A Single Amino Acid Substitution in the Pleckstrin Homology Domain of Phospholipase C δ1 Enhances the Rate of Substrate Hydrolysis*

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The pleckstrin homology (PH) domain has been postulated to serve as an anchor for enzymes that operate at a lipid/water interface. To understand further the relationship between the PH domain and enzyme activity, a phospholipase C (PLC) δ1/PH domain enhancement-of-activity mutant was generated. A lysine residue was substituted for glutamic acid in the PH domain of PLC δ1 at position 54 (E54K). Purified native and mutant enzymes were characterized using a phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2)/dodecyl maltoside mixed micelle assay and kinetics measured according to the dual phospholipid model of Dennis and co-workers (Hendrickson, H. S., and Dennis, E. A. (1984) J. Biol. Chem. 259, 5734–5739; Carmen, G. M., Deems, R. A., and Dennis, E. A. (1995) J. Biol. Chem. 270, 18711–18714). Our results show that both PLC δ1 and E54K bind phosphatidylinositol bisphosphate cooperatively (Hill coefficients, n = 2.2 ± 0.2 and 2.0 ± 0.1, respectively). However, E54K shows a dramatically increased rate of (PI(4,5)P2)-stimulated PI(4,5)P2 hydrolysis (interfacial V_{max} for PLC δ1 = 4.9 ± 0.3 μmol/min/mg and for E54K = 31 ± 3 μmol/min/mg) as well as PI hydrolysis (V_{max} for PLC δ1 = 27 ± 3.4 nmol/min/mg and for E54K = 95 ± 12 nmol/min/mg). In the absence of PI(4,5)P2 both native and mutant enzyme hydrolyze PI at similar rates. E54K also has a higher affinity for micellar substrate (equilibrium dissociation constant, K_{d} = 85 ± 36 μM for E54K and 210 ± 48 μM for PLC δ1). Centrifugation binding assays using large unilamellar phospholipid vesicles confirm that E54K binds PI(4,5)P2 with higher affinity than native enzyme. E54K is more active even though the interfacial binding constant (K_{d}) for E54K (0.034 ± 0.01 mol fraction PI(4,5)P2) is higher than the K_{d} for native enzyme (0.012 ± 0.002 mol fraction PI(4,5)P2), d-Insitol trisphosphate is less potent at inhibiting E54K PI(4,5)P2 hydrolysis compared with native enzyme. These results demonstrate that a single amino acid substitution in the PH domain of PLC δ1 can dramatically enhance enzyme activity. Additionally, the marked increase in V_{max} for E54K argues for a direct role of PH domains in regulating catalysis by allosteric modulation of enzyme structure.

In many cell types, ligand binding to integral membrane receptors leads to an increase in the intracellular second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. This increase results largely from activation of a family of phosphoinositide-specific phospholipase C (PLC) enzymes that hydrolyze polyphosphoinositides such as phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (3). IP_3 releases intracellular Ca^{2+} from the endoplasmic reticulum via interaction with a specific receptor located on the surface of the endoplasmic reticulum (3, 4). Diacylglycerol, as well as increased intracellular Ca^{2+}, activates protein kinase C (PKC) (5). Recent studies suggest that PLC δ1 can be stimulated both by a p122-Rho-GTPase-activating protein (6) and also by G_{12} (transglutaminase II) (7). Recent genetic studies in spontaneously hypertensive rats suggest that activity of this effector enzyme plays an important role in the control of blood pressure (5, 8), and light and electron microscopic studies of PLC δ in neurofibrillar tangles suggest a role in Alzheimer’s disease (9).

PLC δ1 hydrolyzes substrate at a lipid/water interface. Kinetic analyses developed by Dennis and co-workers (1, 2) are used to describe this type of enzymatic activity. According to this dual phospholipid binding model, a soluble enzyme must first associate with lipid (either specifically or nonspecifically) to anchor the enzyme to the lipid/water interface. Enzyme bound to the interface can then subsequently bind its substrate (within the interface) at the catalytic site where substrate hydrolysis occurs. For enzymes operating in the “soothing” or processive mode, multiple catalytic cycles can occur before the enzyme detaches from the interface (10, 11). The pleckstrin homology (PH) domain of PLC δ1 lies within the NH_{2}-terminal region of the enzyme. Both this NH_{2}-terminal region (12, 13) and the PH domain specifically (14) bind to PI(4,5)P2 with high affinity. Further, this binding is important for processive catalysis to occur (13, 14).

This study seeks to determine the mechanisms of PH domain-mediated activation of PLC δ1. A mutant PLC δ1 (E54K) was constructed to generate a PH domain enhancement-of-function mutant. This single amino acid substitution at position 54 of PLC δ1 dramatically increases the activity of this enzyme. Using kinetic analyses we show that changes in both the equilibrium binding constant (K_{d}) and V_{max} contribute to the increased rate of processive hydrolysis, whereas the interfacial binding constant (K_{d}) does not contribute. The position of this substitution within the PH domain of PLC δ1 uniquely demonstrates the functional importance of this domain. Further, the increase in V_{max} for E54K suggests a new role for PH δ1.

The abbreviations used are: IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PKC protein kinase C; PH domain, pleckstrin homology domain; PI, phosphatidylinositol; PE, phosphatidylethanolamine; LUV, large unilamellar vesicle; d-IP_3, d-myoinositol 1,4,5-trisphosphate; PC, phosphatidylcholine; PS, phosphatidylserine.

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domains as allosteric regulatory sites. We show that this PH domain-mediated change in $K_\text{d}$ and $V_{\text{max}}$ is a mechanism for PLC \( \delta 1 \) activation and propose that this may be a general mechanism for mutant enzymes in which overactivation leads to human disease.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phosphatidylinositol (PI) and phosphatidylethanolamine (PE) were from Avanti Polar Lipids. Radiolabeled phospholipids were purchased from DuPont NEN. PI(4,5)P$_2$ was from Calbiochem. PLC \( \delta 1 \) monoclonal antibodies were a gift from Dr. Steven Roffler, Academia Sinica, Taiwan. All other reagents were purchased from Sigma and were of the highest grade possible.

**Mutagenesis of Human PLC \( \delta 1 \)**—Replacement of the single glutamic acid residue at position number 54 with lysine in human PLC \( \delta 1 \) (E54K) was performed using the polymerase chain reaction described previously using the bacterial expression plasmid pRSETAplc (15). The mutant, E54K, was subsequently confirmed by DNA sequence analysis.

**Expression and Purification of PLC \( \delta 1 \)**—Escherichia coli (Novagen) were transformed with mutant as well as with wild-type PLC \( \delta 1 \) pRSETA constructs. At appropriate times, the culture was induced with 10 mM isopropyl-1-thio-\( \beta \)-galactopyranoside at 18 °C for about 8 h. The cells were collected by centrifugation and frozen at -20 °C. The remainder of the purification protocol was performed according to Cheng et al. (15). Briefly, cell pellets were ground to a paste in liquid nitrogen using a mortar and pestle. All subsequent steps were performed at 4 °C using prechilled buffers.

**Kinetics of Activated PLC \( \delta 1 \)**—Using PI(4,5)P$_2$ as a function of bulk lipid concentration in the mixed micelle. Thus, to determine the equilibrium dissociation constant for interfacial binding (denoted here as $K_{\text{m}}$), we measured substrate hydrolysis as a function of bulk substrate concentration while keeping the mol fraction of substrate constant (case I). Once the enzyme-mixed micelle complex forms substrate hydrolysis can then proceed at the catalytic site. In contrast to interfacial binding, interfacial catalysis is not dependent on bulk concentration of substrate but rather is dependent on the mol fraction of substrate in the lipid/mixed micelle. Therefore, to assay substrate binding to the catalytic site as well as the maximum rate of interfacial catalysis, both the interfacial Michaelis constant (denoted here as $K_{\text{m}}$) and interfacial $V_{\text{max}}$ were determined by measuring substrate hydrolysis with increasing mol fractions of substrate while keeping the bulk concentration of substrate constant (case II). Covarying bulk lipid concentration and mol fraction yielded a sigmoidal rate versus [substrate] curve indicative of cooperativity (case III).

**Data from all cases were fit to the Hill equation (16) (Equation 1)** using the program PRISM (Graphpad).

\[
V_{\text{max}} = [S] \\
K + [S] \\

\text{max} \text{ is the interfacial Michaelis-Menten constant, } K_{\text{m}} \text{ is the interfacial } V_{\text{max}} \text{ at a constant PI(4,5)P}_2 \text{ concentration of 100 } \mu \text{M, } X \text{ is the mol fraction of substrate, and } n_{\text{Hill}} \text{ is the Hill coefficient, and } K \text{ is a complex association factor.}
\]

**Determination of $K_{\text{m}}$ and $V_{\text{max}}$**—For case I data, Equation 1 reduces to the Henri-Michaelis-Menten equation or simple rectangular hyperbola with slope, $n$, equal to 1. Data were fit to Equation 2 from which values of the equilibrium dissociation constant ($K_{\text{m}}$) were obtained.

\[
v = \frac{V_{\text{max}} [S]}{K_{\text{m}} + [S]} \\

\text{Equation 2}
\]

**Determination of $K_{\text{m}}$ and $V_{\text{max}}$**—For case II data, a sigmoidal curve fit better than a rectangular hyperbola with slope equal to 1. Therefore, data were fit to Equation 3 using the Hill coefficient derived from case III data (16) and $K_{\text{m}}$ as well as $V_{\text{max}}$ determined.

\[
v = \frac{V_{\text{max}} [X]_{\text{Hill,III}}}{K_{\text{m}} + [X]_{\text{Hill,III}}} \\

\text{Equation 3}
\]

$K_{\text{m}}$ is the interfacial Michaelis-Menten constant, $V_{\text{max}}$ is the interfacial $V_{\text{max}}$ at a constant PI(4,5)P$_2$ concentration of 100 $\mu$M, $X$ is the mol fraction of substrate, and $n_{\text{Hill,III}}$ is the Hill coefficient derived from case III. Reaction was terminated by adding 2 volumes of CHCl$_3$/methanol/concentrated HC1 (100:100:0.6 v/v/v) followed by 1 volume of N HCl containing 5 mM EDTA. After vortexing and centrifugation at 10,000 $\times$ g for 5 min, the aqueous phase was removed and counted.

**Protein-Lipid Vesicle Binding Assay**—20 $\mu$g of PI(4,5)P$_2$ and 400 $\mu$g of PE were mixed and dried down to a thin film under N$_2$. PE at 400 $\mu$g/ml (520 $\mu$M) had previously shown no detectable protein binding. Lipid mixtures were lyophilized for >4 h and stored at -80 °C. Before use, 1 ml of 180 mM sucrose was added to the lipid mixtures and bubbled with N$_2$ for 5 min. One ml of 2 $\times$ binding buffer (100 mM HEPES, pH 7, 200 mM KCl, 10 mM EGTA, 10 mM EDTA) was added. The lipid mixture was centrifuged for 30 min at 2,000 $\times$ g at 4 °C. The pellet was redissolved in 1 ml of 50 mM HEPES, 100 mM KCl, 5 mM EGTA, 5 mM EDTA, and 200 $\mu$g/ml bovine serum albumin. This 1 $\times$ PI(4,5)P$_2$/PE lipid vesicle solution was serially diluted into solution concentrations of 0.07-18 $\mu$M PI(4,5)P$_2$ and 2.0-260 $\mu$M PE (constant mol fractions).

Enzyme was added to 190 $\mu$l of the PI(4,5)P$_2$/PE mixture and allowed to incubate at room temperature for 10 min. Enzyme/lipid mixtures were centrifuged at 4 °C, at 200,000 $\times$ g for 40 min. Supernatants were removed, and the pellet was redissolved in sodium dodecyl sulfate-protein buffer and run in a 15% acrylamide gel. The proteins were then immunoblotted.

**Analysis of Kinetic Data**—The kinetics for the native and mutant enzyme were fit to the dual phospholipid binding model originally used to describe the kinetics of phospholipase $A_2$ (1, 2) (Equation 1).

**RESULTS**

**Mutagenesis of PLC \( \delta 1 \)**—The cdNA for human PLC \( \delta 1 \) was mutated to substitute a lysine for glutamic acid at residue
position 54 (Fig. 1A). This residue lies in the β3/β4 loop of the PH domain of PLC δ1 (17). The backbone carboxyl of Glu-54 interacts with the 5 position of the inositol headgroup via a water-mediated hydrogen bond (17). We hypothesized that lysine substitution at this position would enhance affinity for phosphoinositides. This might occur because lysine is a fully charged species at physiological pH, and this ionic interaction would lead not only to displacement of a water molecule but also to enhanced bonding of the positively charged lysine with the negatively charged oxygen at the 5 phosphate of the inositol head group (Fig. 1B). Purified proteins were assayed for their ability to hydrolyze PI in a sodium deoxycholate/PI mixed micelle assay. Both native and mutant enzyme showed a very similar specific activity toward hydrolysis of PI, 0.95 μmol/min/mg for PLC δ1 and 1.2 μmol/min/mg for E54K, ensuring that the substitution E54K has not globally affected enzyme structure or activity.

**Mutant PLC δ1 E54K Has Increased Hydrolytic Activity—**

PI(4,5)P$_2$ hydrolysis was assayed according to case III conditions as described under "Experimental Procedures." Substrate hydrolysis was measured simultaneously varying the bulk concentration and mol fraction of PI(4,5)P$_2$. This was accomplished using multiple assays where the concentration of dodecyl maltoside was kept constant at 200 μM (to ensure that detergent was greater than the critical micellar concentration) and the PI(4,5)P$_2$ concentration increased from 1 to 75 μM. With dodecyl maltoside as diluent, rates of substrate hydrolysis are linear over 4 min, with $r^2$ values ranging from 0.95 to 0.99. When solid lines were fit to both sets of data using Equation 1 from "Experimental Procedures," the Hill coefficient of PLC δ1 was calculated to be 2.2 ± 0.2 and for E54K was calculated to be 2.0 ± 0.1 (Table I). Further, Fig. 2 shows that E54K has a dramatically enhanced rate of catalysis. Thus, the single substitution in the PH domain of this PLC δ1 dramatically enhances its rate of hydrolysis but does not change the inherent cooperativity in enzyme activity.

**Determination of $K_m$—**

The effect of the mutation E54K on secondary binding within the interface ($K_m$) was determined by assaying native and mutant activity according to case II conditions as described under "Experimental Procedures." Multiple measurements of enzyme activity were made at a constant bulk concentration of PI(4,5)P$_2$ (100 μM) was chosen as this value is similar to the calculated $K_m$ for E54K (Table I) and varying mol fractions of substrate (0.01–0.25 mol fraction PI(4,5)P$_2$ using dodecyl maltoside as diluent (Fig. 3). The mol fraction of PI(4,5)P$_2$ was not increased above 0.25 because the kinetics of PI(4,5)P$_2$ hydrolysis deviated from linearity above 0.25 (data not shown) as has been observed by other investigators (16). Solid lines were fit to the data using Equation 3.}

### Table I

|          | Hill coefficient $n$ | Interfacial $K_{m}$ | $K_s$ | $V_{max}$ |
|----------|----------------------|---------------------|-------|-----------|
| PLC δ1   | 2.2 ± 0.2            | 0.012 ± 0.002       | 210 ± 48 | 4.9 ± 0.3 |
| E54K     | 2.0 ± 0.1            | 0.034 ± 0.01        | 85 ± 36 | 31 ± 3    |

**Fig. 1.** Schematic representation of PLC δ1 and proposed interaction between K54 and IP$_3$. Panel A, PLC δ1 is schematically represented showing locations of X, Y, and PH domains. The glutamic acid residue at position 54 was replaced by a basic lysine residue. Position 54 is within the PH domain (amino acids 16–124). Panel B, the diagram of the PH domain/IP$_3$ binding site is based on the crystal structure of PLC δ1 PH domain complexed with IP$_3$ (17). The substituted lysine residue at position 54 is proposed to interact with an oxygen at the 5 phosphate position of the substrate inositol head group.

**Fig. 2.** PLC δ1 and E54K show the same degree of catalytic cooperativity, but E54K has a higher initial rate of PI(4,5)P$_2$ hydrolysis. Initial rates of substrate hydrolysis are shown for native (■) and mutant (▲) enzyme as a function of both bulk PI(4,5)P$_2$ concentration and mol fraction of substrate (case III). Activity toward PI(4,5)P$_2$/dodecyl maltoside mixed micelles was assayed according to case II conditions as stated under “Experimental Procedures” with the concentration of dodecyl maltoside fixed at 200 μM and the concentration of PI(4,5)P$_2$ varied. Solid lines were fit to raw data using Equation 1 from which Hill coefficients were obtained (Table I). Error bars represent the S.E. (n = 2) and are shown when larger than the symbol.
Kinetics of Activated PLC δ1

Fig. 3. PLC δ1 and E54K have similar $K_m$ values though E54K has a higher $V_{max}$, for PI(4,5)P$_2$ hydrolysis. Initial rates of substrate hydrolysis are shown for native (■) and mutant (▲) enzyme as a function of PI(4,5)P$_2$ surface concentration or PI(4,5)P$_2$ mol fraction (case II). Activity toward PI(4,5)P$_2$/dodecyl maltoside mixed micelles was assayed for case II conditions according to “Experimental Procedures.” The dodecyl maltoside concentration was varied to vary the PI(4,5)P$_2$ mol fraction, and the PI(4,5)P$_2$ concentration was fixed at 100 μM. Solid lines were fit to raw data using Equation 3 from which values of $K_m$ and $V_{max}$ were obtained (Table I). Error bars represent the S.E. ($n = 2$) and are shown when larger than the symbol.

Fig. 4. E54K has both a lower $K_m$ value than PLC δ1 as well as a higher initial rate of PI(4,5)P$_2$ hydrolysis. Initial rates of substrate hydrolysis are shown for native (■) and mutant (▲) enzyme as a function of bulk PI(4,5)P$_2$ concentration (case I). Activity toward PI(4,5)P$_2$/dodecyl maltoside mixed micelles was assayed for case I conditions according to “Experimental Procedures.” PI(4,5)P$_2$ concentration and dodecyl maltoside were covaried while keeping the PI(4,5)P$_2$ mol fraction constant at 0.04. Solid lines were fit to raw data using Equation 2 from which values of $K_m$ were obtained (Table I). Error bars represent the S.E. ($n = 2$) and are shown when larger than the symbol.

other independent measure of the relative affinities of native and mutant enzyme for PI(4,5)P$_2$/PE LUVs. E54K was associated with LUVs at much lower concentrations of PI(4,5)P$_2$ than is the case for PLC δ1 (Fig. 5). At about 1.1 μM PI(4,5)P$_2$ PLC δ1 was no longer associated with LUVs, suggesting that it no longer bound to PI(4,5)P$_2$ at this concentration. However, even at concentrations as low as 0.07 μM PI(4,5)P$_2$ E54K still associated with the LUVs. Neither enzyme bound to PE LUVs alone over the range of PE concentrations used in this assay (data not shown).

$PI(4,5)P_2$ Enhances the Rate of PI Hydrolysis for Both PLC δ1 and E54K—PI hydrolysis was assayed according to case III conditions described under “Experimental Procedures.” The Hill coefficient of PLC δ1 was calculated to be 1.9 ± 0.2 and for E54K was calculated to be 2.0 ± 0.4 (Fig. 6). Fig. 6 shows that both PLC δ1 and E54K have similar rates of catalysis for PI in the absence of PI(4,5)P$_2$ ($V_{max} = 15 ± 2$ nmol/min/mg and $V_{max}$ for E54K = 18 ± 2 nmol/min/mg). Assays were also performed in the presence of 50 μM PI(4,5)P$_2$. Hydrolysis of substrate under these conditions was still cooperative with Hill coefficients of 1.5 ± 0.3 for PLC δ1 and 1.6 ± 0.8 for E54K. In the presence of PI(4,5)P$_2$ (Fig. 6), PLC δ1 activity was enhanced 2-fold ($V_{max} = 27 ± 3$ nmol/min/mg), but E54K activity was enhanced more than 5-fold ($V_{max} = 95 ± 10$ nmol/min/mg).

$\beta$-IP$_3$ Inhibits PLC δ1 and E54K—$\beta$-IP$_3$ inhibited PI(4,5)P$_2$ hydrolysis for both native and mutant enzyme. Dodecyl maltoside/PI(4,5)P$_2$ mixed micelles were assayed according to “Experimental Procedures” with constant PI(4,5)P$_2$ (1 μM) and dodecyl maltoside (200 μM) concentrations. $\beta$-IP$_3$ (0 to 25 μM) was added to each assay prior to addition of enzyme. Both enzymes were inhibited by $\beta$-IP$_3$ in a dose-dependent manner.

(“Experimental Procedures”) which describes case II activity. The data fit a sigmoidal curve better than a hyperbolic one. This result has also been noted when measuring case II activity of PLC δ (16) and phospholipase A$_2$ (1). For PLC δ1, $K_m = 0.012 ± 0.002$, and for E54K $K_m = 0.034 ± 0.01$ (Table I). $V_{max}$ for PLC δ1 was 4.9 ± 0.3 μmol/min/mg and 31 ± 3 μmol/min/mg for E54K (Table I).

Determination of $K_m$—The effect of E54K on initial binding to the interface was determined by assayng enzyme activity according to case I conditions as described under “Experimental Procedures.” Multiple assays were performed with varying bulk concentrations of PI(4,5)P$_2$ (10–325 μM) and PI(4,5)P$_2$ mol fraction constant. This was achieved by increasing the bulk concentration of PI(4,5)P$_2$ (adding more lipid) while simultaneously increasing the concentration of dodecyl maltoside thus ensuring that the PI(4,5)P$_2$ mol fraction was kept constant at 0.04. This value was chosen as it approximates the $K_m$ for E54K. Solid lines were fit to the data using Equation 2 which simply describes a rectangular hyperbola with slope equal to 1 from which values of $K_m$ were determined. E54K was much more active than native enzyme even at relatively low substrate concentrations (Fig. 4). The $K_m$ for PLC δ1 was calculated to be 210 ± 48 μM, whereas the $K_m$ for E54K was only 85 ± 36 μM (Table I). These values were within the range of constants determined previously for PLC δ (16), PLC γ (18, 19), and PLC δ1 (13, 14).

In addition to the kinetic analysis, a thermodynamic equilibrium centrifugation binding assay was done to examine further initial binding to substrate. This assay was not used quantitatively since the kinetic data were more reliable, and the equilibrium association constant $K_a$ (or $K_{eq}$) has not always provided good agreement with affinity constants determined kinetically (13, 16). However, it was used qualitatively as an-
FIG. 5. E54K has a higher affinity for PI(4,5)P₂ in LUVs than native PLC δ1. Native and mutant enzyme were incubated with sucrose-loaded LUVs (20:1 PE/PI(4,5)P₂) with a range of PI(4,5)P₂ concentrations (0.07–18 μM). This was followed by ultracentrifugation as described under “Experimental Procedures.” PLC δ1 and E54K protein remaining in the pellet were quantitated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot and were compared with controls. PLC δ1 bound to LUVs with PI(4,5)P₂ concentrations as low as 1.1 μM. E54K also bound to LUVs but bound at PI(4,5)P₂ concentrations below 1.1 μM even down to 0.07 μM, suggesting that E54K has a much higher affinity for PI(4,5)P₂ in sucrose-loaded LUVs. The experiment shown is representative of multiple experiments that gave relatively similar results within each assay (E54K bound better than PLC δ1), but variability among assays precludes determination of rigorous binding constants.

(Fig. 7). The shift in the dose-response curve to the right for E54K was consistent with data demonstrating higher interfacial binding but might also suggest that this mutant bound D-IP₃ less well than native enzyme.

DISCUSSION

The dual phospholipid model (1, 2) is used extensively to characterize the activity of a number of enzymes that operate processively at a lipid/water interface (1, 16, 19). According to this model, enzymes must first associate with an interface that contains substrate to form an enzyme-interface complex (Reaction 1). Enzyme attachment to the interface has been shown to occur via protein/lipid interactions (13, 14, 16, 20) but could also occur via protein/protein interactions. Interface binding can be due to specific or nonspecific recognition of anchoring ligand, which may or may not be a true substrate for the enzyme. The model necessitates two distinct binding sites: an anchoring and catalytic site. For PLC δ1, these two sites exist in a single molecule (13–15). Interface binding is mediated through an NH₂-terminal PH domain (13, 14). This domain has been shown in PLC δ1 to bind polyphosphoinositides with high affinity and specificity (13, 14, 17, 21). Secondary binding of substrate occurs at the catalytic domain with a lower affinity (100 μM) (13, 14, 17, 21, 22).

The tertiary structure of the PLC δ1 PH domain complexed to IP₃ was recently solved by x-ray crystallography at a resolution of 1.9 Å (17). IP₃ binds to the PH domain of PLC δ1 with very high affinity (Kₐ = 210 nM) (17). Other investigators functionally demonstrate a high affinity interaction among IP₃, PLC δ1 (23), and its PH domain (21). Several residues in the PLC δ1 PH domain interact directly with the phosphates of the inositol headgroup. These include basic residues Lys-30, Lys-32, and Lys-57 as well as a Van der Waals interaction between Trp-36 and the inositol ring (17). In fact, our laboratory has shown the functional importance of many of these residues. Substitution at this position (Fig. 1A) changes the charge state from −1 to +1, providing a positively charged species that may enhance binding to a negatively charged phosphate of the inositol head group for IP₃ as well as other phosphoinositides (Fig.

![Diagram](Image)

FIG. 6. PI(4,5)P₂ enhances PI hydrolysis for PLC δ1 and E54K, but E54K is enhanced more than PLC δ1. Panel A, native enzyme initial rates of substrate hydrolysis are shown as a function of both bulk PI concentration and mol fraction of substrate (case III). Activity toward PI/dodecyl maltoside mixed micelles was assayed according to case III conditions as stated under “Experimental Procedures” with the dodecyl maltoside concentration fixed at 200 μM. Calcium concentration fixed at 1 mM, and PI concentration varied. Solid symbols indicate activity in the absence of any PI(4,5)P₂. Open symbols indicate activity in the presence of 50 μM PI(4,5)P₂. Panel B, mutant enzyme was assayed equivalently. Solid lines were fit to raw data using Equation 1 from which Vₘₐₓ and Hill coefficients were obtained. Error bars represent the S.E. (n = 2) and are shown when larger than the symbol.

1B) (17). We propose that the tighter binding of PI(4,5)P₂ to E54K is because of stronger direct ionic interactions with the lysine and elimination of potentially unfavorable interactions between the negatively charged native glutamic acid and PI(4,5)P₂.

PI hydrolysis in PI/sodium deoxycholate mixed micelles was used to assess PLC δ1 activity during and following purification. Both native (0.95 μmol/min/mg) and mutant (1.2 μmol/min/mg) enzyme have a similar specific activity toward hydrolysis of PI in PI/deoxycholate micelles, indicating that the tertiary structure of E54K has not been adversely affected by the amino acid substitution. A PI(4,5)P₂/dodecyl maltoside mixed micelle assay was used to characterize the kinetics of enzyme activity. This system has been used to characterize PLC δ1, PLC δ2, turkey erythrocyte PLC (16), and PLC δ1 (13).

The main advantage in using dodecyl maltoside is that it is an inherently neutral diluent of PI(4,5)P₂. Increasing mol fractions of detergent are not inhibitory toward PLC activity (16). As reported by other groups (13, 14, 16), we find that so-called neutral diluents such as phosphatidylcholine (PC) and phosphatidylserine (PS) can affect PLC activity with PC being inhibitory and PS being stimulatory (data not shown). This makes these lipids less desirable as diluents of substrate since
they are not true neutral diluents, and observed effects on PI(4,5)P₂ hydrolysis might be attributed to various complex diluent effects instead of substrate effects.

In agreement with several investigators (13, 14, 24), PLC δ1 hydrolysis of PI(4,5)P₂ and PI is processive and cooperative. The Hill coefficients for native and mutant enzyme for hydrolysis of substrates PI(4,5)P₂ and PI range from 1.5 to 2.2 (Table I). This suggests that at least 2 mol of substrate bind per mol of both PLC δ1 and E54K. Further, the similarity among Hill coefficients for native and mutant enzyme for both PI(4,5)P₂ and PI substrate suggests that the E54K mutation has not affected the inherent processivity of PLC δ1. A likely model for this processivity is that one molecule of substrate must bind to the catalytic site with the second molecule of PI(4,5)P₂ binding to the PH domain (13, 14, 16, 25) which serves as an anchor or site of allosteric modulation. Clearly, the PH domain is not solely responsible for processivity even among PLC enzymes. The PI-PLC from Bacillus cereus also hydrolyzes PI processively even though this enzyme contains no PH domain (26). In addition, human PLC δ1 that has 70 amino acids deleted from the PH domain can hydrolyze PI processively (14).

To understand fully the mechanisms of activation of enzymes that act at a lipid/water interface, it is necessary to determine the contribution of interfacial binding and catalysis in overall enzyme activation. Hendrickson and Dennis (20) demonstrate that didecanoyl-PC activation of secretory phospholipase A₂ substrate (thio-PE) hydrolysis is dependent on the surface concentration of didecanoyl-PC rather than on the bulk concentration of this activator. Further, they find that didecanoyl-PC activation of thio-PE substrate results not from an increase in secondary binding within the interface (no change in Kₐ) but by increasing the catalytic rate or efficiency of the enzyme (by increasing the Vₘₐₓ). Studies with the phospholipase A₂ inhibitor mananolide also show that interface binding is not crucial for PC stimulation of PE hydrolysis (27).

Previous studies with PLC enzymes indicate that changes in Kₐ, Kₐ', and Vₘₐₓ mediate enzyme activation. Wahl et al. (19) find that epidermal growth factor-activated enzyme has a 4-fold lower Kₐ for PI(4,5)P₂ hydrolysis as well as a 7.5-fold lower Kₐ. This demonstrates that both interfacial binding as well as secondary substrate binding within the interface mediate the epidermal growth factor stimulation of PLC γ1 PI(4,5)P₂ hydrolysis. The Vₘₐₓ values for activated and inactivated enzymes are equivalent, suggesting that activation does not affect catalytic efficiency. Work from our laboratory (14) shows that PI(4,5)P₂-mediated stimulation of PLC δ1 PI hydrolysis results from decreases in both Kₐ and Kₐ', although the largest magnitude change is in Kₐ. Again, Vₘₐₓ values for activated and unactivated enzymes are equivalent. Jones and Carpenter (18) report that phosphatidic acid enhances PI(4,5)P₂ hydrolysis not by increasing the affinity of PLC γ1 for substrate micelles but by decreasing the Kₐ of the activated enzyme 10-fold and increasing the Vₘₐₓ 4-fold. These data suggest that phosphatidic acid activation of PLC γ1 occurs not by affecting the affinity of enzyme for initial binding to substrate but by affecting secondary binding within the substrate interface or by affecting interfacial catalytic efficiency. βγ subunits of heterotrimeric G-proteins activate the PLC β2 enzyme (28, 29). Romoser et al. (28) find that at activating concentrations of βγ subunits, PLC β2 does not show any increased affinity for PI(4,5)P₂/lipid vesicles (28). Although the authors do not demonstrate the mechanism of activation, their data suggest that interfacial binding does not play a role in activation by βγ subunits.

The results reported here demonstrate that catalytic efficiency (Vₘₐₓ) and interfacial binding (Kₐ) are the primary determinants of PLC δ1 enhanced activity. The PH domain mutant E54K has a markedly enhanced catalytic activity (Fig. 3). Kinetic analyses show that E54K has a much higher Vₘₐₓ (31 μmol/min/mg) than does PLC δ1 (4.9 μmol/min/mg) (Table I). However, the Kₐ for E54K is actually a bit higher (0.034 mol fraction PI(4,5)P₂) than for PLC δ1 (0.012 mol fraction PI(4,5)P₂) (Table I). These data suggest that the affinity for PI(4,5)P₂ binding within the interface at the catalytic site is unchanged or even lower so that, all things equal, E54K and native enzyme should have the same rates of substrate hydrolysis, or E54K should hydrolyze substrate less well. Therefore, increased catalytic efficiency (Vₘₐₓ) is likely a determining factor in the observed enhanced activity of E54K.

E54K shows a reduction of approximately 2.5-fold in the Kₐ (85.0 μM) compared with native enzyme (211.5 μM) (Fig. 4 and Table I). These data suggest that the increased activity of E54K is partially a result of enhanced initial binding to the interface. A centrifugation binding assay with LUVs confirms the enhanced interface binding of E54K. E54K is able to bind to lower concentrations of PI(4,5)P₂ in LUVs than PLC δ1. These data are not examined quantitatively in part because the kinetic constants are more rigorous, and the equilibrium association constant Kₐ (or 1/Kₐ') has not always provided good agreement with affinity constants determined kinetically (13, 16). The poor agreement between Kₐ and Kₐ' may be the result of different lipid interfaces differentially affecting enzyme vesicle binding affinity (14, 16). In addition, the assumption of 1:1 stoichiometry between PI(4,5)P₂ and enzyme might be violated given that results for case II data are sigmoidal, indicating cooperativity of binding within the interface. Among other things this may indicate the presence of another PI(4,5)P₂ binding site within the interface. Some likely structural candidates might be the C2 domain (30) or the gelsolin-like PI binding domain
Both native and E54K enzyme can be inhibited by low concentrations of D-IP3 (Fig. 7). Interestingly, the data show that D-IP3 is less potent at E54K than native PLC η1 (Fig. 7). Although the mechanism for this difference is unclear, it might be because of E54K increased affinity for PI(4,5)P2. These data are somewhat surprising given that D-IP3 and PI(4,5)P2 have been shown to have similar high affinities for the PLC η1 PH domain (21, 35), which suggests that the structural determinants mediating a high affinity interaction between PH domain and the phosphoinositides are in the polar head group (17). The mechanisms of this differential inhibition will be explored in the future.

In summary, we have found that a single amino acid substitution of PLC η1 leads to enzyme activation. The mutation is a lysine for glutamic acid substitution at position 54 within the PH domain. The native and mutant enzymes were characterized using the dual phospholipid binding model of Hendrickson and Dennis (1). The data show that both native and mutant enzymes operate in the same processive mode of hydrolysis, both binding to 2 mol of PI(4,5)P2/mol of enzyme. The interfacial Vmax for E54K is about 6-fold greater than that for native PLC η1. The data also show that E54K shows no increase in substrate binding within the PI(4,5)P2/dodecyl maltoside micelle interface (Ks). However, in both kinetic and thermodynamic assays, E54K has an increased affinity for initial substrate binding. In fact, the Ks for E54K is approximately 2.5-fold lower than that of native enzyme. PI(4,5)P2 also enhances PI hydrolysis for PLC η1 by almost 2-fold but more than 5-fold for E54K. Thus, both increased interfacial binding and increased catalytic efficiency enhance enzyme activity.

The PH domain mediates these effects in PLC η1. It is interesting to speculate that this same mechanism of activation may operate for other enzymes as well. The small nonreceptor tyrosine kinase, Bruton's tyrosine kinase, Bruton's tyrosine kinase, also has a PH domain. In fact, when the conserved mutation E54K is made in this enzyme (E41K), the result is a 100-fold increase in cell growth in NIH-3T3 cells and enhanced Bruton's tyrosine kinase membrane association (36). Although the mechanism for this gain of function is not clear, recent evidence shows that Bruton's tyrosine kinase PH domain containing the E41K mutation binds to inositol 1,2,3,4,5,6-hexakisphosphate 2-fold better than native Bruton's tyrosine kinase PH domain (37). Given that the PH domain is the structural determinant mediating PLC η1 membrane translocation (38) and enzyme activation (14), it is possible that the in vivo transformation and membrane localization seen in the mutant Bruton's tyrosine kinase follow the mechanism we have proposed for E54K/PLC η1. There is already ample evidence demonstrating that the PH domain of various enzymes mediates enzyme translocation to membranes (14, 36, 38, 39). Our data show the importance of membrane association and allosteric modulation in mediating enzyme activation in vitro.

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