Acad. Sci. U.S.A. 80, 999–1002.
Johnson, V. G., Greenwalt, D. E., Madara, P. J., & Mather, I. H. (1988) *Biochem. J. 251*, 507–514.
Keenan, T. W., Mather, I. H., & Dylewski, D. P. (1988) in *Fundamentals of Dairy Chemistry* (Wong, N. P., Ed.) pp 511–582, Van Nostrand Reinhold, New York.
Kieffer, N., Bettaieb, A., Legrand, C., Coulombel, L., Vainchenker, W., Edelman, L., & Breton-Gorius, J. (1989) *Biochem. J. 262*, 835–842.
Laemmli, U. K. (1965) *Nature 227*, 680–685.
Mangino, M. E., & Brunner, J. R. (1977) *J. Dairy Sci. 60*, 841–850.
Mather, I. H. (1987) in *The Mammary Gland: Development, Regulation and Function* (Neville, M. C., & Daniel, C. W., Eds.) pp 217–267, Plenum, New York.
Mather, I. H., & Keenan, T. W. (1983) in *Biochemistry of Lactation* (Mepham, T. B., Ed.) pp 231–283, Elsevier, Amsterdam.
Mather, I. H., Tamplin, C. B., & Irving, M. G. (1980) *Eur. J. Biochem. 110*, 327–336.
McGregor, J. L., Catimel, B., Parmentier, S., Clezardin, P., Dechavanne, M., & Leung, L. L. K. (1989) *J. Biol. Chem. 264*, 501–506.
Ockenhouse, C. F., Tandon, N. N., Magowan, C., Jamieson, G. A., & Chulay, J. D. (1989a) *Science 243*, 1469–1471.
Oquendo, P., Hundt, E., Lawler, J., & Seed, B. (1989) *Cell 58*, 95–101.
Peixoto de Menezes, A., & Pinto da Silva, P. (1978) *J. Cell Biol. 76*, 767–778.
Sage, H., Farin, F. M., Striker, G. E., & Fischer, A. B. (1983) *Biochemistry 22*, 2148–2155.
Shaw, S. (1987) *Immunol. Today 8*, 1–3.
Shimizu, M., & Yamauchi, K. (1982) *J. Biochem. (Tokyo) 91*, 515–524.
Silverstein, R. L., Asch, A. S., & Nachman, R. L. (1989) *J. Clin. Invest. 84*, 546–552.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.
Sullivan, C. H., Mather, I. H., Greenwalt, D. E., & Madara, P. J. (1982) *Mol. Cell. Biol. 44*, 13–22.
Tandon, N. N., Lipsky, R. H., Burgess, W. H., & Jamieson, G. A. (1989a) *J. Biol. Chem. 264*, 7570–7575.
Tandon, N. N., Kralisz, U., & Jamieson, G. A. (1989b) *J. Biol. Chem. 264*, 7576–7583.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Wilkner, N. F., Dixit, V. M., Frazier, W. A., & Clark, R. A. F. (1987) *J. Invest. Dermatol. 88*, 207–211.

Purification and Characterization of the *Tetrahymena pyriformis* P–C Bond Forming Enzyme Phosphoenolpyruvate Phosphomutase†

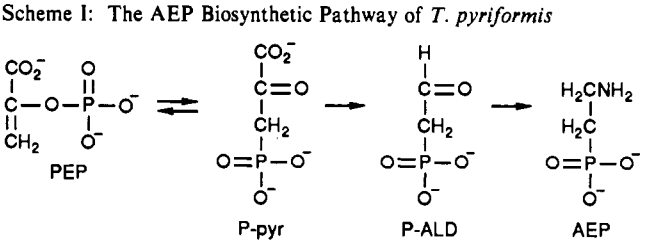
Elise D. Bowman, Michael S. McQueney, Jeffrey D. Scholten, and Debra Dunaway-Mariano*
Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742
Received March 2, 1990; Revised Manuscript Received April 18, 1990

ABSTRACT: In this paper the purification and characterization of the *Tetrahymena pyriformis* enzyme phosphoenolpyruvate phosphomutase are described. PEP phosphomutase was first fractionated from *T. pyriformis* cellular extract by using 70% ammonium sulfate. Chromatography of the crude protein fraction on a DEAE-cellulose column followed by phenyl-Sepharose column chromatography and then Bio-Gel P-200 column chromatography afforded pure PEP phosphomutase in an approximate overall yield of 70 units/150 g of cells. The maximum turnover number observed for PEP phosphomutase catalysis of the phosphonopyruvate → PEP reaction is 38 s^{-1} (25 °C). The enzyme was shown to be a homodimer of 38 000-dalton subunits and to require a divalent metal ion for activity. Mg^{2+} (relative V_m = 1), Co^{2+} (rel V_m = 0.5), Zn^{2+} (rel V_m = 0.4), and Mn^{2+} (rel V_m = 0.3) each satisfied the cofactor requirement. Binding of the physiological cofactor, Mg^{2+} (K_i = 0.3 mM at pH 7.5), and phosphonopyruvate (K_m = 2 μM at pH 7.5) was found to be ordered, with cofactor binding preceding substrate binding. Within the pH range of 5–9 catalysis (V_m) was found to be pH independent, while phosphonopyruvate binding dropped at acidic and basic pH.

Following the first discovery of a P–C bond containing natural product that took place over 30 years ago (Horiguchi & Kandatsu, 1959) the presence of phosphonates and phosphinates in a variety of biological systems has been firmly established [for reviews on this topic see Hori et al. (1984), Hilderbrand (1983), and Mastelerz (1984)]. To this day, however, we know very little about how these reduced phosphorus compounds are synthesized, how they are degraded, and how they serve the organisms in which they are found.

Recently, our laboratory set out to investigate the modes of biosynthesis and biodegradation of this interesting class of natural products. In an earlier paper (Barry et al., 1988) we reported on the steps of the biosynthetic pathway which lead, in *Tetrahymena pyriformis*, to the most ubiquitous of the phosphonate natural products, (2-aminoethyl)phosphonate (AEP).[1] We had found, true to an early hypothesis (Warren,

Scheme I: The AEP Biosynthetic Pathway of *T. pyriformis*



†This work was supported by NIH Grant GM 28688.
* Author to whom correspondence should be addressed.

0006-2960/90/0429-7059$02.50/0

1968; Trebst & Geike, 1967), that the P–C bond present in AEP is formed by the rearrangement of the high-energy phosphate ester phosphoenolpyruvate (PEP) to phosphonopyruvate (P-pyr) (see Scheme I). Importantly, this novel P–C bond forming reaction does not appear to be unique to *T. pyriformis* but has also been detected as a key step in the elaborate biosynthetic pathway of *Streptomyces hygroscopicus* that leads to the antibiotic bialaphos (Seto, 1986; Anzai et al., 1987). In the present paper we extend our earlier communication on the discovery of PEP phosphomutase (EC 5.4.2.9) in *T. pyriformis* (Bowman et al., 1988) to present the purification and physical and kinetic properties of this novel P–C bond forming enzyme.

Materials and Methods

Cell Culture. *T. pyriformis* (W), obtained from the American Type Culture Center (ATCC No. 151905), was grown at 29 °C in sterilized medium containing bactopeptone (20 g/L), yeast extract (5 g/L), dextrose (2 g/L), NaCl (0.1 g/L), KCl (4 mg/L), and $CaCl_2$ (8 mg/L). Five-milliliter, 4-day-old stock cultures were used to inoculate 125-mL portions of media. After 2 days of mixing on a gyratory shaker (110 rpm) the 125-mL cultures were used to inoculate 3-L portions of fresh media in 6-L flasks. After 3–4 days of growth (at which point the absorbance of the culture at 530 nm had reached 1 OD unit) the cells were harvested by centrifugation (10 min at 6000 rpm). Approximately 5 g of cells were obtained per liter of culture.

Cell Lysis and Fractionation. One hundred twenty grams of *Tetrahymena* cells were suspended in 500 mL of suspension buffer containing 50 mM Hepes (pH 7.5), 1 mM EDTA, 50 μM phenylmethanesulfonyl fluoride, 50 μg/mL trypsin inhibitor (type II-S, Sigma Chemical Co.), 1 mM 1,10-phenanthroline, 0.4% (w/v) bovine serum albumin, 1 mM benzamidine hydrochloride, and 10 mM 2-mercaptoethanol. The cell suspension was passed through a French press at 16 000 psi and then centrifuged at 17000*g* and 4 °C for 10 min. Streptomycin sulfate (1.5% w/v) was added to the supernatant, and the resulting mixture was stirred for 30 min at 4 °C and then centrifuged (10 min, 17000*g*). Ammonium sulfate was added to the supernatant to give a final concentration of 45% (w/v). The precipitate formed was removed by centrifugation, and ammonium sulfate was added to the supernatant fraction to give the final concentration of 70% (w/v). The resulting protein precipitate was collected by centrifuging the mixture at 17000*g* for 30 min.

Purification of PEP Phosphomutase. (*A*) *DEAE-Cellulose Chromatography.* Six grams of crude protein pellet (see above) was dissolved in buffer (50 mM triethanolamine, 5 mM $MgCl_2$, and 0.5 mM DTT, pH 7.5) and dialyzed for 9 h at 4 °C against 2 L of buffer (which was replaced with fresh buffer every third hour). The remaining purification steps were all carried out at 4 °C. The dialyzed protein was loaded onto a 22 cm × 3.5 cm DEAE-cellulose column (Sigma Chemical Co.) that had been equilibrated with 50 mM triethanolamine (pH 7.5) containing 5 mM $MgCl_2$ and 0.4 mM DTT. Isocratic elution of the PEP phosphomutase from the column was carried out with the equilibration buffer.

(*B*) *Phenyl-Sepharose Chromatography.* The PEP phosphomutase active fractions obtained from the DEAE-cellulose chromatography were made 25% w/v in ammonium sulfate and then applied to a 26 × 2.5 cm phenyl-Sepharose (CL-4B, Sigma Chemical Co.) column equilibrated with 50 mM triethanolamine (pH 7.5) containing 25% (w/v) ammonium sulfate, 5 mM $MgCl_2$, and 0.5 mM DTT. The column was eluted with a 1.5-L linear gradient of 25%–0% ammonium sulfate [in 50 mM triethanolamine (pH 7.5), 5 mM $MgCl_2$, and 0.5 mM DTT].

(*C*) *Bio-Gel P-200 Chromatography.* The PEP phosphomutase containing fractions obtained from the phenyl-Sepharose chromatography were pooled, concentrated to 10 mL with an Amicon ultrafiltration apparatus, and then loaded onto a 90 × 4.5 cm Bio-Gel P-200 (Bio-Rad Chemical Co.) column. The column was eluted at 4 °C with 50 mM triethanolamine (pH 7.5) containing 5 mM $MgCl_2$ and 0.5 mM DTT. The PEP phosphomutase containing fractions were made 10% (w/v) in glycerol and frozen in a dry ice–acetone bath before storing at −20 °C.

Enzyme Assay. Where noted, the PEP phosphomutase activity was measured by using a direct spectrophotometric assay wherein the increase in the absorptivity of the reaction solution resulting from the conversion of phosphonopyruvate (Anderson et al., 1984) to PEP was monitored at 233 nm ($\Delta\epsilon$ = 1500 at pH 7.5). Otherwise, the pyruvate kinase/NADH–LDH coupled spectrophotometric assay was employed wherein the decrease in the absorbance (at 340 nm; $\Delta\epsilon$ = 6220) of a 3-mL assay solution, containing PEP phosphomutase and phosphonopyruvate in 50 mM K^+Hepes (pH 7.5), 0.2 mM NADH, 40 units/mL pyruvate kinase, 20 units/mL lactate dehydrogenase, and specified concentrations of $MgCl_2$ and ADP, was monitored.

PEP Phosphomutase Molecular Weight and Amino Acid Composition Determination. The molecular weight of the native enzyme was estimated by using size exclusion chromatography on a Blue Dextran 2000 calibrated Sephadex G-100 column [1.8 × 1000 cm; 50 mM triethanolamine (pH 7.5), 0.5 mM DTT, and 5 mM $MgCl_2$ as eluant]. The molecular weight of the PEP phosphomutase was estimated from the plot of retention volume vs log (molecular weight) of the protein molecular weight standards. Ribonuclease A, chymotrypsinogen A, ovalbumin, and bovine serum albumin (MW = 13 700, 25 000, 43 000, and 67 000, respectively) were used as molecular weight markers. The molecular weight of denatured PEP phosphomutase was measured by using SDS–PAGE techniques (7% polyacrylamide slab gel). The PEP phosphomutase was chromatographed in 200 mM phosphate buffer (pH 7.0) containing 0.1% SDS along with the protein molecular weight standards. The molecular weight of denatured PEP phosphomutase was determined from a plot of R_f vs log (molecular weight) of the protein standards. The amino acid analysis of the purified protein was performed at the Protein/DNA Facility at the University of Maryland.

Kinetic Mechanism. The initial velocity of the PEP phosphomutase catalyzed conversion of phosphonopyruvate to PEP was measured at pH 7.5 as a function of the concentration of phosphonopyruvate and uncomplexed Mg^{2+}. PEP formation was monitored by using the pyruvate kinase/lactate dehydrogenase coupled assay. All reaction cells contained 0.2 mM ADP, 0.2 mM NADH, and 50 mM K^+Hepes (pH 7.5). The concentration of free Mg^{2+} in each reaction was calculated from the amount of $MgCl_2$ added minus the amount of Mg^{2+} complexed to the ADP (K_s for MgADP = 12.9 mM^{-1}; Pecoraro et al., 1984). The initial velocity data obtained were

[1] Abbreviations: AEP, (2-aminoethyl)phosphonate; PEP, phosphoenolpyruvate; P-pyr, phosphonopyruvate; DTT, dithiothreitol; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LDH, lactate dehydrogenase; NADH, dihydronicotinamide adenine dinucleotide; ADP, adenosine 5′-diphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

Table I: Purification of Phosphoenolpyruvate Phosphomutase

step	total protein[a] (mg)	total units[b] (mol/min)	specific activity [mol/(min·mg of protein)]	% yield	purification (x-fold)
extract from 150 g of cells	13200[c]	420	0.032	100	
15% (w/o) streptomycin, sulfate	12270	386	0.030	92	0.9
ammonium sulfate fraction (45–70%)	6060	291	0.048	69	1.5
DEAE-cellulose column	2500	204	0.082	49	2.6
phenyl-Sepharose column	25.9	145	5.60	35	175
Bio-Gel P-200 column	3.7	66	22.4	16	700

[a] Determined from A_{280} (1 = 1 mg/mL). [b] Determined as described under Materials and Methods. [c] Approximately 15% of this weight results from the BSA added to the cells prior to lysing them in the French press (at 16000 psi).

analyzed according to eq 1 (where V_0 = initial velocity, V_m = maximal velocity, K_B = Michaelis constant for B, K_{iA} = dissociation constant of A, and [A] and [B] refer to the concentrations of the reactants) by using the Fortran computer program EQORDO (Cleland, 1979).

$$V_0 = \frac{V_m[A][B]}{K_B[A] + [A][B] + K_{iA}K_B} \quad (1)$$

pH Dependency of the PEP Phosphomutase Catalyzed Reaction. Initial velocity data were measured at 25 °C as a function of phosphonopyruvate concentration at a fixed, saturating concentration of Mg^{2+} (8 mM) by using the coupled assay (2 mM ADP, 0.4 mM NADH, 40 units/mL pyruvate kinase, 30 units/mL lactate dehydrogenase, and 100 mM K^+Mes, K^+Hepes, or K^+Ches). The apparent pK_a values of the ionizing groups were calculated by using eq 2 (where $Y = V_m$ or V_m/K_m, c = pH-independent value of Y, and K_1 and K_2 = dissociation constants of groups that ionize) and the BELL computer program (Cleland, 1979).

$$\log Y = \log [c/(1 + H/K_1 + K_2/H)] \quad (2)$$

Results

Solubilization of PEP Phosphomutase from T. pyriformis Membranes. T. pyriformis cells, harvested during log-phase growth, were lysed in the presence of BSA and protease inhibitors with a tissue homogenizer. Centrifugation of the homogenate at 17000*g* divided the phosphomutase activity almost equally between the pellet and supernatant fractions. Centrifugation of the supernatant fraction at 110000*g* to separate the microsomal fraction resulted in ca. 20% loss of activity to the pellet. On the basis of the observed partitioning of phosphomutase activity between membrane and supernatant fractions we surmised that the phosphomutase is loosely associated with the cellular membrane and we, therefore, sought an efficient method to dislodge it. We found that further homogenization of the 17000*g* pellet in the presence of 0.1% Triton X-100 solubilized 60% of the enzyme activity present. Fortunately, the use of detergent to extract the mutase activity from the membrane could be avoided by using a French press rather than a tissue homogenizer to lyse the cells. Specifically, opening the cells under pressure (16000 psi) solubilized 85% of the phosphomutase activity.

Purification and Characterization of the PEP Phosphomutase. Purification of PEP phosphomutase which had been precipitated from the cellular extract (French press) with 70% ammonium sulfate was attempted on DEAE-, CM-, and phosphate–cellulose columns, and on hydroxylapatite, phenyl-Sepharose, butyryl-Sepharose, acridine dye, Sephadex 100, and Bio-Gel P-200 columns. The phosphomutase was not retained by the CM- or phosphate–cellulose columns, nor did it bind tightly to the DEAE-cellulose column. In contrast, the phosphomutase was tightly absorbed by the hydroxylapatite column. Unfortunately, our attempts to elute the phosphomutase from this column with a phosphate gradient or with NaCl in phosphate buffer resulted in relatively pure, but inactive enzyme.[2] Of the hydrophobic absorbants, the phenyl-Sepharose was found most effective in fractionating the phosphomutase, and of the two gel filtration resins, Bio-Gel P-200 produced the best results.

Table II: Amino Acid Composition of PEP Phosphomutase As Determined with an Automated Amino Acid Analyzer

amino acid	no. of residues/ 38000 Da	amino acid	no. of residues/ 38000 Da
His	5	Val	24
Arg	13	Met	5
Lys	25	Ile	18
Asx	34	Leu	26
Glx	21	Pro	16
Ser	12	Cys	6
Thr	12	Phe	19
Gly	34	Tyr	18
Ala	38	Trp[a]	11

[a] Determined according to the method of Bencze and Schmid (1957).

The purification scheme found to be most effective for PEP phosphomutase purification is summarized in Table I. Enzyme prepared in this manner migrated as a single protein band on SDS–PAGE gels and most typically showed a specific activity in the range of 20–30 units/mg of protein, with the highest activity observed at 60 units/mg.[3] After quick freezing in a buffered solution (pH 7.5) containing 10% glycerol (v/v), 50 mM triethanolamine, 0.5 mM DTT, and 5 mM $MgCl_2$, the phosphomutase could be stored at −20 °C without causing significant loss of activity (<20% activity loss over a 6-week period).

The molecular weight of the denatured phosphomutase was determined by SDS–PAGE analysis to be ~38000. The molecular weight of the native protein, a homodimer, was determined by using gel filtration techniques to be ~81000 [in reasonable agreement with the molecular weight of 69000 reported by Seidel et al. (1988)]. The amino acid composition of the phosphomutase, based on a subunit weight of 38000 daltons, is shown in Table II. For the most part, the amino acid composition conforms to that of the "average" protein (Klapper, 1977) with the exception of the hydroxylic amino acids (Ser and Thr), which are present in half the expected amount, and the aromatic residues (Phe and Trp), which are present in twice the expected amount.

PEP Phosphomutase Kinetic Mechanism. Mg^{2+} was found to be a required cofactor for PEP phosphomutase. The kinetic

[2] Seidel et al. (1988) reported the effective use of hydroxylapatite column chromatography in the purification of PEP phosphomutase. Details of the chromatography were not presented in their communication, so it is unclear at this time as to why their findings are different from our own.

[3] Seidel et al. (1988) reported a specific activity of 74 units/mg.

Table III: Kinetic Constants for PEP Phosphomutase Metal Ion Activation[a]

metal ion	$V_m^{M^{2+}}/V_m^{Mg^{2+}}$	K_m (μM)
Mg^{2+}	1.0	5.8 ± 0.8
Mn^{2+}	0.3	7 ± 1
Co^{2+}	0.5	2 ± 1
Zn^{2+}	0.4	7 ± 1

[a] Reaction solutions contained 100 μM phosphonopyruvate in 100 mM K^+Hepes (pH 7.5, 25 °C).

constants for the formation and turnover of the enzyme·Mg^{2+}·phosphonopyruvate complex were determined by measuring the initial velocity of the catalyzed reaction as a function of Mg^{2+} and phosphonopyruvate concentration. A double-reciprocal plot of the initial velocity vs Mg^{2+} concentration at four different levels of phosphonopyruvate intersected at the left of the $1/v$ axis. On the other hand, the same data plotted as a function of phosphonopyruvate concentration intersected on the $1/v$ axis, suggesting that Mg^{2+} binds to the enzyme before phosphonopyruvate (Dixon & Webb, 1979).

Computer-aided analysis of the initial velocity data according to eq 1, which describes a rapid equilibrium ordered reaction, allowed us to calculate the following kinetic constants for the phosphomutase reaction at pH 7.5 and 25 °C: (trail I) $V_m = 13.0 \pm 0.6$ s^{-1}, $K_i^{Mg} = 300 \pm 200$ μM, $K_m^{P\text{-}pyr} = 2 \pm 1$ μM; (trail II) $V_m = 20 \pm 1$ s^{-1}, $K_i^{Mg} = 300 \pm 100$ μM, $K_m^{P\text{-}pyr} = 4 \pm 2$ μM.

Metal Ion Specificity of PEP Phosphomutase. Metal ion activation of PEP phosphomutase was measured in the presence of 100 μM phosphonopyruvate and 100 mM K^+Hepes (pH 7.5, 25 °C) by using the direct spectrophotometric assay. The V_m and apparent K_m values, measured for the Mg^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+} and presented in Table III, reveal a low degree of specificity of the enzyme toward these metal ions.

pH Dependence of PEP Phosphomutase Catalysis. The kinetic constants for the phosphomutase reaction were evaluated at acidic, neutral, and alkaline pH. Because of the difficulty encountered in measuring meaningful initial velocity data for reactions at alkaline or acid pH which contained low levels of both substrate and cofactor, a full kinetic analysis, of the type described above, was deemed impossible. The V_m and V_m/K_m values were, instead, determined from initial velocity data measured as a function of phosphonopyruvate concentration at a saturating level of Mg^{2+}. The V_m and V_m/K_m pH profiles are shown in Figure 1. Analysis of the V_m/K_m pH data with eq 2 gave apparent pK_a values for the ionizing groups of 6.2 ± 0.1 and 8.4 ± 0.1.

Discussion

While the presence of PEP phosphomutase in *T. pyriformis* had been suspected for many years (Warren, 1968), the actual discovery of this enzyme in cell extracts awaited the discovery (Barry et al., 1988) that the solution equilibrium position of the PEP ⇌ phosphonopyruvate reaction heavily favors PEP (i.e., by at least 500:1; Bowman et al., 1988). Once an assay for enzyme activity that was based on PEP production from phosphonopyruvate was applied, isolation of the enzyme from *T. pyriformis* (Bowman et al., 1988; Seidel et al., 1988) as well as from *S. hygroscopicus* (Hidaka et al., 1988) soon followed.

In developing a procedure for the purification of the PEP phosphomutase from *T. pyriformis*, we found that it was necessary to first dislodge the enzyme from the cellular membrane. The most convenient way to accomplish this was to break open the cells under high pressure. In this manner we were able to obtain essentially all of the phosphomutase activity in the "soluble" fraction of the cellular homogenate. The purification scheme that was selected (Table I) capitalized on the enzyme's neutral charge and high degree of hydrophobicity. Specifically, a buffered solution of the 45–70% ammonium sulfate protein cut was first passed through a DEAE-cellulose column to remove anionic proteins. The enriched protein sample was fractionated on a phenyl-Sepharose column. The phosphomutase was found among the group of proteins that were bound most tightly to the column. The mixture of nonpolar proteins that coeluted with the PEP phosphomutase were removed during the final purification step, which employed size exclusion chromatography.

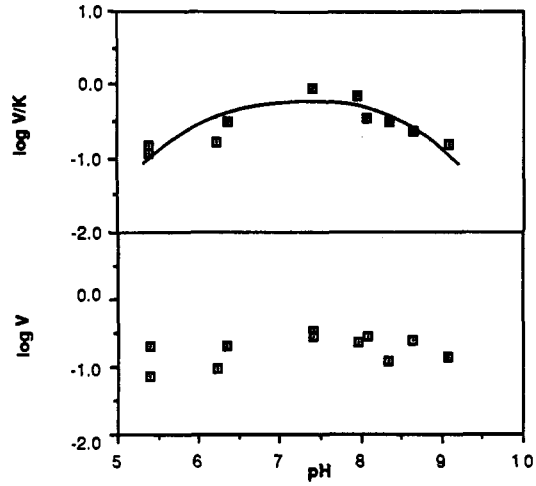


FIGURE 1: pH–rate profile for the PEP phosphomutase catalyzed conversion of phosphonopyruvate to PEP in the presence of saturating Mg^{2+}. See Materials and Methods for experimental details.

Having homogeneous PEP phosphomutase in hand, it was possible to characterize the pH and metal ion dependency of substrate (viz. phosphonopyruvate) binding and catalysis. Like most phosphoryl transferring enzymes, PEP phosphomutase requires a divalent metal ion for activity. While Mg^{2+} would fill the cofactor role under physiological conditions, Co^{2+}, Mn^{2+}, and Zn^{2+} were found to be just as effective as activators (Table III). Analysis of the initial velocity data measured with Mg^{2+} serving as cofactor and phosphonopyruvate as substrate reveals that substrate binding both follows and tightens metal ion binding to the free enzyme. Results obtained from equilibrium dialysis experiments carried out with Mn^{2+} and PEP (Devlin and Dunaway-Mariano, unpublished results) lead to the same conclusion for the binding of the cofactor and physiological substrate. Whether or not the enzyme-bound metal cofactor also coordinates to the substrate is currently under investigation in our laboratory.

The maximum velocity pH profile (Figure 1) shows no evidence of ionizing groups in the regions studied that are kinetically significant and might act as acid–base catalysts. The V_m/K_m pH profile (Figure 1), on the other hand, indicates that at very low phosphonopyruvate concentration catalysis is reduced at acidic and basic pH. One interpretation of this observation is that substrate binding is inhibited by the protonation of one group (apparent pK_a = 6.4) and deprotonation of a second group (apparent pK_a = 8.4). Alternatively, the two ionizing groups may be functioning as acid–base catalysts but go undetected by the V_m pH profile analysis as a result of rate-limiting product dissociation. Identification of these ionizing groups awaits future investigations.

References

Anderson, V. E., Weiss, P. M., & Cleland, W. W. (1984) *Biochemistry 23*, 2779.

Anzai, H., Murkakami, T., Imai, S., Satoh, A., Nagaoka, K., & Thompson, C. J. (1987) *J. Bacteriol. 169*, 3482.
Barry, R. J., Bowman, E., McQueney, M., & Dunaway-Mariano, D. (1988) *Biochem. Biophys. Res. Commun. 153*, 177.
Bencze, W. L., & Schmid, K. (1957) *Anal. Chem. 29*, 1193.
Bowman, E., McQueney, M., Barry, R. J., & Dunaway-Mariano, D. (1988) *J. Am. Chem. Soc. 110*, 5575.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 84.
Dixon, M., & Webb, E. C. (1979) *Enzymes*, 3rd ed., p 92, Academic Press, New York.
Hidaka, T., & Seto, H. (1989) *J. Am. Chem. Soc. 111*, 8012.
Hidaka, T., Mori, M., Imai, S., Hara, O., Nagaoka, K., & Seto, H. (1989) *J. Antibiot. 42*, 491.
Hilderbrand, R. C., Ed. (1983) *The Role of Phosphonates in Living Systems*, CRC Press, Boca Raton, FL.
Hori, T., Horiguchi, M., & Haysohi, A. (1984) in *Biochemistry of Natural C–P Compounds*, Maruzen, Tokyo.
Horiguchi, M., & Kandatsu, M. (1959) *Nature 184*, 901.
Klapper, M. H. (1977) *Biochem. Biophys. Res. Commun. 78*, 1018.
Mastalerz, P. (1984) in *Natural Products Chemistry* (Zalewski, R. I., & Skolik, J. J., Eds.) Elsevier, Amsterdam.
Pecoraro, U. L., Hermes, J. D., & Cleland, W. W. (1984) *Biochemistry 23*, 5262.
Schendel, P. F., & Wells, R. D. (1973) *J. Biol. Chem. 248*, 8319.
Seidel, H. M., Freeman, S., Seto, H., & Knowles, J. R. (1988) *Nature 335*, 457.
Seto, H. (1986) in *Mycotoxins and Phycotoxins* (Steyn, P. S., & Vleggaar, R., Eds.) p 77, Elsevier, Amsterdam.
Trebst, A., & Geike, F. (1967) *Z. Naturforsch. 22b*, 989.

Differential Scanning Calorimetry Study of Mixed-Chain Phosphatidylcholines with a Common Molecular Weight Identical with Diheptadecanoylphosphatidylcholine†

Hai-nan Lin, Zhao-qing Wang, and Ching-hsien Huang*
Department of Biochemistry, Health Sciences Center, University of Virginia, Charlottesville, Virginia 22908
Received December 20, 1989; Revised Manuscript Received April 20, 1990

ABSTRACT: To examine the thermotropic phase behavior of various mixed-chain phosphatidylcholines in excess water and to compare it with the known behavior of identical-chain phosphatidylcholines, we have carried out high-resolution differential scanning calorimetric (DSC) studies on aqueous dispersions of 10 different mixed-chain phosphatidylcholines. These lipids, C(16):C(18)PC, C(18):C(16)PC, C(15):C(19)PC, C(19):C(15)PC, C(14):C(20)PC, C(20):C(14)PC, C(13):C(21)PC, C(21):C(13)PC, C(12):C(22)PC, and C(22):C(12)PC, have a common molecular weight which is the same as that of C(17):C(17)PC, an identical-chain phosphatidylcholine with a molecular weight of 762.2. When the values of any of the thermodynamic parameters (T_m, ΔH, and ΔS) of the mixed-chain phosphatidylcholines and C(17):C(17)PC are plotted against the normalized chain-length difference ($\Delta C/CL$), a linear function with negative slope is obtained provided that the value of $\Delta C/CL$ is within the range of 0.09–0.4. The linear relationship suggests that these mixed-chain phospholipids are packed in the gel-state bilayer similar to the bilayer structure of C(17):C(17)PC at $T < T_m$; however, the negative slope suggests that the conformational statistics of the hydrocarbon chain and the lateral lipid–lipid interactions of these phosphatidylcholines in the gel-state bilayer are perturbed proportionally by a progressive increase in the chain-length inequivalence between the two acyl chains within each lipid molecule. When the value of $\Delta C/CL$ for mixed-chain phosphatidylcholines reaches the range of 0.44–0.55, the thermotropic phase behavior deviates markedly from that of less asymmetric phosphatidylcholines, suggesting that these highly asymmetric lipids are packed into mixed interdigitated bilayers at $T < T_m$. The heating and cooling pathways of aqueous dispersions prepared from the 10 mixed-chain phospholipids are also discussed.

In recent years, high-resolution differential scanning calorimetry (DSC)[1] has been widely used to characterize the thermotropic phase behavior of aqueous dispersions of mixed-chain phospholipids [for a recent review, see Huang and Mason (1986)]. In most of these DSC studies, however, the calorimetric experiments were carried out primarily in an ascending temperature mode. Although calorimetric cooling scans of aqueous dispersions of mixed-chain phospholipids were known for quite a few lipid species (Boggs & Mason, 1986; Mattai et al., 1987), they were limited to runs performed at relatively high scanning rates. Now, high-resolution DSC instruments with cooling scan capability are commercially available. This new accessory enables us to scan the aqueous lipid sample in both the ascending and descending temperature modes at low scanning rates; consequently, the phase transition

† This research was supported in part by U.S. Public Health Service Grant GM-17452 from the National Institute of General Medical Sciences, NIH, Department of Health and Human Services. This paper is dedicated to Professor T. E. Thompson on the occasion of his 65th birthday.

[1] Abbreviations: C(*X*):C(*Y*)PC, saturated L-α-phosphatidylcholine having *X* carbons in the *sn*-1 acyl chain and *Y* carbons in the *sn*-2 acyl chain; DSC, differential scanning calorimetry.

0006-2960/90/0429-7063$02.50/0