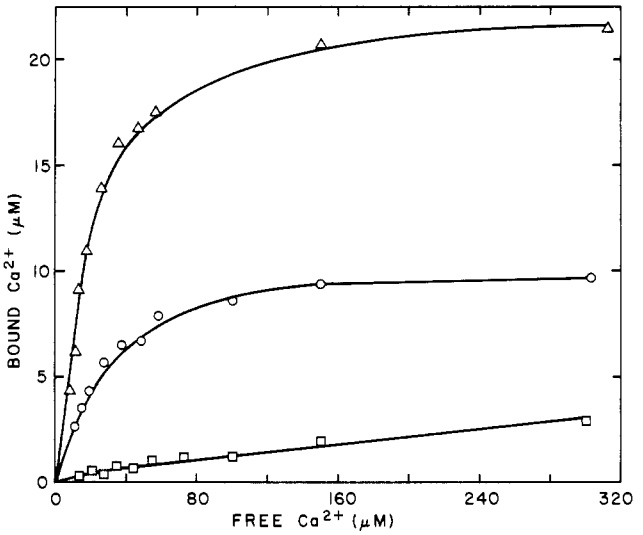

FIGURE 8: Binding of Ca^{2+} to pyruvate dehydrogenase phosphatase in the absence and presence of dihydrolipoyl transacetylase. The samples contained phosphatase (1.53 mg/mL) (○), or transacetylase (3.2 mg/mL) (□), or both proteins (△) in 0.2 mL of 50 mM Pipes buffer, pH 7.0, 0.5 mM dithiothreitol, and 2 μM $^{45}Ca^{2+}$ (400000 cpm). Ca^{2+} binding was measured by the flow dialysis procedure as described under Experimental Procedures.

0.2 M KCl, the phosphatase bound only one Ca^{2+} per molecule, even in the presence of E_2. It appears that binding of Ca^{2+} at the "intrinsic" site on the phosphatase was not affected, other than a 2-fold increase in K_d. However, the second Ca^{2+}-binding site, requiring the presence of both the phosphatase and E_2, was apparently altered.

Ca^{2+} binding to the phosphatase was also studied by using the Ca^{2+} chelator EGTA in conjunction with flow dialysis (Haiech et al., 1980). This procedure allowed rapid determination of Ca^{2+}-binding parameters without prior Ca^{2+} removal from the relatively unstable phosphatase. Phosphatase

Table II: Binding Parameters of Phosphatase for Ca^{2+}

conditions	K_d[a]	n[b]
low I, pH 7.0[c]	35 ± 3 (3)	0.92 ± 0.13
low I, pH 7.0, E_2	24 ± 1 (3)	1.75 ± 0.15
high I, pH 7.0[d]	62 ± 7 (2)	1.12 ± 0.08
low I, pH 7.0, Mg^{2+} [e]	55 (1)	0.93
low I, pH 7.0, Mg^{2+}, E_2	41 (1)	1.83
low I, pH 7.6, EGTA[f]	7.5 (1)	0.96
low I, pH 7.6, EGTA, E_2	5 (1)	2.31

[a] Dissociation constant (units are micromolar); average value and number of experiments are given. [b] Moles of Ca^{2+} bound per unit of M_r 150000. [c] I is ionic strength; 50 mM Pipes and 0.5 mM dithiothreitol, pH 7.0; E_2 is dihydrolipoyl transacetylase. [d] 50 mM Mops, 0.5 mM dithiothreitol, and 0.2 M KCl, pH 7.0. [e] 50 mM Pipes, 0.5 mM dithiothreitol, and 1.5 mM $MgCl_2$, pH 7.0. [f] 50 mM Pipes, 20 mM Mops, and 1 mM dithiothreitol, pH 7.6. Data from Figure 9B.

samples, loaded with $^{45}Ca^{2+}$, were back-titrated with EGTA in a buffer containing 50 mM Pipes, 20 mM Mops, pH 7.6, 1 mM dithiothreitol, and 0.1 mM $MgCl_2$. The apparent stability constant of the Ca^{2+}–EGTA complex at pH 7.6 was taken to be 8.13×10^7 M^{-1} (Bartfai, 1979). Scatchard plots of the data (Figure 9B) confirmed the existence of one Ca^{2+}-binding site on the uncomplexed phosphatase, with a K_d value of about 8 μM. In the presence of E_2, two equivalent and apparently noninteracting Ca^{2+}-binding sites with K_d's of about 5 μM were detected per unit of M_r 150000.

Discussion

The procedure described is applicable to purification of pyruvate dehydrogenase phosphatase from both heart and kidney mitochondria. Heart mitochondria contain at least 3 times as much phosphatase as kidney mitochondria and are the preferred source for isolation of the phosphatase. After extraction of the pyruvate dehydrogenase complex from mitochondria, 40–60% of the phosphatase is associated with the membrane fraction. The remainder of the phosphatase co-

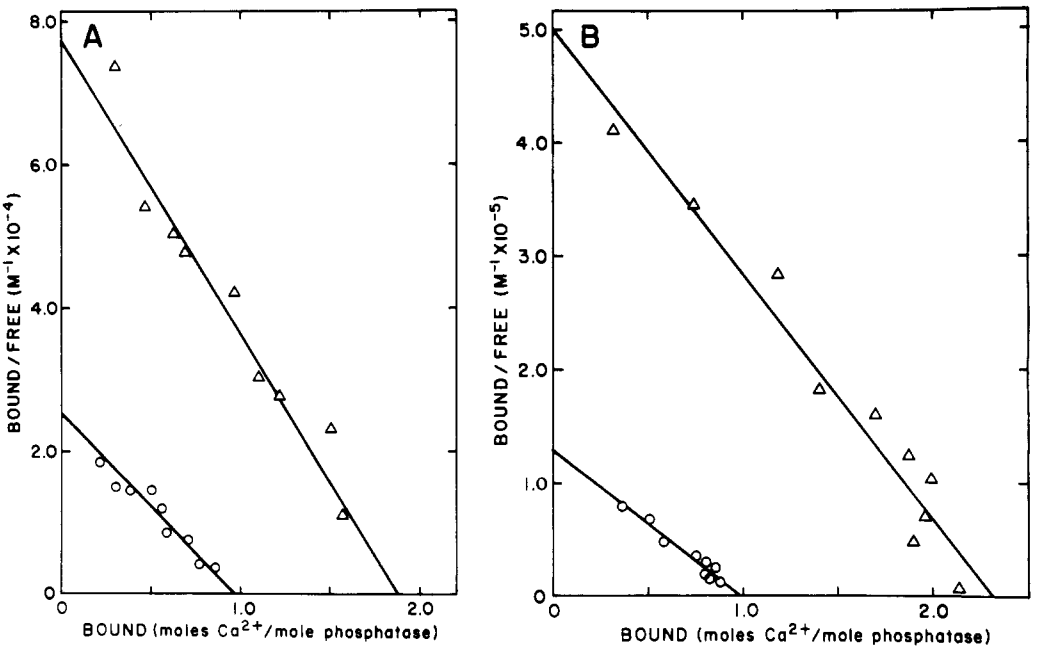

FIGURE 9: Scatchard plots of Ca^{2+} binding to pyruvate dehydrogenase phosphatase. (A) Ca^{2+} binding measured in the absence (○) and presence (△) of dihydrolipoyl transacetylase. The data points correspond to those presented in Figure 8. (B) Ca^{2+} binding measured by using EGTA in conjunction with the flow dialysis technique. The upper chamber of the flow dialysis cell contained phosphatase (1.5 mg/mL) (○) or phosphatase (0.91 mg/mL) and dihydrolipoyl transacetylase (4.62 mg/mL) (△) in 50 mM Pipes, 20 mM Mops, pH 7.6, 1 mM dithiothreitol, 0.1 mM $MgCl_2$, and 118 μM $^{45}Ca^{2+}$. Aliquots of 1 or 10 mM EGTA were added at intervals of every six fractions. The average of radioactivities in the last two fractions before each addition of EGTA, after correction for dilution and loss of radioactive calcium, was used to calculate the concentrations of free Ca^{2+} and the Ca^{2+}–EGTA complex. Other conditions were as described under Experimental Procedures.

purifies with the complex and is separated from the latter by ultracentrifugation in the presence of EGTA. This portion of the total phosphatase is combined with that extracted from the membrane fraction. A key step in the purification procedure is affinity chromatography on E_2 coupled to Sepharose 4B. In the presence of Ca^{2+}, the phosphatase binds to E_2 (Pettit et al., 1972) and is subsequently released in the presence of EGTA.

Difficulties were encountered in characterization of pyruvate dehydrogenase phosphatase due to the relatively small amount of enzyme available and to its relative instability. Nevertheless, it was possible to demonstrate by sedimentation equilibrium, gel-permeation chromatography, and $NaDodSO_4$ gel electrophoresis that this phosphatase is a heterodimer of M_r about 150000. The two subunits have M_r's of about 97000 and 50000, respectively. Evidence is presented that phosphatase activity is associated with the latter subunit, which is sensitive to proteolysis. The finding that the highly purified phosphatase contains one FAD per molecule of M_r 150000 is surprising. The FAD is apparently associated with the M_r 97000 subunit. The function of this subunit remains to be established.

Pyruvate dehydrogenase phosphatase preparations from bovine heart and kidney (Linn et al., 1972) and from pig heart (Siess & Wieland, 1972) were reported previously to have an M_r of 95000–106000 as estimated by gel-permeation chromatography on Sephadex G-100. The preparations showed a major band on $NaDodSO_4$ gel electrophoresis, with an estimated M_r of 92000–100000, and one or two minor bands. The specific activity of those phosphatase preparations was <10% of the activity of the homogeneous preparation described here. It appears that the earlier preparations were deficient in the protease-sensitive subunit of M_r 50000 and that the M_r of the active species was underestimated by gel-permeation chromatography on Sephadex G-100.

Pyruvate dehydrogenase phosphatase activity toward its protein substrate (phosphorylated E_1) and tryptic phosphopeptides derived therefrom requires Mg^{2+} or Mn^{2+} (Hucho et al., 1972; Siess & Wieland, 1972; Davis et al., 1977). Phosphatase activity toward phosphorylated E_1 is stimulated about 10-fold by Ca^{2+}, provided E_2 is present (Pettit et al., 1972). However, phosphatase activity toward phosphopeptide substrates is not affected by Ca^{2+}, whether or not E_2 is present (Davis et al., 1977). These observations indicate that Ca^{2+} is not directly involved in phosphatase catalysis. In the presence of Ca^{2+}, the phosphatase binds to E_2, and its apparent K_m for phosphorylated E_1 is decreased from about 58 μM to about 2.9 μM, with little change in V_{max}, if any (Pettit et al., 1972). We suggested that Ca^{2+} plays a structural role, perhaps by altering the conformation of the phosphatase or serving as a bridging ligand, permitting specific binding of this converter enzyme to E_2 (Pettit et al., 1972; Reed, 1974). Favorable topographical positioning of the phosphatase and phosphorylated E_1 on E_2 apparently facilitates the Mg^{2+}-dependent dephosphorylation.

To gain further insight into the role of Ca^{2+} in phosphatase action, we studied Ca^{2+} binding to the phosphatase with the flow dialysis technique (Colowick & Womack, 1969; Feldmann, 1978). This procedure requires minimal amounts of enzyme and is relatively rapid. In 50 mM Pipes, pH 7.0, the phosphatase bound approximately 1 mol of Ca^{2+} per mol of enzyme (M_r 150000) with a K_d of about 35 μM. In the presence of both the phosphatase and E_2, two equivalent and noninteracting Ca^{2+} binding sites with a K_d of about 24 μM were detected per unit of M_r 150000. In the presence of 0.2 M KCl, which produced virtually complete inhibition of phosphatase activity, the enzyme bound only one Ca^{2+} per molecule even in the presence of E_2. $^{45}Ca^{2+}$ binding to the phosphatase was also studied by using EGTA in conjunction with flow dialysis (Haiech et al., 1980). An advantage of this procedure is that it permits relatively rapid determination of Ca^{2+}-binding parameters without prior removal of $^{40}Ca^{2+}$ from the phosphatase. In 50 mM Pipes and 20 mM Mops, pH 7.6, the phosphatase exhibited one Ca^{2+}-binding site with a K_d of about 8 μM. In the presence of both the phosphatase and E_2, two equivalent and noninteracting Ca^{2+}-binding sites with a K_d of about 5 μM were detected per unit of M_r 150000. These results confirm those obtained in the absence of EGTA with respect to the number of Ca^{2+}-binding sites. The basis of the 4-fold difference in the K_d values obtained in the two sets of experiments is not clear but may be due, at least in part, to differences in pH. A pH of 7.6, rather than 7.0, was chosen for the latter experiments to ensure essentially complete association of Ca^{2+} and EGTA.

We interpret these results to indicate that the phosphatase possesses an "intrinsic" Ca^{2+}-binding site and that a second Ca^{2+}-binding site is produced when both the phosphatase and E_2 are present. The second site is apparently altered by increasing the ionic strength, with a concomitant decrease in phosphatase activity. Localization of the second Ca^{2+}-binding site remains to be established. An attractive possibility is that this second site is at the interface between the phosphatase and E_2, with Ca^{2+} acting as a bridging ligand for specific attachment of the phosphatase to E_2. Alternatively, the second Ca^{2+}-binding site may be on either the phosphatase or E_2, produced by a conformational change in either enzyme when both are present. It is interesting to note that preliminary studies on the binding stoichiometry of the phosphatase to E_2 indicate that there may be as few as 5–6 binding sites for the phosphatase on the 60-subunit E_2 (Wu, 1982).

It is of interest to compare the K_d values of the phosphatase for Ca^{2+} (5–24 μM at pH 7.0–7.6) with reported K_m values for Ca^{2+}. Using Ca–EGTA buffers and computed free Ca^{2+} concentrations, Randle et al. (1974) reported an apparent K_m for Ca^{2+} of about 0.7 μM. In the absence of EGTA, the apparent K_m value was approximately 100-fold greater. Using Ca–EGTA buffers and pyruvate dehydrogenase complex prepared by a different method, Kerbey & Randle (1979) reported K_m values for Ca^{2+} that averaged about 50 μM.

Because the pyruvate dehydrogenase complex and its two regulatory enzymes are located within the inner membrane–matrix compartment of mitochondria, changes in mitochondrial matrix free Ca^{2+} concentrations could play an important role in regulation of pyruvate dehydrogenase activity. Denton and co-workers (McCormack & Denton, 1980; Denton et al., 1980) reported a matrix free Ca^{2+} concentration of about 1 μM, based upon determination, using Ca–EGTA buffers, of the concentration of Ca^{2+} required for half-maximal stimulation of the activities of pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase complex, and NAD^+-isocitrate dehydrogenase in intact mitochondria from rat adipose tissue and heart. Similar results were reported by Hansford (1981) for intact rat heart mitochondria. However, Williamson & Murphy (1980), using metallochromic indicators and a null-point technique, reported a matrix free Ca^{2+} concentration of about 60 μM in respiring rat liver mitochondria. In view of this uncertainty, the relationship between intramitochondrial Ca^{2+} levels and regulation of pyruvate dehydrogenase complex activity remains to be established.

Although the data on substrate specificity are limited, these data, together with the mitochondrial localization of pyruvate

dehydrogenase phosphatase, its M_r of 50000, its physical association with a flavoprotein of M_r 97000, its requirement for Ca^{2+} as well as Mg^{2+} (or Mn^{2+}), its sensitivity to inactivation by trypsin, and the lack of inhibition by protein phosphatase inhibitors 1 and 2, appear to distinguish this phosphoprotein phosphatase from other well-documented phosphoprotein phosphatases (Lee et al., 1980; Cohen, 1978; Brautigan et al., 1982; Yang et al., 1980). No evidence was obtained in this investigation for an active species of M_r 35000 present in or derived from preparations of pyruvate dehydrogenase phosphatase. However, it should be noted that an M_r 35000 form of phosphorylase phosphatase from rabbit liver showed significant activity in dephosphorylating and reactivating phosphorylated pyruvate dehydrogenase complex from bovine kidney (Reed et al., 1980).

Much of the current interest in pyruvate dehydrogenase phosphatase comes from reports that insulin stimulates release from plasma membranes of a substance that increases pyruvate dehydrogenase complex activity of mitochondrial preparations (Jarett & Seals, 1979; Larner et al., 1979; Kiechle et al., 1981; Seals & Czech, 1981; Saltiel et al., 1981); this putative chemical mediator is proposed to act by stimulating pyruvate dehydrogenase phosphatase activity (Mukherjee & Jungas, 1975; Popp et al., 1980). However, the limited data presented thus far do not permit an unequivocal distinction between stimulation of pyruvate dehydrogenase phosphatase activity or inhibition of pyruvate dehydrogenase kinase activity as the cause of the insulin-mediated increase in pyruvate dehydrogenase complex activity (Pettit et al., 1982a).

Acknowledgments

We thank Debra Smith and Russel Walker for skilled technical assistance and Dr. Stephen Yeaman, Dr. Daniel Ziegler, and Dr. Marvin Hackert for advice and assistance.

References

Babul, J., & Stellwagen, E. (1969) *Anal. Biochem. 28*, 216–221.

Bartfai, T. (1979) *Adv. Cyclic Nucleotide Res. 10*, 219–242.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brautigan, D. L., Ballou, L. M., & Fischer, E. H. (1982) *Biochemistry 21*, 1977–1982.

Burton, K. (1955) *Methods Enzymol. 2*, 199–204.

Cohen, P. (1978) *Curr. Top. Cell. Regul. 14*, 117–196.

Colowick, S. P., & Womack, F. C. (1969) *J. Biol. Chem. 244*, 774–777.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Davis, P. F., Pettit, F. H., & Reed, L. J. (1977) *Biochem. Biophys. Res. Commun. 75*, 541–549.

Denton, R. M., Randle, P. J., & Martin, B. R. (1972) *Biochem. J. 128*, 161–163.

Denton, R. M., McCormack, J. G., & Edgell, R. M. (1980) *Biochem. J. 190*, 107–117.

DeRosier, D. J., Munk, P., & Cox, D. J. (1972) *Anal. Biochem. 50*, 139–153.

Feldmann, K. (1978) *Anal. Biochem. 88*, 225–235.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766.

Haiech, J., Vallet, B., Aquaron, R., & Demaille, J. G. (1980) *Anal. Biochem. 105*, 18–23.

Hansford, R. G. (1981) *Biochem. J. 194*, 721–732.

Hucho, F., Randall, D. D., Roche, T. E., Burgett, M. W., Pelley, J. W., & Reed, L. J. (1972) *Arch. Biochem. Biophys. 151*, 328–340.

Ingebritsen, T. S., Foulkes, J. G., & Cohen, P. (1980) *FEBS Lett. 119*, 9–15.

Jarett, L., & Seals, J. R. (1979) *Science (Washington, D.C.) 206*, 1407–1408.

Kerbey, A. L., & Randle, P. J. (1979) *FEBS Lett. 108*, 485–488.

Kiechle, F. L., Jarett, L., Kotagal, N., & Popp, D. A. (1981) *J. Biol. Chem. 256*, 2945–2951.

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

Larner, J., Galasko, G., Cheng, K., DePaoli-Roach, A. A., Huang, L., Daggy, P., & Kellogg, J. (1979) *Science (Washington, D.C.) 206*, 1408–1410.

Lee, E. Y. C., Silberman, S. R., Ganapathi, M. K., Petrovic, S., & Paris, H. (1980) *Adv. Cyclic Nucleotide Res. 13*, 95–131.

Linn, T. C. (1971) *Arch. Biochem. Biophys. 161*, 505–514.

Linn, T. C., Pelley, J. W., Pettit, F. H., Hucho, F., Randall, D. D., & Reed, L. J. (1972) *Arch. Biochem. Biophys. 148*, 327–342.

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

McCormack, J. G., & Denton, R. M. (1980) *Biochem. J. 190*, 95–105.

Mukherjee, C., & Jungas, R. L. (1975) *Biochem. J. 148*, 229–235.

Munk, P., & Halbrook, M. E. (1976) *Macromolecules 9*, 568–574.

Ornstein, L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321–349.

Pettit, F. H., & Reed, L. J. (1982) *Methods Enzymol. 89*, 376–386.

Pettit, F. H., Roche, T. E., & Reed, L. J. (1972) *Biochem. Biophys. Res. Commun. 49*, 563–571.

Pettit, F. H., Humphreys, J., & Reed, L. J. (1982a) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3945–3948.

Pettit, F. H., Yeaman, S. J., & Reed, L. J. (1982b) *Methods Enzymol.* (in press).

Popp, D. A., Kiechle, F. L., Kotagal, N., & Jarett, L. (1980) *J. Biol. Chem. 255*, 7540–7543.

Randle, P. J., Denton, R. M., Pask, H. T., & Severson, D. L. (1974) *Biochem. Soc. Symp. No. 39*, 75–87.

Reed, L. J. (1974) *Acc. Chem. Res. 7*, 40–56.

Reed, L. J. (1981) *Curr. Top. Cell. Regul. 18*, 95–106.

Reed, L. J., Pettit, F. H., Yeaman, S. J., Teague, W. M., & Bleile, D. M. (1980) in *Enzyme Regulation and Mechanism of Action* (Mildner, P., & Ries, B., Eds.) pp 47–46, Pergamon Press, Oxford.

Saltiel, A., Jacobs, S., Siegel, M., & Cuatrecasas, P. (1981) *Biochem. Biophys. Res. Commun. 102*, 1041–1047.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Seals, J. R., & Czech, M. P. (1981) *J. Biol. Chem. 256*, 2894–2899.

Siess, E. A., & Wieland, O. H. (1972) *Eur. J. Biochem. 26*, 96–105.

Stepp, L. R., Bleile, D. M., McRorie, D. K., Pettit, F. H., & Reed, L. J. (1981) *Biochemistry 20*, 4550–4560.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Williamson, J. R., & Murphy, E. (1980) *Adv. Exp. Med. Biol. 132*, 671–688.

Wu, T. L. (1982) Doctoral Dissertation, University of Texas at Austin, Austin, TX.

Yang, S. D., Vandenheede, J. R., Goris, J., & Merlevede, W. (1980) *J. Biol. Chem. 255*, 11759–11767.

Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.

Ziegler, D. M., & Pettit, F. H. (1966) *Biochemistry 5*, 2932–2938.