Bimodal Lipid Substrate Dependence of Phosphatidylinositol Kinase†

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ABSTRACT: Phosphatidylinositol (PI) kinase activity was solubilized from rat liver microsomes and partially purified by chromatography on hydroxyapatite and Reactive Green 19–Superose. Examination of the ATP dependence using a mixed micellar assay gave a K_m of 120 μM. The dependence of reaction rate on PI was more complicated. PI kinase bound a large amount of Triton X-100, and as expected for a micelle-associated enzyme utilizing a micelle-associated lipid substrate, the reaction rate was dependent on the micellar mole fraction, PI/(PI + Triton X-100), with a K_m of 0.02 (unitless). Activity showed an additional dependence on bulk PI concentration at high micelle dilution. These results demonstrated two kinetically distinguishable steps leading to formation of a productive PI/enzyme(/ATP) complex. The rate of the first step, which probably represents exchange of PI from the bulk micellar pool into enzyme-containing micelles, depends on bulk PI concentration. The rate of the second step, association of PI with enzyme within a single micelle, depends on the micellar mole fraction of PI. Depression of the apparent V_{max} at low ionic strength suggested that electrostatic repulsion between negatively charged PI/Triton X-100 mixed micelles inhibits PI exchange, consistent with a model in which intermicellar PI exchange depends on micellar collisions.

Identification of the biochemical significance of inositol phospholipid metabolism in transmembrane signaling has brought about renewed interest in the enzymology of the metabolic pathway referred to as the phosphatidylinositol (PI)[1] cycle [for recent reviews, see Catt and Balla (1989), Downes (1989), and Putney et al. (1989)]. Knowledge about the metabolism of the soluble inositol polyphosphates has increased rapidly (Shears, 1989), though their functions are still not clear. Characterization of metabolism of the lipid intermediates has proceeded more slowly due to problems inherent in the study of membrane-bound enzymes with insoluble substrates.

We are interested in the enzymes that metabolize lipid components of the PI cycle, and initial studies have focused on rat liver PI kinase. This membrane-bound enzyme interacts with its substrate by free lateral diffusion through the two-dimensional continuum of cellular membranes. To study the kinetic properties of the isolated enzyme requires knowledge of the aggregation state of the enzyme and its lipid substrate. Earlier studies often used ultrasonic dispersions of enzyme and PI to measure activity, which were adequate for qualitative analyses. However, such a system does not clarify the rate-limiting molecular processes, since neither enzyme nor PI can diffuse freely in aqueous solutions.

Including a detergent such as Triton X-100 was found to enhance reaction rates, presumably by solubilizing the enzyme and its lipid substrate (Kai et al., 1966; Tou et al., 1969; Jergil & Sundler, 1983; Collins & Wells, 1983). Though detergent is essential for solubilizing PI kinase and enhancing the reaction rate, it also introduces kinetic artifacts that are only beginning to be understood. In this paper, we describe studies of the solubilization and partial purification of rat liver PI kinase. Kinetic analyses reveal a complex lipid substrate dependence, with the reaction rate sensitive to both bulk aqueous concentration and micellar mole fraction.

Materials and Methods

Materials. Phosphatidylinositol (bovine liver) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Hydroxyapatite (Bio-Gel HTP) was from Bio-Rad Laboratories (Richmond, CA), and Superose 6 prep grade was from Pharmacia (Piscataway, NJ). C_8E_4 was obtained from Bachem Bioscience (Philadelphia, PA). Dye–ligand agaroses, octyl- and phenyl-Sepharose, reactive triazine dyes, and other reagents were from Sigma Chemical Co. (St. Louis, MO) and Aldrich Chemical Co. (Milwaukee, WI). [γ-^{32}P]ATP was purchased from NEN Research Products (Boston, MA).

Dye–ligand matrices were prepared from reactive triazine dyes and either 4% cross-linked agarose or Superose 6 prep grade as described by Heyns and DeMoor (1974). Octyl- and 2-ethylhexyl-Superose were prepared from octyl glycidyl ether (Ulbrich et al., 1964) and 2-ethylhexyl glycidyl ether (Aldrich) as described by Hjerten et al. (1974).

PI Kinase Assay. Enzyme activity was measured in a 0.1-mL reaction volume containing 50 mM Bis-Tris-HCl, pH 6.5, 200 mM NaCl, 10 mM $MgCl_2$, 10% (w/v) glycerol, 10 mM 2-mercaptoethanol, 1% (w/v) Triton X-100, 1.6 mM PI (mole fraction = 0.09), 1 mM ATP (1–2 μCi of [γ-^{32}P]ATP per assay, 20–50 cpm/pmol), and up to 25 μg of protein. Reactions were started by ATP addition, and stopped after 5- or 10-min incubation at 30 °C by adding 3 mL of chloroform/methanol (1:2 v/v). Labeled product was extracted by sequentially adding 0.7 mL of 1 M $MgCl_2$/0.2 M HCl, 1 mL of chloroform, and 1 mL of 1 M $MgCl_2$/0.2 M HCl. Tubes were sealed with Teflon-lined caps, and the contents were mixed vigorously. Phases were separated by brief centrifugation. The lower phase was washed twice with 2-mL portions of preformed upper phase. Of the resulting 2 mL of lower

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[1] Abbreviations: C_8E_4, *n*-octyl tetraethylene glycol monoether; $C_{13}E_{10}$, isotridecyl decaethylene glycol monoether; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; PI, phosphatidylinositol.

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phase, 1 mL was removed and dried, and radioactive product was determined by liquid scintillation counting. Soybean phospholipid (1 mg) was added as a carrier during the extraction. Recovery of product by the extraction procedure was greater than 95%. Assays were carried out under conditions where the reaction was linear with respect to time and protein. A unit of activity is defined as the amount of enzyme catalyzing the formation of 1 micromole of product per minute under the above conditions. Data presented are the averages of duplicate determinations which usually differed by less than 5%.

Reaction products were analyzed by thin-layer chromatography on oxalate-impregnated silica gel 60 plates (E. Merck) using the solvent system chloroform/methanol/4 M ammonia (9:7:2 v/v). The only product detectable by thin-layer chromatography was phosphatidylinositol monophosphate. The position of the monophosphate on the inositol ring was not determined but is presumed to be the 4-hydroxyl, since the enzyme preparation was inhibited by adenosine, indicative of type II, PI-4-kinase activity (Whitman et al., 1987, 1988).

Controlling the micellar ratio of PI/Triton X-100 was important for kinetic analysis. The critical micelle concentration of Triton X-100 was determined in assay buffer by plotting surface tension (measured by the du Nouy ring method) against the logarithm of concentration. The result (0.015%) is identical with that reported elsewhere for Triton X-100 in water (Mukerjee & Mysels, 1970). Assuming a mean molecular weight of 625, this corresponds to 0.24 mM (Hendrickson & Dennis, 1984). In all assays, the monomer concentration of Triton X-100 was subtracted from the total to give the relevant micellar concentration. The free monomer concentration of PI was assumed to be negligible.

Partial Purification of Rat Liver Microsomal PI Kinase. Liver microsomes were isolated from female Sprague-Dawley rats as described by Sedgwick and Hubscher (1965). PI kinase activity was solubilized at a final protein concentration of 2.5 mg/mL in 160 mL of buffer A (20 mM Bis-Tris·HCl, pH 6.5, 200 mM NaCl, 10% glycerol, and 10 mM 2-mercaptoethanol) containing 50 mM potassium phosphate and 5% $C_{13}E_{10}$. This detergent is very similar to Triton X-100 in terms of physicochemical properties, as well as PI kinase solubilization (Ganong & Lu, 1989) and stability, but is UV-invisible.

After centrifugation at 105000*g* for 1 h, the supernatant was applied to a 2.5 × 15 cm column of hydroxyapatite equilibrated with buffer A containing 50 mM potassium phosphate and 0.05% $C_{13}E_{10}$. The column was washed with the same buffer until the absorbance returned to base line, and bound protein was eluted with a 360-mL potassium phosphate gradient from 50 to 400 mM. Fractions of 10 mL were collected.

Pooled active fractions from the hydroxyapatite runthrough were applied to a Reactive Green 19–Superose column (2.5 × 11 cm) equilibrated with buffer A containing 0.05% $C_{13}E_{10}$. The column was washed with the same buffer until the absorbance dropped to base line. A gradient from 0.2 to 2 M NaCl was followed by 2 M NaCl. Fractions of 10 mL were collected. Reactive Green 19 coupled to Superose 6 prep grade gave substantially better resolution than the same dye coupled to 4% agarose.

Dialysis of the pooled active fractions from the Green 19–Superose column overnight against 20 mM Bis-Tris·HCl, pH 6.5, did not cause appreciable aggregation or loss of activity. The dialyzate was concentrated 5-fold using Omega ultrafiltration membranes before being stored at −80 °C. Activity was stable, with a half-life of several months. Enzyme activity was absolutely dependent on added PI (detection limit <1%).

Gel filtration chromatography (Sepharose CL-6B, Sephacryl S-300, TSK 3000 SW, Superose 6 prep grade, and Zorbax GF-450), hydrophobic interaction chromatography (octyl- and phenyl-Sepharose, octyl- and 2-ethylhexyl-Superose 6), poly(ethylene glycol) precipitation, and aqueous polymer phase separation using poly(ethylene glycol)/dextran mixtures did not result in increased specific activity. Ammonium sulfate precipitated activity, but only at concentrations which caused the precipitated enzyme to float, making quantitative recovery difficult. With small samples, thermal Triton X-114 phase separation gave approximately a 2-fold purification, but scaleup was unsuccessful. Activity did not bind to PI–agarose, even when excess detergent was removed with Bio-Beads SM-2 (Saltiel et al., 1987). Neither significant protein nor activity was retained by concanavalin A– or wheat germ agglutinin–agarose.

Sucrose Gradient Ultracentrifugation. Sucrose gradients (4.5 mL; 5–20% w/v) were prepared over a cushion of 60% sucrose (0.5 mL) in 20 mM Na-Hepes, pH 7.5, 200 mM NaCl, and 0.05% (w/v) Triton X-100, using either H_2O or D_2O. Samples (100 μL) of standard proteins (200 μg) or solubilized microsomes (250 μg of protein) were applied to the tops of parallel gradients and centrifuged for 21 h at 4.0 °C at 48 000 rpm in an SW50.1 rotor using a Beckman L8-80M ultracentrifuge. The tubes were fractionated from the tops, and protein or enzyme was assayed in fractions to determine the distance migrated.

Protein standards were lysozyme ($s_{20,w}$ = 1.91 S, $\bar{v}$ = 0.703; Sophianopoulos et al., 1962), trypsinogen ($s_{20,w}$ = 2.48 S, $\bar{v}$ = 0.73; Tietze, 1953), carbonic anhydrase ($s_{20,w}$ = 2.8 S, Petermann & Hakala, 1942; $\bar{v}$ assumed 0.73), ovalbumin ($s_{20,w}$ = 3.6 S, Kegeles & Gutter, 1951; $\bar{v}$ = 0.748, Dayhoff et al., 1952), and bovine serum albumin ($s_{20,w}$ = 4.30 S, Miller & Golder, 1952; $\bar{v}$ = 0.734, Dayhoff et al., 1952). Lysozyme activity was measured spectrophotometrically using a suspension of *Micrococcus lysodeikticus* as substrate, and other proteins were determined colorimetrically (Peterson, 1977). Densities were measured gravimetrically.

Results

Solubilization of Rat Liver PI Kinase. In initial attempts to solubilize PI kinase, rat liver microsomes were incubated at 0 °C with nonionic (Triton X-100, Tergitol TMN-10, C_8E_4, octyl glucoside) and zwitterionic detergents (CHAPS, Zwittergent 3-12) at various detergent:protein ratios. Less than 80% of enzyme activity was recovered in a high-speed supernatant under all conditions. With Triton X-100, the efficiency of solubilization decreased as the weight ratio of detergent to protein increased, as was also observed by Bostwick and Eichberg (1981). This result suggests that noncovalent polar interactions promote membrane protein aggregation in solutions of nonionic or zwitterionic detergents.

We tested the possibility that aggregation was caused by electrostatic interactions by measuring solubilization at different salt concentrations. At salt concentrations over 200 mM, greater than 95% of activity was consistently solubilized (Ganong & Lu, 1989). Solubilization was rapid; no preincubation was required. Prewashing microsomes with 1 M NaCl did not circumvent the need for high salt during solubilization. The effect seems to be due to increased ionic strength and not to depend on specific ions, since 100 mM sodium sulfate or ammonium sulfate (ionic strength 250 mM) was able to promote complete solubilization. This ionic strength requirement for solubilization was seen with other

polyoxyethylene detergents (Ganong & Lu, 1989) and with CHAPS.

Anionic detergents (deoxycholate, laurate, and dodecyl sulfate) mixed with Triton X-100 at mole fractions of 10–40% also solubilized over 95% of enzyme activity, even in the absence of added electrolyte. Use of ionic detergents alone markedly decreased the enzyme's stability. Thus, when addition of ionic detergents increases ionic strength in the vicinity of the micelle/water interface, rather than the bulk solution, the effect on solubilization is similar.

Size Analysis of PI Kinase/Triton X-100 Aggregates. Samples of enzyme solubilized with Triton X-100, with or without 200 mM NaCl, were analyzed by gel filtration at 4 °C on a Sephacryl S-300 column. Activity solubilized without salt emerged from the column in the void volume, implying an aggregate molecular weight greater than 10^6. In contrast, enzyme solubilized in the presence of 200 mM NaCl was included in the column and eluted just before pure Triton X-100 micelles, consistent with a single polypeptide per protein/detergent(/lipid) mixed micelle. When solubilized enzyme was desalted on a Sephadex G-25 column, the enzyme aggregated, appearing in the void volume of the Sephacryl column. It appears that the effect of salt is not to increase the rate of solubilization, but rather to render the enzyme in a more uniformly disaggregated state.

Comparison of PI kinase elution in 200 mM NaCl with globular protein standards indicated an apparent molecular weight of about 220000. Solubilized membrane proteins bind a variable amount of nonionic detergent, which contributes significantly to the solubilized enzyme's aggregate size. Since bound detergent has a different partial specific volume than protein, its presence markedly affects a solubilized protein's sedimentation behavior (Meunier et al., 1972). When the mobility of PI kinase was examined in sucrose gradients prepared in H_2O, activity migrated at a rate consistent with a globular protein of approximate M_r 39000 (Figure 1A). When the same experiment was repeated using gradients prepared in D_2O (Figure 1B), the solubilized enzyme migrated at a molecular weight less than 10000. The discrepancy between these values and that from gel filtration indicates substantial detergent binding. The partial specific volume of the enzyme/detergent complex was calculated from the data of Figure 1 as described by Meunier et al. (1972). The value obtained, 0.87 cm^3/g, suggests that over half the particle's mass is contributed by detergent.

Partial Purification of PI Kinase. Preliminary studies with the enzyme indicated rat liver PI kinase was unstable when solubilized from microsomes with Triton X-100 at pH 7.5 ($t_{1/2}$ of 27 min at 30 °C). Its stability was not influenced by several protease inhibitors, including leupeptin (10 μg/mL), pepstatin (10 μg/mL), aprotinin (10 μg/mL), TLCK (50 μg/mL), TPCK (100 μg/mL), and EGTA (5 mM). Specific thiol protease inhibitors were not used, as PI kinase appears to be inhibited by thiol reagents (Colodzin & Kennedy, 1965; Kai et al., 1966; Tou et al., 1969). Stability was enhanced at pH 6.5 in the presence of 10% glycerol and 10 mM 2-mercaptoethanol.

Because ionic strength is easier to control than anionic/nonionic detergent mole ratios, we included 200 mM sodium chloride in all buffers to prevent enzyme aggregation during the initial steps of purification. Solubilized activity was not retained by DEAE-Sephacel, phosphocellulose, polylysine–agarose, Q or S Sepharose, or a MonoQ column, presumably due to the high salt concentration. PI kinase bound to hydroxyapatite at 200 mM NaCl and 1 mM potassium phosphate, and was eluted early in a potassium phosphate gradient, but tailing of the peak led to poor resolution and recovery. However, PI kinase was efficiently recovered in the runthrough when solubilized microsomes were applied in 50 mM potassium phosphate. The runthrough contained 90% of the recovered activity and 30% of total protein. Recovery of 60% of the applied activity resulted in 1.5–2-fold purification.

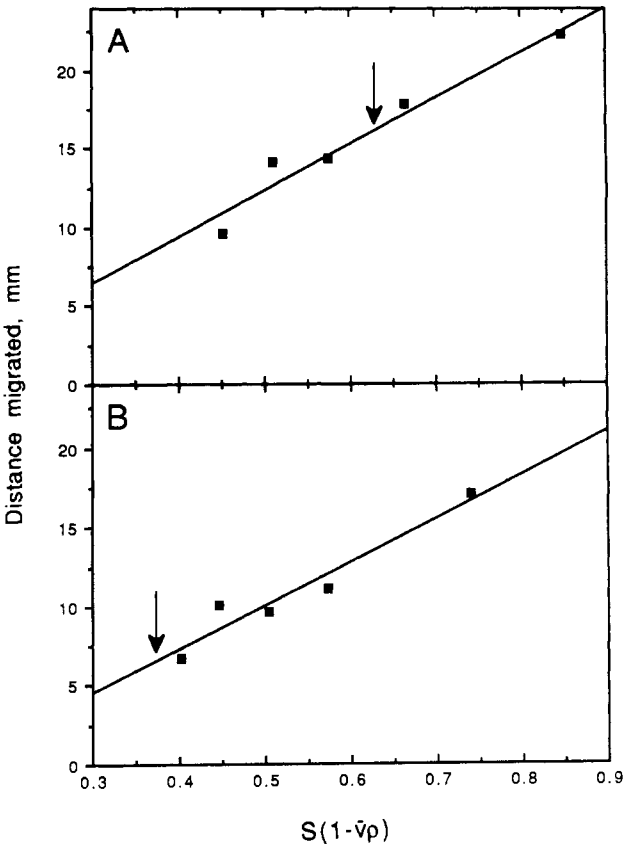

FIGURE 1: Sucrose density gradient centrifugation of solubilized PI kinase. Sucrose gradients were prepared and analyzed as described under Materials and Methods, using H_2O (panel A) or D_2O (panel B). Migration distance is plotted against the standard proteins' $s_{20,w}$ values, corrected for the buoyant density factor $(1 - \bar{v}\rho)$. Arrows indicate position of PI kinase activity. Protein standards, from left to right, are lysozyme, trypsinogen, carbonic anhydrase, ovalbumin, and bovine serum albumin.

Fifteen reactive triazine dyes coupled to agarose were tested for binding and elution of PI kinase activity in a linear 0.2–2 M NaCl gradient. No activity was recovered from Reactive Blue 2, Reactive Blue 5, Reactive Blue 160, Reactive Brown 10, Reactive Green 5, Reactive Red 4, Reactive Red 120, or Reactive Yellow 2. Activity did not bind to Reactive Yellow 3 or Reactive Yellow 86, but was recovered with most of the applied protein in the runthrough. With Reactive Blue 72, Reactive Green 19, and Reactive Orange 14, activity bound and was eluted during the gradient. However, poor recoveries were obtained from Reactive Blue 72, and Reactive Orange 14 leached from the matrix too rapidly to be useful. Reactive Green 19, on the other hand, has given reproducibly good purifications. All of the PI kinase activity bound at 200 mM NaCl, whereas 65% of the protein ran through. The most active fractions contained 90% of recovered enzyme activity, and 10% of total protein. Loss of 50–70% of the total applied activity resulted in 3–5-fold purification.

Several other chromatographic and solution phase purification methods were attempted unsuccessfully (see Materials and Methods). Hydroxyapatite and Green 19–Superose chromatography were the only two approaches that reprodu-

Table I: Partial Purification of Rat Liver Microsomal Phosphatidylinositol Kinase

step	total protein (mg)	total act. (milliunits)	sp act. (milliunits/mg)	purification (x-fold)	yield (%)
solubilized microsomes	210	343	1.6	1.0	100
hydroxyapatite	73	216	3.0	1.8	63
Green Superose	8	92	11.5	7.0	27

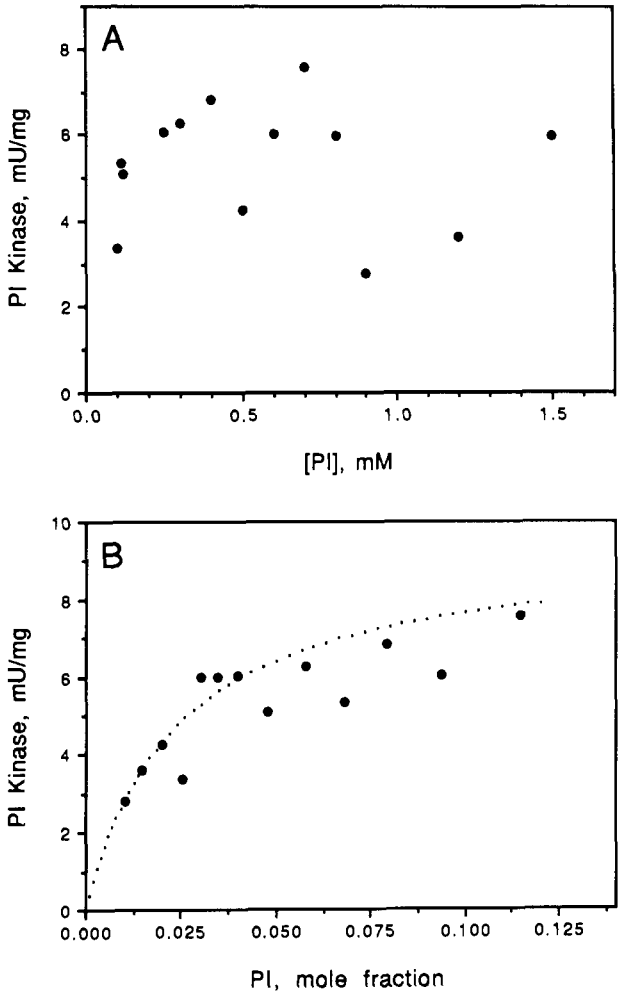

FIGURE 2: Dependence of PI kinase activity on PI, bulk concentration compared with micellar mole fraction. PI and Triton X-100 concentrations were varied independently. (A) PI concentration expressed as bulk concentration (millimolar). (B) PI concentration expressed as mole fraction [PI/(PI + Triton X-100), unitless]. The dotted line shows the best fit for assays in which [PI] was greater than 0.25 mM (see text). ATP concentration was 1.0 mM.

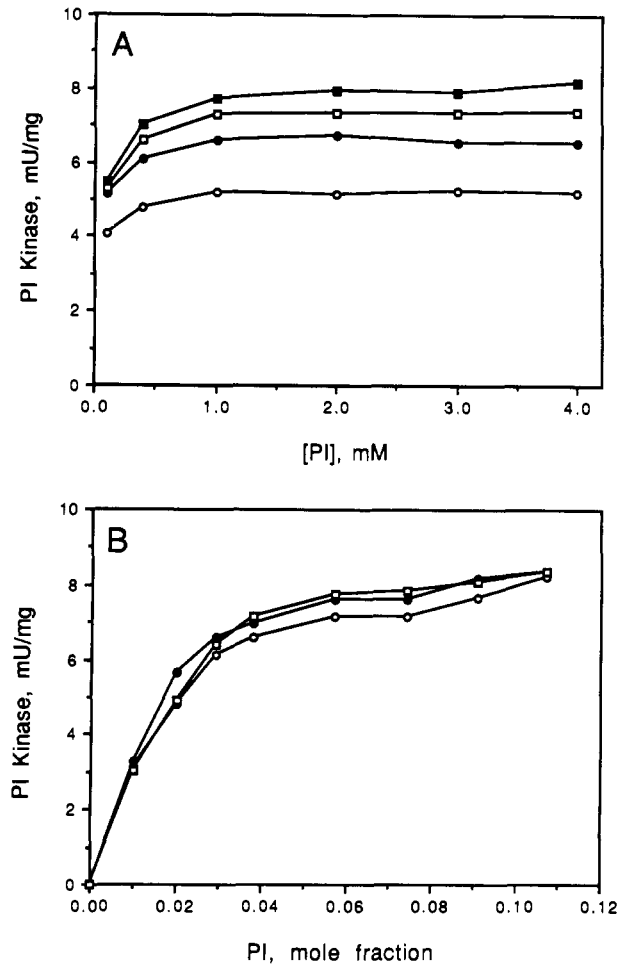

FIGURE 3: Dependence of PI kinase activity on micellar PI mole fraction. (A) Dependence of activity on bulk concentration at four PI mole fractions [(○) 0.02; (●) 0.04; (□) 0.06; (■) 0.08]. (B) Dependence of activity on PI mole fraction at three Triton X-100 concentrations [(○) 0.5%; (●) 1.0%; (□) 2.0%]. ATP concentration was 1.0 mM.

cibly yielded an increase in PI kinase specific activity. A summary of a typical partial purification is presented in Table I. This enzyme preparation was used to examine the reaction's substrate dependence.

ATP Dependence. Dependence of activity on ATP concentration was measured in assays containing 1% Triton X-100 and 1.78 mM PI (mole fraction 0.10, 5 times the K_m, see below). The ATP dependence showed typical Michaelis–Menten kinetics. Double-reciprocal analysis gave an apparent K_m of 120 μM.

PI Dependence. PI kinase presumably interacts with PI within a detergent/PI/enzyme mixed micelle. Since PI is diluted by detergent in a uniform population of mixed micelles, the PI dependence should be a function of the PI:detergent ratio rather than the bulk aqueous PI concentration. We tested this hypothesis by the experiment shown in Figure 2. ATP concentration was held constant at 1.0 mM, and PI and Triton X-100 were varied independently and randomly. When activity was expressed as a function of bulk PI concentration (Figure 2A), there was no clear correlation. When the same data were replotted with PI concentration expressed as the mole fraction PI/(PI + detergent), activity showed a hyperbolic dependence (Figure 2B).

These results are consistent with the hypothesis that PI kinase activity is a function of the micellar PI mole fraction. Most of the data points closely fit a hyperbolic curve (Figure 2B, broken line), but four points fell significantly below the curve. Those points correspond to the four assays with the lowest bulk PI concentration. This suggested some dependence of activity on bulk concentration, contrary to the hypothesis. Therefore, we tested the bulk concentration dependence over a 40-fold range, at four fixed molar ratios of PI/(PI + detergent) (Figure 3A). At each molar ratio, the activity increased between 0.1 and 1 mM PI and then remained essentially constant to 4 mM PI.

These data clearly show the reaction rate is sensitive to bulk PI concentration. In this experiment, the plateau values increased with a nonlinear dependence on mole fraction, as

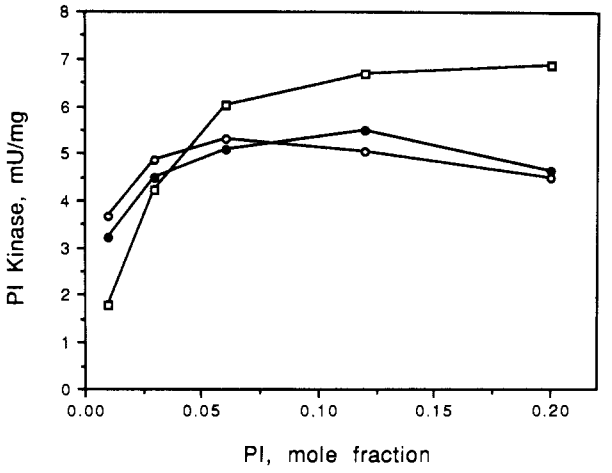

FIGURE 4: Effect of ionic strength on PI dependence. Concentration of Triton X-100 was 1.0%; ATP concentration was 1.0 mM. Sodium chloride concentration was 0 (○), 40 (●), or 400 (□) mM.

expected from Figure 2B. The mole fraction dependence was examined in more detail at three detergent concentrations (Figure 3B). The similarity of the curves, despite 4-fold differences in bulk PI concentration, confirmed the enzyme's dependence on the micellar mole fraction of PI with respect to total amphiphile. Double-reciprocal analysis indicated a K_m of 0.02 (unitless). Thus, there appear to be two distinct modes of PI concentration dependence: a bulk dependence observable at low concentrations, superimposed on a mole fraction dependence.

Ionic Strength Dependence. In experiments similar to that presented in Figure 3B, activity decreased at PI mole fractions greater than 0.15, suggesting classical substrate inhibition. A second possibility was that electrostatic repulsion of negatively charged PI/Triton X-100 mixed micelles was inhibiting intermicellar substrate exchange at high PI mole fractions. That possibility was tested by examining the PI dependence of the reaction at different ionic strengths (Figure 4). In the absence of NaCl, with only the buffer contributing to ionic strength, activity increased as the PI mole fraction increased to 0.06 and then decreased with increasing mole fraction. With the addition of 40 mM NaCl, activity increased up to a mole fraction of 0.12 and then decreased. When the salt concentration was increased to 400 mM, no decline in activity occurred up to a mole fraction of 0.20. At high ionic strength, the data more nearly resemble a Michaelis–Menten hyperbola.

Discussion

A fundamental requirement for purifying a membrane protein is solubilization into discrete protein/detergent mixed micelles. To achieve solubilization of rat liver microsomal PI kinase using nonionic or zwitterionic detergents, which do not contribute net charge, requires an ionic strength of about 200 mM. The same is true for total microsomal protein solubilization. This phenomenon has not been systematically studied. In these experiments, gel filtration chromatography has proven a better indicator of solubilization than has sedimentation.

Dependence on salt for solubilization is an equilibrium rather than a kinetic effect, as salt removal causes protein reaggregation. It suggests that microsomal proteins aggregate via electrostatic interactions in the matrix of the nonionic amphiphile. These interactions may occur in the interfacial region between the hydrophobic micelle core and bulk solution, where the dielectric constant would be lower than that of bulk water, but where interactions could still be influenced by the solution's ionic strength. This seems to be supported by the fact that increased proportions of anionic detergents in mixed micelles with nonionics cause a similar increase in solubilization. Such an approach to solubilization of PI kinase has been employed by others (Suarez-Quian et al., 1987; Porter et al., 1988).

Either high ionic strength or maintenance of an optimal anionic:nonionic detergent ratio seems to be necessary initially to prevent enzyme aggregation and loss of activity. Critical micelle concentrations of anionic detergents are generally much larger, as well as more sensitive to pH and ionic strength, than those of nonionic detergents. This makes it difficult to control micellar mole ratios as bulk detergent concentrations are varied in chromatographic buffers of varying pH and ionic strength. Therefore, we chose to maintain the solubility of PI kinase during purification by including 200 mM NaCl along with nonionic detergent in all chromatographic buffers.

Our two-column procedure yields a preparation of PI kinase free of endogenous substrates. We have used this partially purified enzyme to study the enzyme's substrate dependences. Dependence on ATP was as expected for interaction between an enzyme and a water-soluble substrate, but the PI dependence was more complex. Phospholipids are amphiphilic compounds and aggregate spontaneously in aqueous solutions. The aggregation state is usually undefined and difficult to control. Aggregation of amphiphilic lipid substrates could affect rates of enzyme-catalyzed reactions in many ways: for example, by introducing artifactual rate-limiting steps (interaggregate substrate diffusion) or by altering apparent substrate site affinity (steric effects). These are important considerations in analyzing enzymological data that involve lipid substrates, though they are often ignored. Most such problems are overcome by including an excess of detergent in assay buffers, provided the enzyme is stable in its presence. Detergents form a stable amphiphile dispersion in which molecular organization of other amphiphiles (e.g., lipid, protein) is dominated by the detergent's aggregation properties.

An aqueous solution of Triton X-100 and phospholipid is composed of mixed micelles with phospholipid uniformly distributed (Robson & Dennis, 1978). Comparison of the apparent molecular weight of PI kinase determined by gel filtration chromatography with that measured by sucrose gradient ultracentrifugation indicates PI kinase binds a large amount of Triton X-100 in the absence of added PI. Uncertainty exists as to whether membrane proteins bind an amount of nonionic detergent equivalent to a single micelle (Le Maire et al., 1983). Without implying anything about the quantity of detergent bound, it is likely that interaction of PI with PI kinase occurs within a single detergent/enzyme/lipid mixed micelle in a manner topographically similar to that which occurs in native membranes. Since in terms of interaction of PI with the enzyme's active site, the substrate is diluted by detergent rather than water, it is reasonable to expect that the reaction rate depends on the molar ratio of PI and detergent, rather than on the lipid concentration relative to bulk aqueous phase. This rationale has been applied to other enzymes utilizing a micellar dispersion of lipids as substrates or activators, including phospholipase A_2 (Deems et al., 1975; Hendrickson & Dennis, 1984), diacylglycerol kinase (Walsh & Bell, 1986), and protein kinase C (Hannun et al., 1985, 1986). In all cases, the catalytic rate was sensitive to the lipid:detergent molar ratio. This is true for PI kinase as well (Figure 3). PI kinase activity was insensitive to bulk PI concentration (or micelle number) above 1 mM, if the mole fraction of PI relative to total amphiphile was held constant. However, activity was sensitive to changes in this mole fraction, yielding a K_m of 0.02 (unitless).

An important question is whether such a micellar dispersion permits sufficiently rapid intermicellar lipid exchange such that catalysis, and not exchange, limits the observed reaction rate. This problem is emphasized by consideration of the relevant stoichiometries. A conservative estimate suggests that, under our assay conditions, micelles lacking enzyme are in excess of enzyme-containing micelles by at least 4 orders of magnitude. Phosphorylation of even a fraction of a percent of PI requires substantial intermicellar redistribution of substrate and product. From studies of diacylglycerol kinase (Walsh & Bell, 1986), it was concluded that diacylglycerol exchange was significantly faster than the catalytic rate. We began our studies with the assumption that the same would be true for PI kinase over a wide range of conditions; that is, that the horizontal lines in Figure 3A would extend essentially to the vertical axis. However, we found that at any fixed PI mole fraction, enzyme activity decreased with decreasing bulk PI concentration below 1 mM. These results are similar to those presented by Belunis et al. (1988) and Porter et al. (1988), studying PI kinase purified from yeast and bovine uterus, respectively. Our data suggest that there are two kinetic steps affecting interaction of PI with enzyme: one that is sensitive to bulk concentration and one that is sensitive only to the molar ratio of micellar amphiphiles. Exchange of PI among the micelle population is an artifactual step resulting from the use of a micellar pseudophase, and is not relevant to in vivo kinetics. Interaction of PI with the enzyme's active site within a single micelle is the step that is sensitive to the mole fraction. This is the catalytically relevant step which corresponds to enzyme–substrate interaction in native membranes. If this interpretation is correct, analyzing PI dependence at a fixed PI:detergent molar ratio would give information about kinetics of intermicellar lipid exchange, but would tell nothing about substrate–enzyme interaction.

It is not obvious why intermicellar substrate exchange should limit PI kinase activity, but not diacylglycerol kinase activity. Exchange might occur either by monomer diffusion or by collisions between micelles or submicellar aggregates. The monomer solubilities of diacylglycerol and PI are not known, though they would be expected to be very low and to depend on the acyl chain composition. Nevertheless, because of its charged, polar headgroup, PI should have the higher solubility. If intermicellar exchange occurs by monomer diffusion and the rate of diffusion depends simply on lipid monomer solubility, then PI should exchange more rapidly than diacylglycerol. However, the equilibrium monomer concentration cannot be assumed to be an indication of the rate of interaggregate exchange, which is a function only of the off rate from mixed micelles. It would be difficult to predict how that rate might differ between the two lipids.

However, if intermicellar lipid transfer occurs by a collisional exchange mechanism, it is possible to make predictions about differences between these two lipids. Since PI is charged, unlike diacylglycerol, increasing the PI mole fraction increases the net micellar charge. This would result in significant electrostatic repulsion, decreasing intermicellar interactions. This mechanism has been demonstrated in kinetic studies using pure detergents. For ionic detergents at low ionic strength, intermicellar exchange occurs only by way of monomer diffusion due to electrostatic repulsion of micelles (Kahlweit, 1982). On the other hand, nonionic detergents are exchanged predominantly by micellar collisions irrespective of ionic strength. Thus, as the PI mole fraction increases, there should be an initial increase in the reaction rate in response to higher micellar PI concentrations. At higher mole fractions when the buffer ionic strength is low, electrostatic repulsion would lead to progressive inhibition as intermicelle exchange becomes more rate-limiting. This inhibition was observed experimentally (Figure 4), and resembles substrate inhibition. At high ionic strength, micelles would be shielded from this repulsion. Increasing ionic strength abrogated the inhibition at high mole fraction, and the substrate dependence curve resembles the classical Michaelis–Menten hyperbola. The inhibitory effect of high ionic strength at low mole fractions (Figure 4) is more difficult to explain. If there is an important electrostatic component to the association of PI with the enzyme's active site, then increasing the ionic strength might inhibit that association, most noticeably at high substrate surface dilution. There are other possible explanations for these effects of ionic strength on enzyme activity, but the data of Figure 4 are consistent with a model for intermicellar exchange via micellar collisions. It would be harder to reconcile these data with a model in which monomer diffusion limits reaction rates. Increasing ionic strength would be expected to decrease both monomer solubility ("salting out" effect; Tanford, 1980) and the off rate constants (by minimizing electrostatic contributions), giving results contrary to those observed.

These considerations are irrelevant to mixed micellar assays of diacylglycerol kinase, or phospholipase A_2 hydrolysis of phosphatidylcholine and phosphatidylethanolamine, since none of these lipid substrates contributes net charge to nonionic detergent micelles. In the case of protein kinase C activation by mixed micelles of phosphatidylserine and diacylglycerol (Hannun et al., 1985, 1986), once an active lipid/calcium/enzyme complex forms, there is no reason to expect that the rate of phosphorylation of soluble protein substrates by ATP should be affected by rates of intermicellar lipid exchange. A bulk concentration dependence such as we observe in assays of PI kinase should be most prominent for enzymes that metabolize anionic lipids such as CDP-diacylglycerol, phosphatidic acid, and the inositides.

The kinetic model for PI kinase described above resembles the dual phospholipid model for phospholipase A_2 activity proposed by Hendrickson and Dennis (1984) in that there are two substrate-dependent steps, one dependent on bulk concentration and the other on mole fraction. A key conceptual difference is that in the dual phospholipid model, the bulk concentration dependence reflects diffusion and binding of soluble enzyme to lipid substrate at a micelle surface. In contrast to phospholipase A_2, PI kinase binds a micelle of detergent in the absence of added substrate (Figure 1), and its bulk concentration dependence probably reflects diffusion of lipid to this micelle-associated enzyme from the bulk micellar pool.

It is unfortunate but unavoidable that using detergents in studies of membrane-bound enzymes introduces artifacts that can confuse kinetic studies. Hopefully, more detailed study of the structure and dynamics of detergent micelles will lead to better understanding of the quantitative effects of these artifacts, in particular, how they may be accounted for in enzymatic rate equations.

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References

Belunis, C. J., Bae-Lee, M., Kelley, M. J., & Carman, G. M. (1988) *J. Biol. Chem. 263*, 18897–18903.

Bostwick, J. R., & Eichberg, J. (1981) *Neurochem. Res. 6*, 1053–1065.

Catt, K. J., & Balla, T. (1989) *Annu. Rev. Med. 40*, 487–509.
Collins, C. A., & Wells, W. W. (1983) *J. Biol. Chem. 258*, 2130–2134.
Colodzin, M., & Kennedy, E. P. (1965) *J. Biol. Chem. 240*, 3771–3780.
Dayhoff, M. O., Perlmann, G. E., & MacInnes, D. A. (1952) *J. Am. Chem. Soc. 74*, 2515–2517.
Deems, R. A., Eaton, B. R., & Dennis, E. A. (1975) *J. Biol. Chem. 250*, 9013–9020.
Downes, C. P. (1989) *Biochem. Soc. Trans. 17*, 259–268.
Ganong, B. R., & Lu, C.-M. (1989) *Anal. Biochem. 179*, 66–71.
Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1985) *J. Biol. Chem. 260*, 10039–10043.
Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1986) *J. Biol. Chem. 261*, 7184–7190.
Hendrickson, H. S., & Dennis, E. A. (1984) *J. Biol. Chem. 259*, 5734–5739.
Heyns, W., & DeMoor, P. (1974) *Biochim. Biophys. Acta 358*, 1–13.
Hjerten, S., Rosengren, J., & Pahlman, S. (1974) *J. Chromatogr. 101*, 281–288.
Jergil, B., & Sundler, R. (1983) *J. Biol. Chem. 258*, 7968–7973.
Kahlweit, M. (1982) *J. Colloid Interface Sci. 90*, 92–99.
Kai, M., White, G. L., & Hawthorne, J. N. (1966) *Biochem. J. 101*, 328–337.
Kegeles, G., & Gutter, F. J. (1951) *J. Am. Chem. Soc. 73*, 3770–3777.
Le Maire, M., Kwee, S., Andersen, J. P., & Moller, J. V. (1983) *Eur. J. Biochem. 129*, 525–532.
Meunier, J. C., Olsen, R. W., & Changeux, J. P. (1972) *FEBS Lett. 24*, 63–68.
Miller, G. L., & Golder, R. H. (1952) *Arch. Biochem. Biophys. 36*, 249–258.
Mukerjee, P., & Mysels, K. J. (1970) *Critical Micelle Concentrations of Aqueous Surfactant Systems*, U.S. Government Printing Office, Washington, DC.
Petermann, M., & Hakala, N. (1942) *J. Biol. Chem. 145*, 701–705.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.
Porter, F. D., Li, Y.-S., & Deuel, T. F. (1988) *J. Biol. Chem. 263*, 8989–8995.
Putney, J. W., Jr., Takemura, H., Hughes, A. R., Horstman, D. A., & Thastrup, O. (1989) *FASEB J. 3*, 1899–1905.
Robson, R. J., & Dennis, E. A. (1978) *Biochim. Biophys. Acta 508*, 513–524.
Saltiel, A. R., Fox, J. A., Sherline, P., Sahyoun, N., & Cuatrecasas, P. (1987) *Biochem. J. 241*, 759–763.
Sedgwick, B., & Hubscher, G. (1965) *Biochim. Biophys. Acta 106*, 63–77.
Shears, S. B. (1989) *Biochem. J. 260*, 313–324.
Sophianopoulos, A. J., Rhodes, C. K., Holcomb, D. N., & Van Holde, K. E. (1962) *J. Biol. Chem. 237*, 1107–1112.
Suarez-Quian, C. A., O'Shea, J. J., & Klausner, R. D. (1987) *Biochem. Biophys. Res. Commun. 143*, 512–516.
Tanford, C. (1980) *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, Wiley, New York.
Tietze, F. (1953) *J. Biol. Chem. 204*, 1–11.
Tou, J.-S., Hurst, M. W., & Huggins, C. G. (1969) *Arch. Biochem. Biophys. 131*, 596–602.
Ulbrich, V., Makes, J., & Jurecek, M. (1964) *Collect. Czech. Chem. Commun. 29*, 1466–1475.
Walsh, J. P., & Bell, R. M. (1986) *J. Biol. Chem. 261*, 6239–6247.
Whitman, M., Kaplan, D., Roberts, T., & Cantley, L. (1987) *Biochem. J. 247*, 165–174.
Whitman, M., Downes, C. P., Keeler, M., Keller, T., & Cantley, L. (1988) *Nature 332*, 644–646.