Analysis of PI-PLC Binding to PC and PMe Vesicle Surfaces Using EPR and NMR

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Introduction

Phosphatidylinositol-specific phospholipase C (PI-PLC) is an enzyme with an important role in membrane-associated signal transduction in eukaryotes. Bacterial PI-PLC are secreted and have a simpler structure than the eukaryotic enzymes (1). These peripheral membrane proteins, about 35 kD in size are considered to be possible factors in pathogenicity in bacteria such as *Bacillus cereus* and *Listeria monocytogenes* (2). PI-PLC is a water-soluble enzyme that cleaves the natural membrane lipid phosphatidyl inositol (PI) (2), converting it to diacylglycerol (DAG) and the water-soluble head group, cIP (D-myo-inositol 1,2-cyclic-phosphate) through an intramolecular phosphotransferase step (1). There is then a cyclic phosphodiesterase reaction that converts cIP to I-1-P (D-myo-inositol 1-phosphate). PI hydrolysis may occur through general acid and base catalysis using two histidine residues (5).

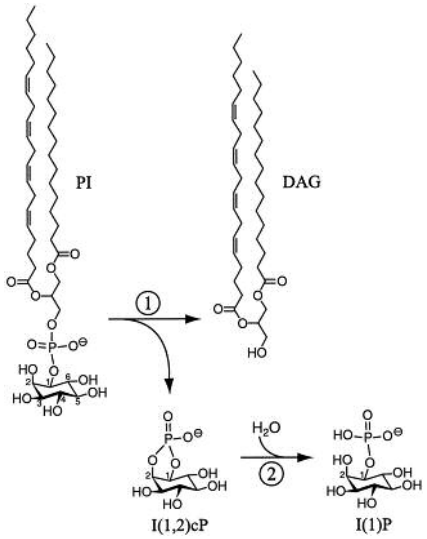

Figure 1. The reaction catalyzed by bacterial PI-PLC enzymes. The primary reaction occurs at the lipid-water interface of the PI contained in a vesicle. cIP is released into the aqueous phase and the second reaction, hydrolyzing cIP to I-1-P, occurs 10^{-3} times slower than the first.

PI-PLC has been shown to prefer micellar PI to monomeric PI (5), and the enzyme is activated for cleavage of PI and hydrolysis of cIP by interfaces of PC, a nonsubstrate that does not bind at the active site (4). Studies involving PC/PI vesicles have demonstrated that at the PI interface, PC vesicles allosterically bind PI-PLC. This

attaches the enzyme to the PI interface in an active conformation (6). PC has also been shown to activate PI-PLC toward cIP hydrolysis to I-1-P (7).

The crystal structure of the similar PI-PLC from *Bacillus cereus* shows that PI-PLC is composed of a distorted $(\alpha\beta)_6$ barrel (Figure 10) (2, 3). The rim of the active site has a short helix B and a particular mobile loop composed of seven hydrophobic amino acids fully exposed to solvent (4). Several studies have examined the role of tryptophan residues in helix B and the mobile rim loop (1, 4). The secondary structure features could be important in binding the fatty acyl chains of PI and the PC activator. PI-PLC mutants with replacement of the bulky hydrophobic amino acids show reduced affinity for PC surfaces. Tryptophan-242 is solvent exposed in PI-PLC and has been implicated in inserting into bilayers. It is an important residue in binding interfaces, both activating zwitterionic and substrate anion surfaces (4). This characteristic makes the tryptophan residue an interesting site when examining PI-PLC docking to the membrane. Trp 242 and Tyr 57, a residue that is farther away from the active site and is thought to be more distant from the phospholipid bilayer, were replaced with cysteines for introduction of a spectroscopic probe (Figure 17). Reaction of the Cys thiol with a nitroxide spin label introduces an unpaired electron at a site that should sense membrane docking of the protein. In this experiment, phosphatidyl-methanol (PMe) was used as a stand-in for the negatively charged PI substrate, and a comparison of PI-PLC interaction with this phospholipid and with the zwitterionic PC nonsubstrate was done in order to better understand the surface interaction of PI-PLC.

Examining protein docking, however, presents some challenges. High-resolution structure determination cannot visualize the interface between protein and the

phospholipid bilayer. Previous studies have shown that use of EPR site-directed spin labeling can provide a structural analysis of this interface (8). By replacing a residue with a cysteine, a disulfide bond can form via a disulfide exchange reaction between this residue and a methanethiosulfonate spin-label (Figure 11). EPR provided some information as to mobility of the protein at the position of the spin label. This information showed that the protein was slightly less mobile at position 242 than 57 upon addition of vesicles, indicating that perhaps the spin label at W242C was partitioned into the vesicle and therefore slightly more immobilized. Difficulties with aggregation of protein during this procedure made further analysis necessary in order to draw any definite conclusions.

NMR has also been used to examine phospholipid dynamics (9). Changes in linewidths of both ^{1}H and ^{31}P resonances of the phospholipids can indicate interactions between the protein and phospholipids. In this experiment with spin-labeled PI-PLC, NMR was useful in examining how PI-PLC binds to POPC and DOPMe vesicles. My NMR results indicate that PI-PLC may have greater interactions with the PMe phosphorus moiety than with the PC phosphorus. The ^{1}H data suggest that the $–OCH_3$ group on PMe is more greatly affected by the presence of the spin label than the $-N(CH_3)_3$ group. It also appears from this data that the spin labeled W242C amino acid inserts into the bulk methylene region of the vesicles. Results concerning the affect of the spin labeled Y57C mutant showed similar results, although linewidth changes exhibited larger than expected errors. Based on these results, it does appear that the amino acid at 242 interacts strongly with the PMe substrate analogue. This suggests that the mobile loop is key to orienting the substrate in the active site.

Materials and Methods

Chemicals. 1-palmitoyl-2-oleoylphosphatidylcholine (POPC or PC), dioleoylphosphatidylmethanol (DOPMe), diC_7PC (1,2-diheptanoylphosphatidylcholine), and PI were purchased from Avanti Polar Lipids. 1-Oxyl 2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methylmethanethiosulfonate (MTSL) was purchased from Toronto Research Chemicals. SDS-PAGE molecular weight markers and Coomassie brilliant blue 250 were obtained from Sigma. The Q-Sepharose fast flow resin and Phenyl-Sepharose resin were obtained from Pharmacia. BL21(DE3)-RIL cells (4) were purchased from Stratagene. Plasmids with mutations at positions Y57C and W242C for PI-PLC were obtained from graduate student Xin Zhang.

Media and Plates. Luria-Bertani (LB) broth has 5 g yeast extract, 10 g NaCl, and 10 g of tryptone per liter and 34 μg/ml chloroamphenicol and 100 μg/ml ampicillin. LB agar plates had 5 g yeast extract, 10 g NaCl, 10 g tryptone, and 20 g agar per liter and 34 μg/ml chloroamphenicol and 50 μg/ml ampicillin. SOC broth, which is used for growth of high efficiency competency bacterial cells, was prepared from 10 ml of 2 M filter-sterilized glucose per liter, and SOB broth, which is made from 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl along with 10 ml 1 M $MgSO_4$ per liter.

Transformation and Over expression of Recombinant PI-PLC Protein. The recombinant plasmids were transformed into BL21(DE3)-RIL competent cells for expression. 1 μl recombinant plasmid (Y57C or W242C plasmids) was added to 100 μl chilled competent cells and placed on ice for 30 minutes. The competent cells were then heat shocked in a 42.1 °C water bath for 40 seconds, and then was immediately placed on ice. 500 μl SOC media was added to transformed cells, and this was shaken at 200 rpm

for one hour. 200 µl of this transformation was spread onto LB agar plates and this was incubated overnight at 37 °C. A single colony from the plate was then grown in 5 ml LB medium prepared as described at 37 °C until the optical density at 600 nm was 0.6. 2 mL of this culture were used to inoculate 2 L of LB medium containing antibiotics, which was then incubated at 37 °C. When the optical density of the culture was 0.7 at 600 nm, IPTG was added to a final concentration of 0.4 mM. Incubation was continued another 3 hours until optical density reached 1.5. Cultured cells were then harvested by centrifugation at 5000 rpm for 10 minutes and stored at -20 °C until used.

Purification of PI-PLC Protein from Cell Pellets. 15 ml of 20 mM Tris buffer, pH 8.9, were added to cell pellet, which was then dispersed and sonicated. After sonication, cells were centrifuged at 15,000 rpm for 50 minutes. The supernatant was removed and dialyzed overnight against 20 mM Tris, pH 8.9. The resulting protein solution was then applied to a Q-Sepharose Fast Flow column and eluted with a gradient of 0.0-0.6 M NaCl in 20 mM Tris-HCl pH 8.8 (80 ml total). The fractions containing PI-PLC were detected through use of SDS-PAGE (12% cross-linked gels were used). After staining for one hour with Coomassie blue, the gels were destained overnight. The PI-PLC band appeared at about 36 kD, and fractions containing the protein (~12 ml) were collected and then passed through a Phenyl-Sepharose column. The protein was eluted with a gradient of 0.6-0.0 M NaCl in 20 mM Tris-HCl, pH 8.8 (80 ml total). Fractions were then analyzed on 12% SDS gels again, and the fractions containing PI-PLC (~25 ml) were set aside for determination of concentration.

Lowry Assay for Protein Concentration. A protein standard was prepared with 0.4 mg/ml bovine serum albumin (BSA). 12 different concentrations were created with

varying volumes of 50 mM Tris buffer pH 8 and 2 ml of Lowry reagent (2% sodium carbonate in 0.1 N NaOH with 1.25 ml of 1% sodium tartrate and 1.25 ml of copper sulfate) in each. 200 µl of 1:1 Folin reagent:water was then added to each. The absorbance at 750 nm was measured (Spectronic 20 Genesys spectrophotometer) for each sample to generate a standard curve. 4 samples of each mutant PI-PLC of varying concentrations were mixed with the Lowry reagents, and the absorbance was measured at 750 nm. Protein concentrations were determined based on the standard curve.

Modification with MTSL. It was necessary to use a 5-10 fold excess of spin label compared to protein in order to modify the Cys on all protein. A 1.4 mg/ml stock solution of spin label in buffer was used. Half of the necessary spin label was added, vortexed shortly, and then kept at -4 °C for three hours. The other half of the spin label was then added, vortexed shortly, and kept at -4 °C overnight. 12 m l of 50 mM Tris, pH 8.0, was then added to each of the protein samples, and the samples were centrifuged at 5000 rpm for 30 minutes. This step was repeated three more times. Originally, no additional preparative step was used, but EPR results showed that free spin label was present, so dialysis was used to remove free nitroxide. The protein was dialyzed against 50 mM Tris buffer at both pH 8.0 and then pH 7.0, against 50 mM Tris with 50 mM myo-inositol (competitive inhibitor) at both pH 8.0 and then pH 7.0, and against 50 mM Tris with 100 mM NaCl at both pH 8.0 and then pH 7.0, and finally against 50 mM Tris pH 8.0 again. The resulting protein was concentrated in a VivaScience Vivaspin 20 ml Concentration filter at 5000 rpm for 30 minutes until the concentration was greater than 5 mg/ml. Protein concentration was again analyzed by the Lowry Assay.

Circular Dichroism Spectroscopy. Secondary structure of mutant PI-PLC and mutant PI-PLC with spin label was measured by CD spectroscopy using an AVIV 202 spectrophotometer. Wavelength scans were done between 190 nm and 260 nm, and were performed at 25 °C. CD scans showed that both the spin labeled and unlabeled proteins for both mutants were folded correctly.

Kinetic Analysis of PI PLC Mutants for Activity. Activity of the proteins was assayed by use of ^{31}P NMR spectroscopy with a Varian INOVA 500 NMR spectrometer. A solution containing 160 µl of 20 mM PI with 80 mM diC_7PC was diluted to a final concentration of 8 mM PI with 32 mM diC_7PC with 4 µl BSA, 226 µl 50 mM Hepes, pH 7.5, 250 µl D_2O, and EDTA (1 mM) was prepared for a total volume of 640 µl. A 400 µl aliquot was analyzed as a control. 10 µg of the spin labeled enzyme was then added and NMR scans were accumulated in 5 minute increments for 30 minutes. Both mutant PI-PLC proteins were analyzed in this manner. The rate of decrease of the substrate (PI) and rate of increase to cIP and I-1-P was then determined and activity of each enzyme could be compared. The assays showed that both spin labeled Y57C and W242C were active, with the spin labeled Y57C mutant converting PI to cIP at approximately 2.3 times the rate of the spin labeled W242C mutant.

PC Vesicle Binding Study. Centricon centrifugal filters were used to analyze PI-PLC binding to PC vesicles. A stock of PC small unilamellar vesicles (SUVs) in 10 mM Tris, pH 7.5, was prepared through sonication. PC samples were prepared to concentrations of 0.01 mM, 0.02 mM, 0.05 mM, 0.10 mM, and 0.20 mM total phospholipid. Two ml of the solutions were added to the filters, and enzyme was added to a final concentration of 20 µg/ml. Solutions were centrifuged at 5000 rpm for 60

minutes. The eluant was collected, frozen in liquid nitrogen, and lyophilized for two days. 45 µl of sample buffer was added to the resulting protein and 12% PAGE was used to analyze the results. The results demonstrated that the two mutants had a very similar affinity for PC surfaces as there was a loss of free PI-PLC as vesicle concentration increased.

Electron Paramagnetic Resonance (EPR). EPR was accomplished using a Bruker EPR spectrometer with a 10-inch magnet, variable temperature control, and microwave bridges at the Whitaker Cardiovascular Institute at Boston University Medical Center with the assistance of Dr. Jason Viereck. Samples were prepared using dialysis and centrifugation to concentrate protein as described before. Samples were concentrated to a concentration greater than 5 mg/ml. EPR spectra were then acquired for both spin-labeled protein in solution, and spin-labeled protein with 6.25 mM POPC or DOPMe SUVs added. The POPC and DOPMe solutions were taken from sonicated concentrated stocks (~50 mM) in order to minimize the dilution of the protein. Eight scans, taking ~80 seconds each, were acquired to provide a minimum signal-to-noise.

Nuclear Magnetic Resonance (NMR). NMR experiments were carried out using a Varian INOVA 500 NMR spectrometer. 10 ml of 50 mM imidazole, pH 7.0, with 0.5 mM EDTA was frozen in liquid nitrogen, lyophilized for two days, and then re-hydrated with 10 ml of D_2O (99%). The protein and POPC and DOPMe vesicles were also frozen and lyophilized and re-hydrated with 0.5 mL D_2O and 0.5 mL of the lyophilized buffer. SUV preparations were made by sonication of the re-suspended lipids in D_2O containing buffer. ^{1}H and ^{31}P spectra of the protein solution, with no added vesicles, were acquired to measure background contribution from buffer and protein. For ^{1}H spectra 128

transients were acquired (Figures 20, 21); for ^{31}P spectra, 1024 transients were acquired (Figures 18, 19). Pulsing conditions included a delay of 2 seconds, a 15 μsec (90°) pulse width, and 1 second of data collection. A 1:1 mixture of POPC and DOPMe was incrementally added to 0.5 mM, 1 mM, 2 mM, 4 mM, and 8 mM in the presence of enzyme. At each concentration ^{1}H and ^{31}P spectra were obtained. Additional concentrations of 0.8 mM, 1.4 mM, 1.8 mM, 2.5 mM, 3.5 mM, and 5 mM vesicles were also analyzed for the W242C mutant.

EPR Results

Initial results from EPR were difficult to interpret. Addition of vesicles to a concentrated solution of PI-PLC did not show any appreciable difference. An overlay of the results showed negligible differences between free protein and protein bound with vesicles. The initial EPR spectra did not show any difference when vesicles were added. There were also no noticeable differences between the spin-labeled Y57C and W242C mutants. PC, PMe, and mixed vesicles were added, but there was no discernible difference in any of the EPR spectra. A possible explanation was that excess spin label was present in the solutions and the spectrum for free spin label dwarfed the broader enzyme-bound nitroxide spectrum. Extensive dialysis was used in an effort to rid the solution of the free spin label.

Further EPR experiments with extensively dialyzed protein yielded some information. Y57C in solution seemed slightly more mobile than W242C. Addition of a PC/PMe vesicle mix to a concentration of 6.25 mM of each caused a difference that was observed in an overlay of the results of both mutants in solution compared to the mutants bound to vesicles. Though the differences were slight, it was shown through EPR that

the spin label was slightly more immobilized upon binding to the PC/PMe vesicles for both Y57C and W242C. It also showed a slight difference in W242C binding to vesicles from Y57C binding to vesicles. In solution alone, Y57C appeared slightly more mobile than W242C. When bound to PC/PMe vesicles, W242C appeared to be slightly less mobile than Y57C still (Figures 2 and 3).

The spectra, though, still showed the superposition of two populations. The differences when protein was bound to vesicles were noticeable, but not sufficient to draw an accurate conclusion about PI-PLC binding to PC compared to binding to PMe. Previous studies have indicated that EPR could be useful in investigating proteins docking to membranes. Penetration depth and angular orientation of the peripheral protein cytosolic phospholipase A_2 ($cPLA_2$) when docking with membranes has been accurately described using EPR by Malmberg et al (8). In that system, twenty-four residues were examined by EPR, allowing for a greater determination of the trends of the different helices' interactions with the phospholipid bilayer. A docking mechanism of $cPLA_2$ was discerned from the EPR data in this study. Examining PI-PLC binding to PC and PMe proved to be more difficult in this case, at least with these initial two nitroxide positions.

NMR Results

^{31}P Vesicle Titration. The vesicle titration experiment provided information through examination of linewidths of the PC and PMe peaks. The linewidth was measured at the half-height of each peak. Before the linewidths of PC and PMe were measured with protein, however, the linewidths of the SUVs were observed without

protein in order to establish a limiting ‘free vesicle’ linewidth, which was determined to be 80 Hz.

W242C was first examined, both with and without the spin labeled enzyme. Vesicles were titrated in at concentrations of 0.5 mM, 1 mM, 2 mM, 4 mM, and 8 mM to a fixed concentration of 28.5 μM PI-PLC. Both PC and PMe phosphorus resonances were broadened significantly by the presence of the spin label. This indicates spin label proximity to both the PMe and PC phosphate groups (Figures 4 and 5). What was interesting was that the linewidths for PMe were broadened to a greater extent than the linewidths for PC (Figure 6). This would suggest that spin label is closer to the PMe phosphorus than to the PC phosphorus, and therefore that the protein interacts more with the PMe head group than the PC head group. Additional ^{31}P studies of W242C at PC/PMe vesicle concentrations of 0.8 mM, 1.4 mM, 1.8 mM, 2.5 mM, 3.5 mM, and 5 mM showed similar trends, although absolute line-broadening values were different than in the first titration. The data again showed a greater interaction of protein with PMe than with PC head groups, although there was not a large difference between the spin labeled protein and the unlabeled protein.

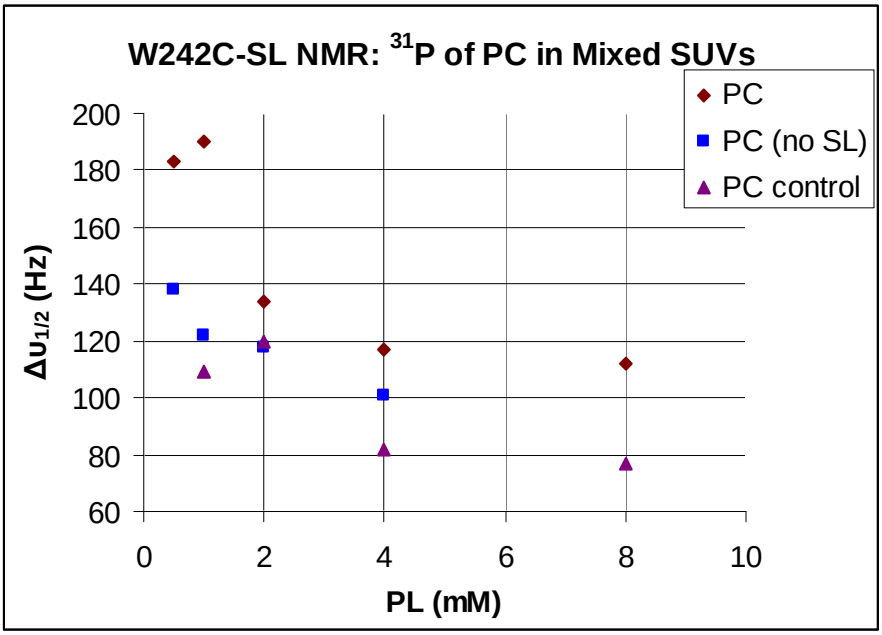

Figure 4: ^{31}P for PC with spin -labeled W242C in red and unlabeled W242C in blue

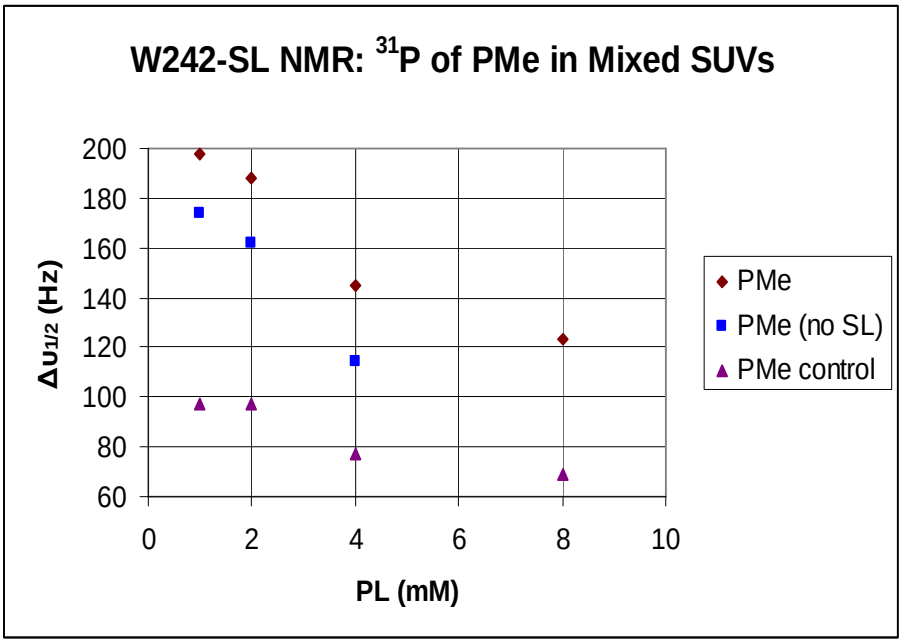

Figure 5: ^{31}P for PMe with spin-labeled W242C in red and unlabeled W242C in blue

PL	App K_d (mM)	Δ_b (Hz)
PC + W242C-SL	1.4±0.8	7400±2700
+ W242C	1.7±0.6	4300±1000
PMe + W242C-SL	3.0±0.9	18000±4000
+ W242C	1.5±1.6 (not enough data)	9300±5000

Figure 6: Kd and linewidths for W242C-SL and W242C with PC and PMe

The apparent K_d's were determined by the equation:

$\Delta\upsilon_{obs} = (\Delta_b * [E]_0) / (K_D + [PL]_0) + \Delta\upsilon_0$ where Δ_b is the bound linewidth, K_D is the apparent dissociation constant in terms of total phospholipid, $[PL]_0$ is the total phospholipid concentration, and $\Delta\upsilon_0$ is the linewidth without protein

The ^{31}P data for Y57C at PC/PMe vesicle concentrations of 0.5 mM, 1 mM, 2 mM, 4 mM, and 8 mM yielded slightly different results. The spin labeled protein again significantly broadened both PC and PMe resonances when compared with linewidths of PC and PMe mixed with unlabeled protein (Figures 12, 13). As with W242C-SL, the spin label at residue 57 also relaxes the phosphorus nuclei. However, Y57C-SL broadens the PC phosphorus resonance to the same extent or even to a greater amount than the PMe phosphorus. The results of these readings indicate that the protein interacts with the PC phosphorous an equal or even greater amount than with the PMe phosphorous.

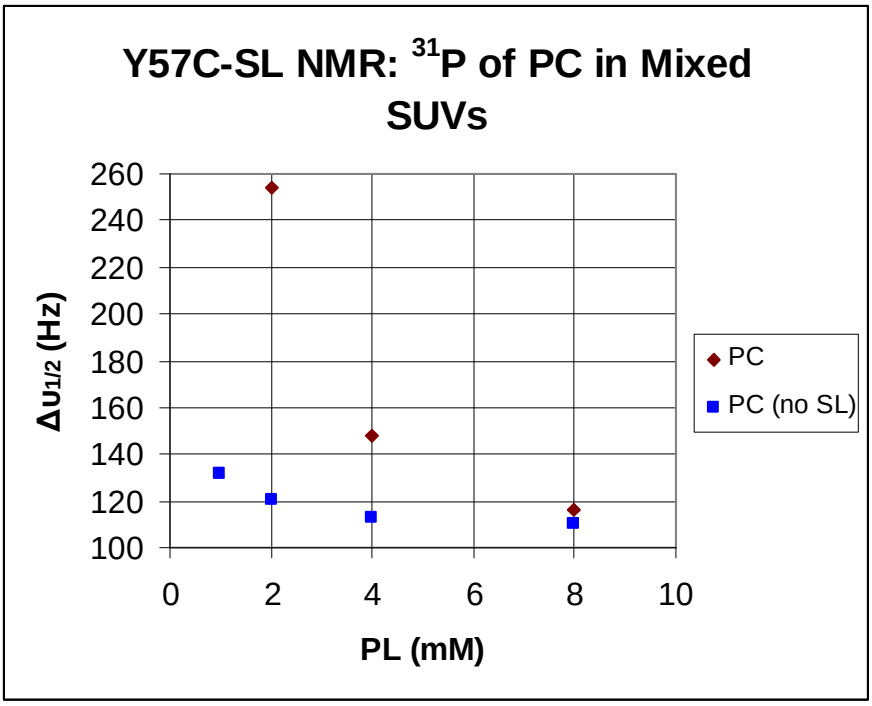

Figure 12: ^{31}P for PC with spin -labeled Y57C in red and unlabeled Y57C in blue

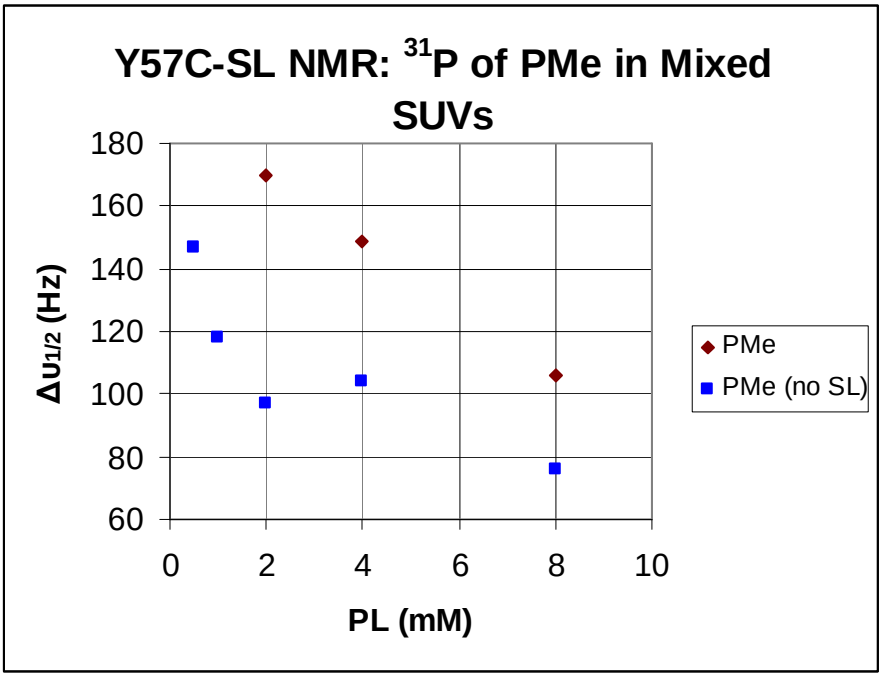

Figure 13: ^{31}P for PMe with spin-labeled Y57C in red and unlabeled Y57C in blue

^{1}H Vesicle Titration. The data from the ^{1}H resonance proved to be very interesting. Both head group and acyl chain resonances were examined. The largest linewidth change occurred with the bulk methylene peak $(CH_2)_n$. With unlabeled W242C, the bulk methylene protons exhibited a linewidth of approximately 49 Hz. This value was unchanged as the concentrations of vesicles increased (Figure 7). The spin labeled protein broadened this peak significantly (Figure 7). The extrapolated bound linewidth was 460 ± 130 Hz. This data suggests that the spin label inserts into the bilayer so that the middle of the chain is relaxed. Additional data points for W242C confirmed this trend, with the linewidth of the bulk methylene peak of the spin labeled protein decreasing as vesicle concentration increased while the $(CH_2)_n$ linewidth remained constant at 49 Hz.

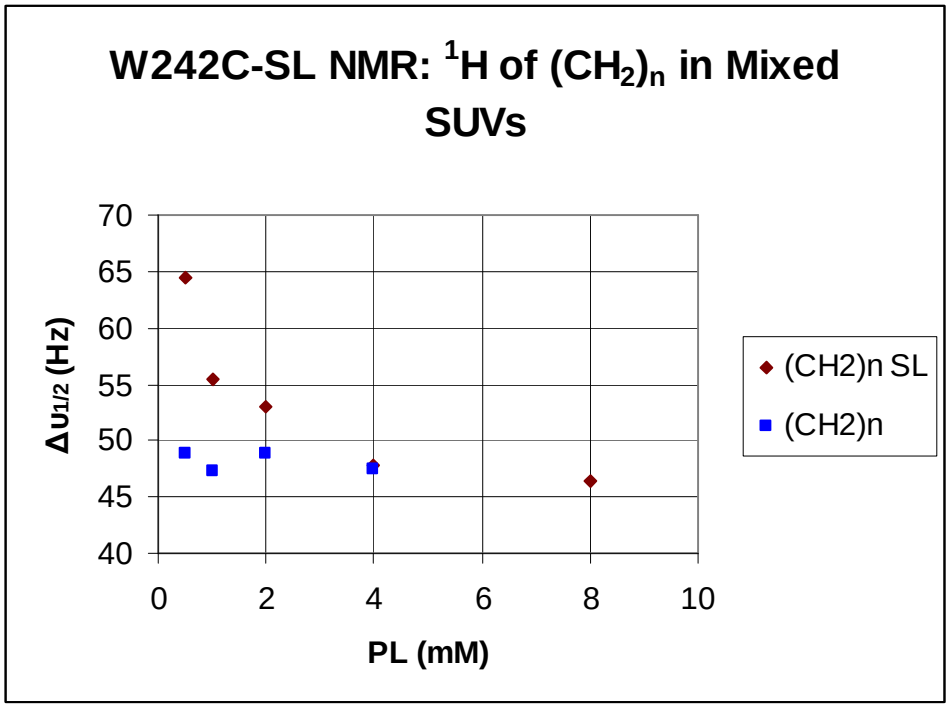

Figure 7: ^{1}H data for $(CH_2)_n$ with spin labeled and unlabeled W242C

The results with the comparison between the $–OCH_3$ group and the $-N(CH_3)_3$ group were also of interest. The results showed that the $-OCH_3$ group sensed the unpaired electron of the spin label, while the $- N(CH_3)_3$ resonance showed little change between the spin labeled and unlabeled W242C. The decrease in linewidth as vesicle concentration increased was nearly negligible for $-N(CH_3)_3$, while the $–OCH_3$ linewidth decreased dramatically (Figure 8). This would indicate that perhaps the $–OCH_3$ interacts with the protein around the residue 242, while the $-N(CH_3)_3$ group is spatially removed from that site.

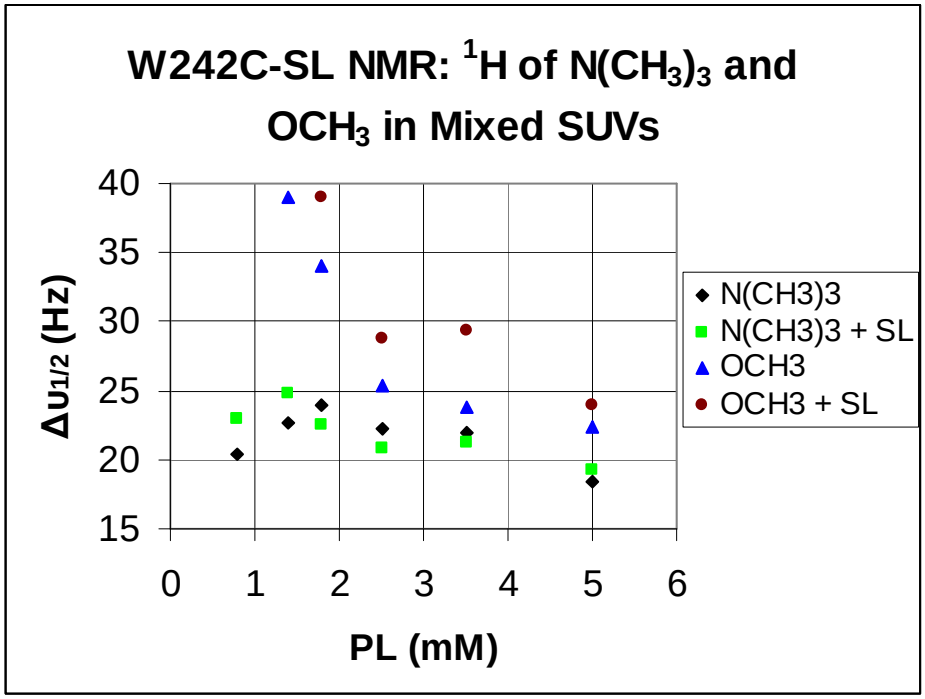

Figure 8: ^1H data for $–N(CH_3)_3$ and –OMe with W242C

Other resonances also showered differential broadening for the α-CH_2 group then mixed with spin-labeled protein which also indicated some increased interaction with the

spin label at W242C. Results consistently showed a somewhat larger linewidth for this peak in the presence of the spin labeled protein (Figure 9), which may suggest again that the spin-labeled protein is interacting with this region of the phospholipid.

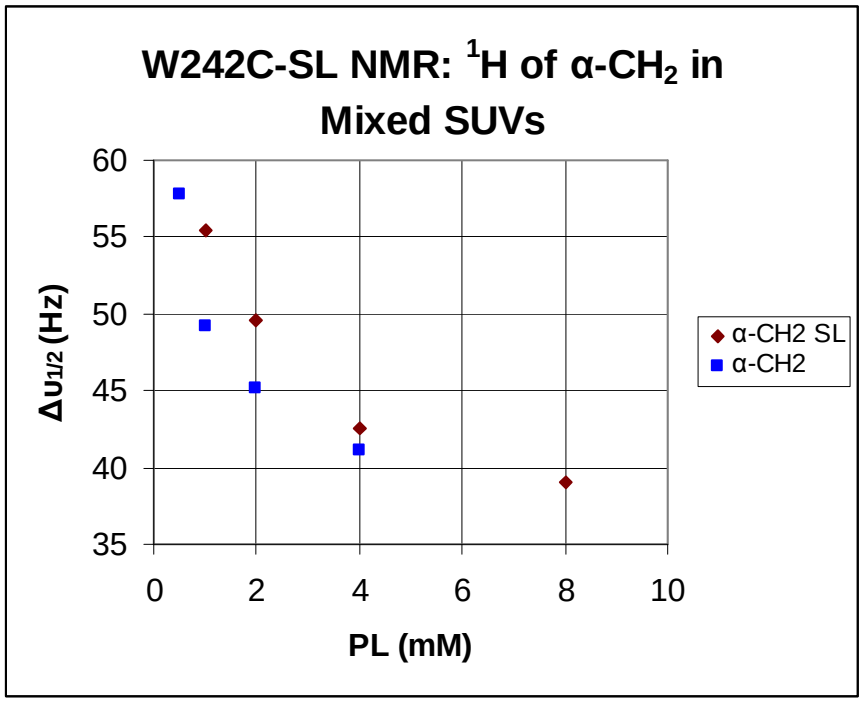

Figure 9: ^{1}H data for α-CH_2 with spin labeled and unlabeled W242C

The ω-CH_3 resonance did not show any appreciable difference in linewidth when spin label was added, and this would appear to indicate that this region of the bilayer has little or no interaction with the protein.

Limited measurements of ^{1}H peaks in a vesicle titration experiment with Y57C yielded some intriguing results. There seemed to be a similar effect in the bulk methylene region, where unlabeled PI-PLC consistently produced a phospholipid $(CH_2)_n$ linewidth of approximately 48 Hz, while the spin labeled protein led to an increased linewidth (66 Hz at a 0.5 mM concentration of PC/PMe, which decreased to 47 Hz at an

8.0 mM concentration, Figure 14). It again appeared that the $-OCH_3$ group showed a greater interaction with the protein spin label than the $-N(CH_3)_3$ group as there was a somewhat larger linewidth for this group (Figure 15). The α-CH_2 group may also have shown some interaction with the protein, though peaks in the 0.5 mM to 1 mM range were too broad with the superposition of contaminants to get an accurate measurement of the changes in linewidths (Figure 16).

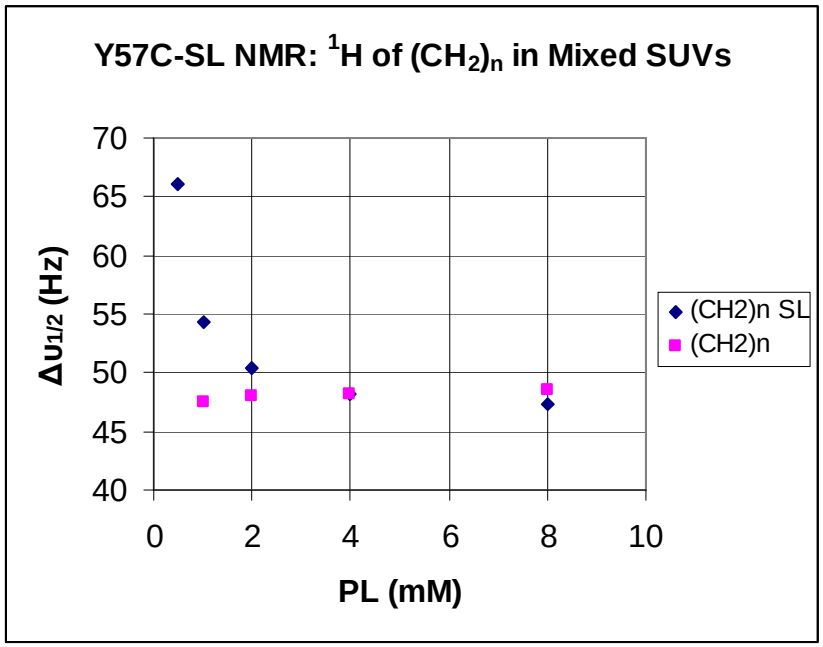

Figure 14: ^1H data for $(CH_2)_n$ with spin labeled and unlabeled Y57C

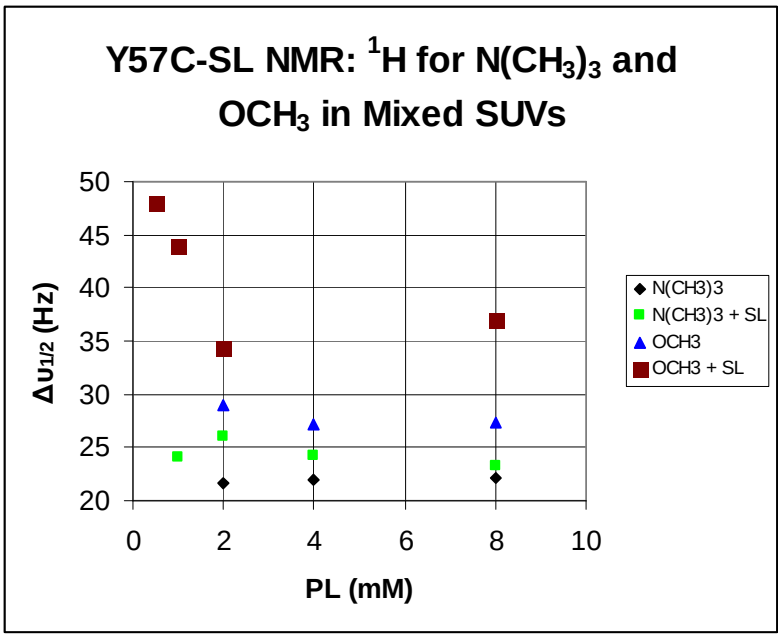

Figure 15: ^{1}H data for $-N(CH_3)_3$ and –OMe with Y57C

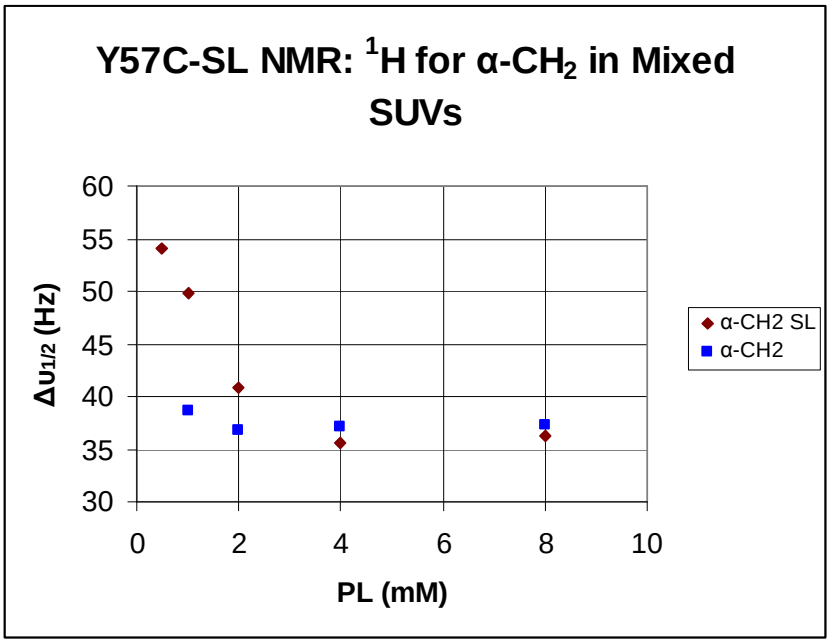

Figure 16: ^{1}H data for α-CH_2 with spin labeled and unlabeled Y57C

Conclusions and Future Experiments

From the EPR results gathered, it appears that the spin label of W242C might be more immobile than that of Y57C when added to a PC/PMe mixed vesicle. This is consistent with the hypothesis that the side chain of residue 242 inserts into the phospholipid bilayer. Such a conformation would constrain movement of the spin label. Other spectra comparing PC or PMe showed little difference. Though at first this was thought to be due to free spin label in the solution causing a superposition of two populations (free and vesicle bound enzyme), it now appears thatthe high protein concentrations required for EPR may have caused some aggregation of the protein This PI-PLC aggregate appeared to weaken binding of PI-PLC to PC and PMe.

The NMR results provided a much clearer picture of PI-PLC interactions with PC/PMe vesicles. The ^{31}P experiments with W242C showed that the negatively charged substrate analogue, PMe, was closer to the spin label than PC. The linewidth data indicated that the protein interaction is greater with the PMe phosphorus than with the PC phosphorus. The same titrations with Y57C indicated that the spin label is not very close to the phosphorus, an observation consistent with Y57C positioned much farther from the interactions with the interface. Y57C perturbed PC to a greater extent than PMe. In contrast to the ^{31}P experiments, the ^{1}H data seems to show that the $-OCH_3$ is closer to the spin label on W242C than is the $-N(CH_3)_3$ group. The increased linewidth of $(CH_2)_n$ suggested there might be some protein penetration into the bulk methylene region. The puzzling fact is that the ^{1}H data for Y57C appears to give similar results to that of W242C. The results for Y57C again show a preference for the $-OCH_3$ group over the $-N(CH_3)_3$ group, an interaction in the bulk methylene area, and possibly some interaction

with the α-CH_2. This might indicate that the presence of the spin label may have a similar effect on linewidths at any position on the protein.

Although discrete distances and proximities for docking the protein to the membrane could not be extracted, the methodology was shown to be feasible. Clearly, additional data with Y57C-SL is necessary. Additionally, it would be valuable to repeat the EPR and NMR experiments presented here with additional cysteine mutants. It would be interesting to examine the results of these experiments if a cysteine replaced an amino acid on the opposite side of the enzyme from the phospholipid interface, at positions such as Glu169 or Leu143. This might confirm that the W242C results do in fact show an interaction between protein and membrane by showing that a spin label on the opposite side of the interface has no effect on linewidths. Cysteine residues could also be introduced near the mutations examined here in order to confirm that the results obtained are valid. Mutations at positions such as Arg56 and Asp54, which are near Tyr57, and at Trp47 and Ile43, which are near Trp242, might confirm this data. Field cycling experiments (9) might also be performed to gain a better understanding of the interactions between PI-PLC and phospholipids. Field cycling has been shown to be effective in monitoring association of PI-PLC with PC and PMe and demonstrating PC activation of PI-PLC toward PI (9). This technique could be useful in examining and characterizing spin label interaction with the phosphorus of PC and PMe.

Figures

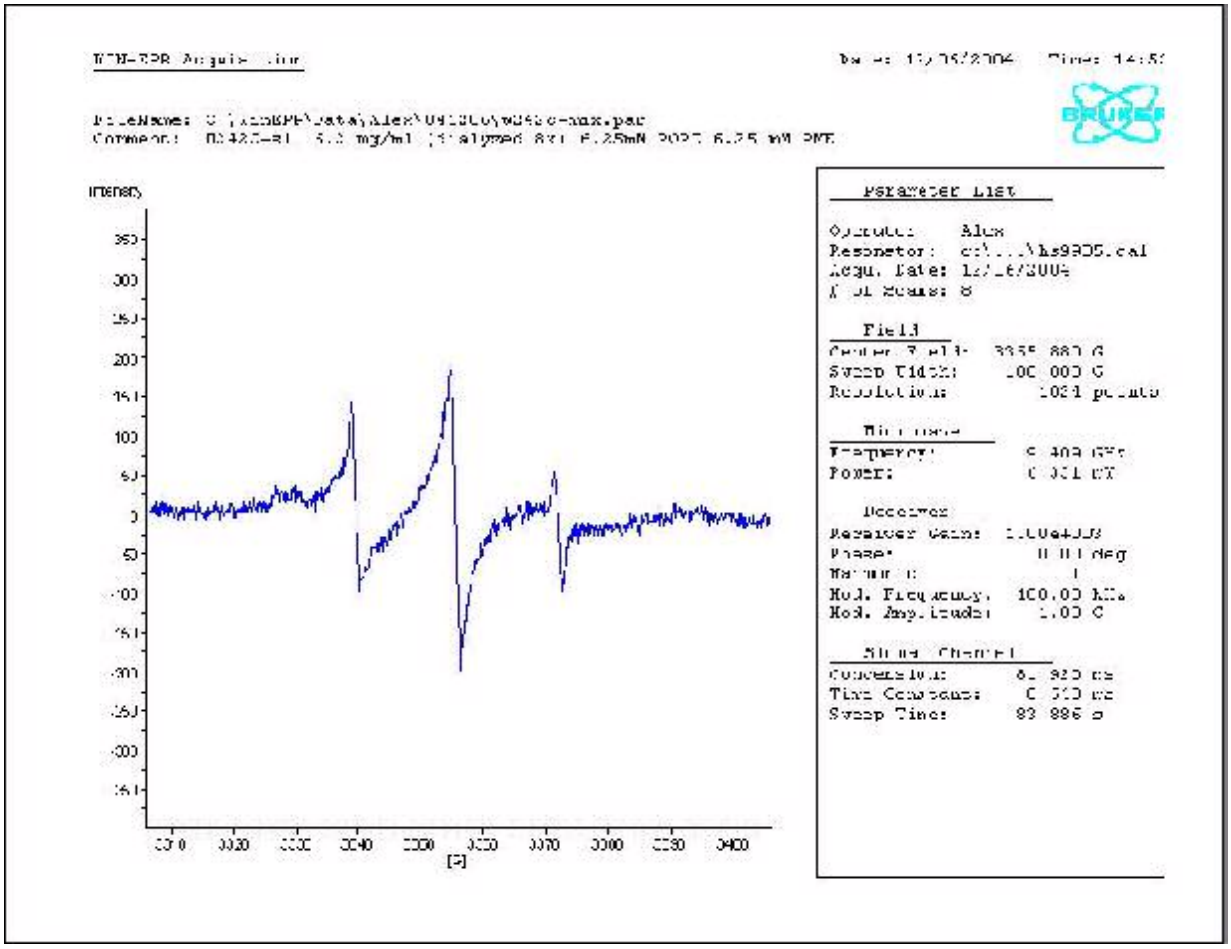

Figure 2: EPR results for W242C in solution (6.2 mg/ml) in 6.25 mM PC | 6.25 mM PMe

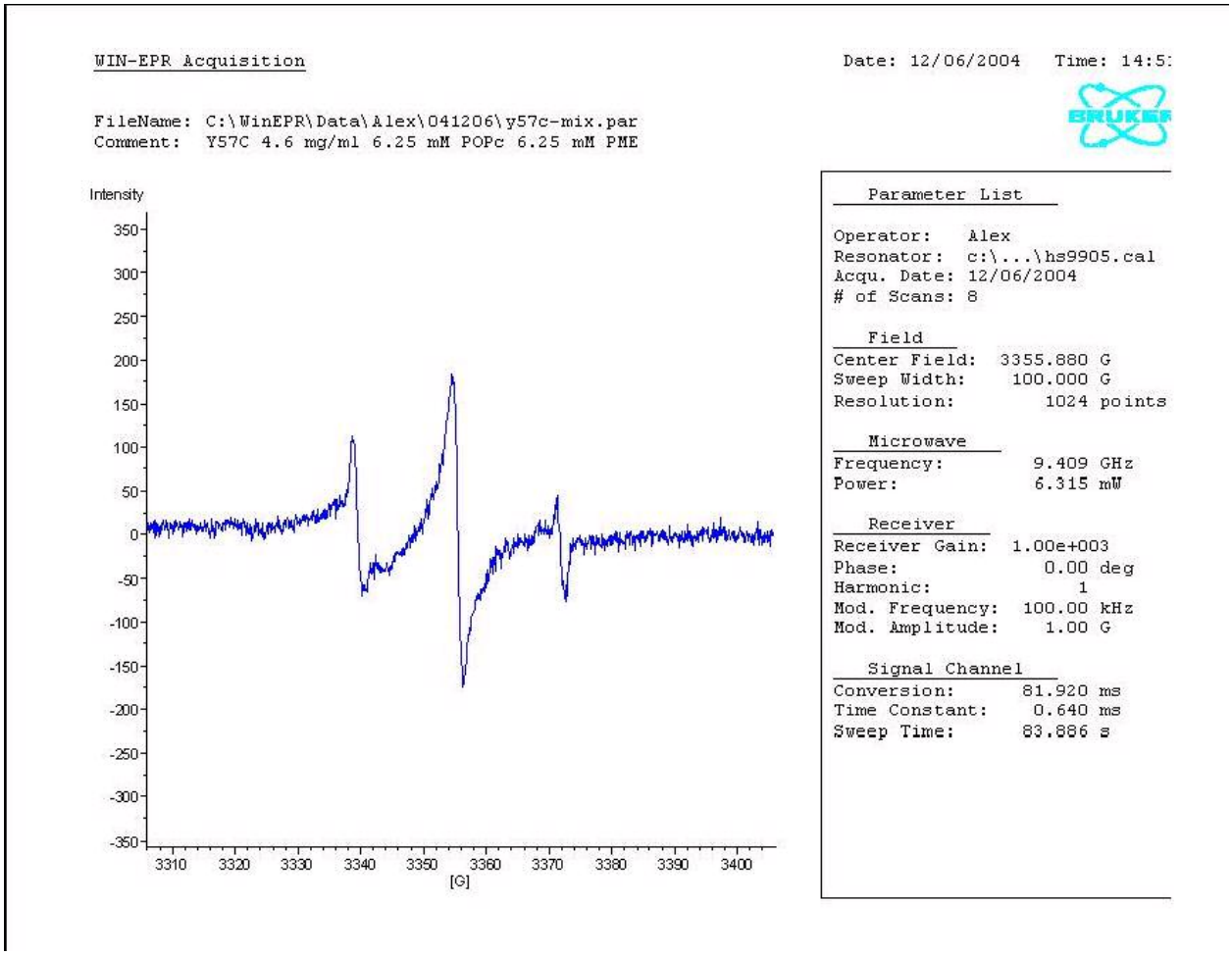

Figure 3: EPR results for Y57C in solution (4.6 mg/ml) in 6.25 mM PC | 6.25 mM PMe

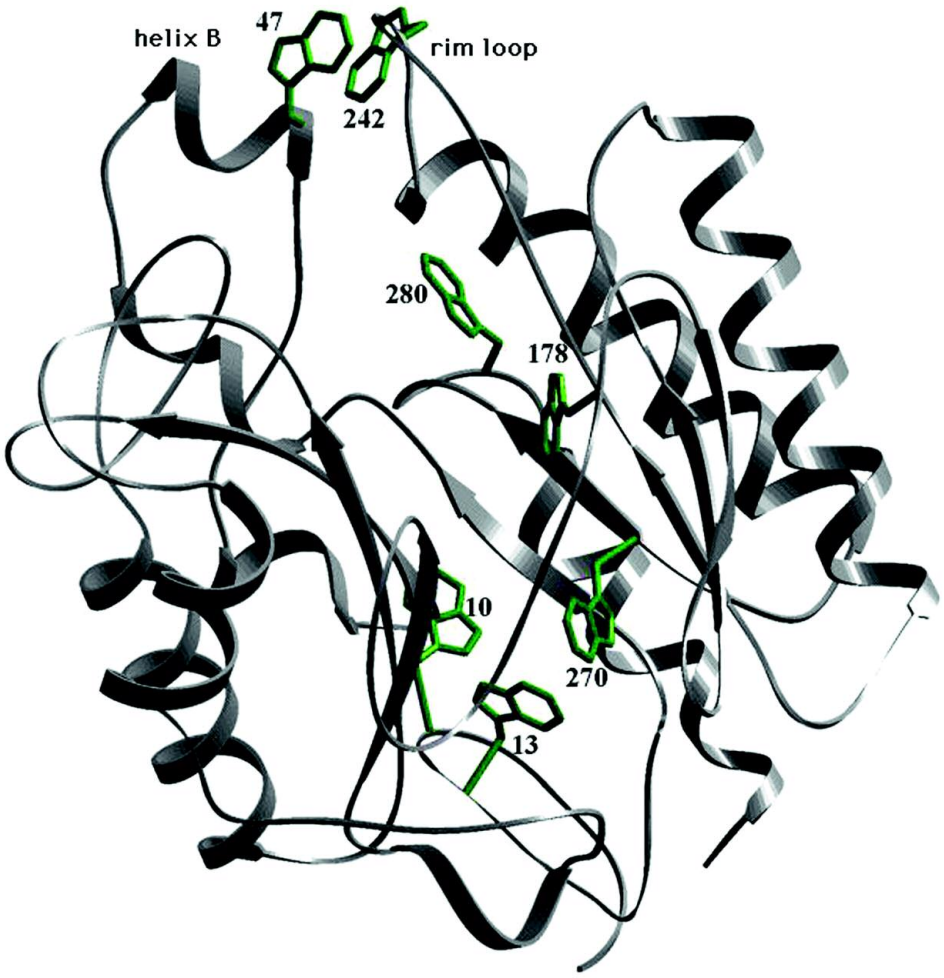

Figure 10: Ribbon diagram of PI-PLC showing rim tryptophans (Trp47 in helix B and Trp242 in the rim loop) (4)

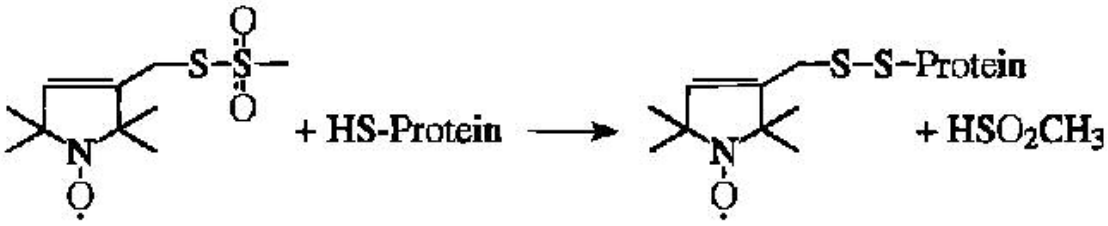

Figure 11: Structure of spin label MTSL and disulfide exchange reaction with cysteine residue of protein (10)

Figure 17: Two views of PI-PLC crystal structure from *Bacillus cereus*, with the two residues examined in this experiment, Trp242 and Tyr57 highlighted in wire-frame

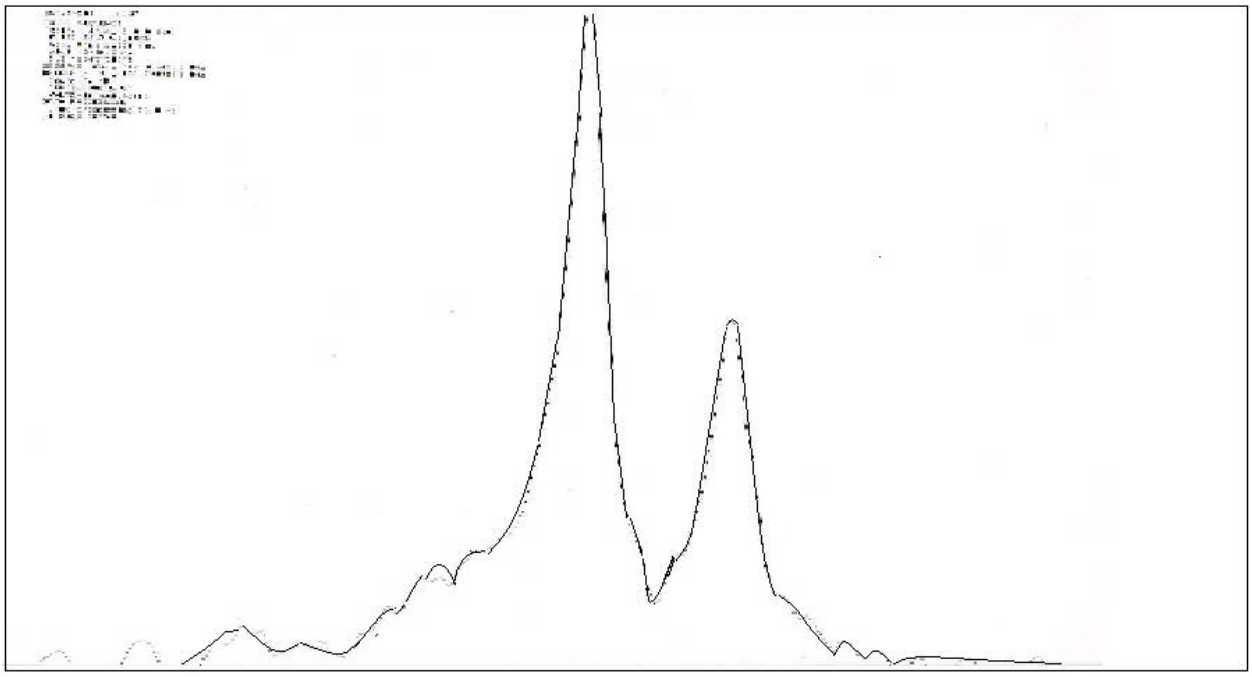

Figure 18: ^{31}P NMR spectra for unlabeled W242C in 2.5 mM PC/PMe. The central peak is the PMe phosphorus, the peak to the right is the PC phosphorus

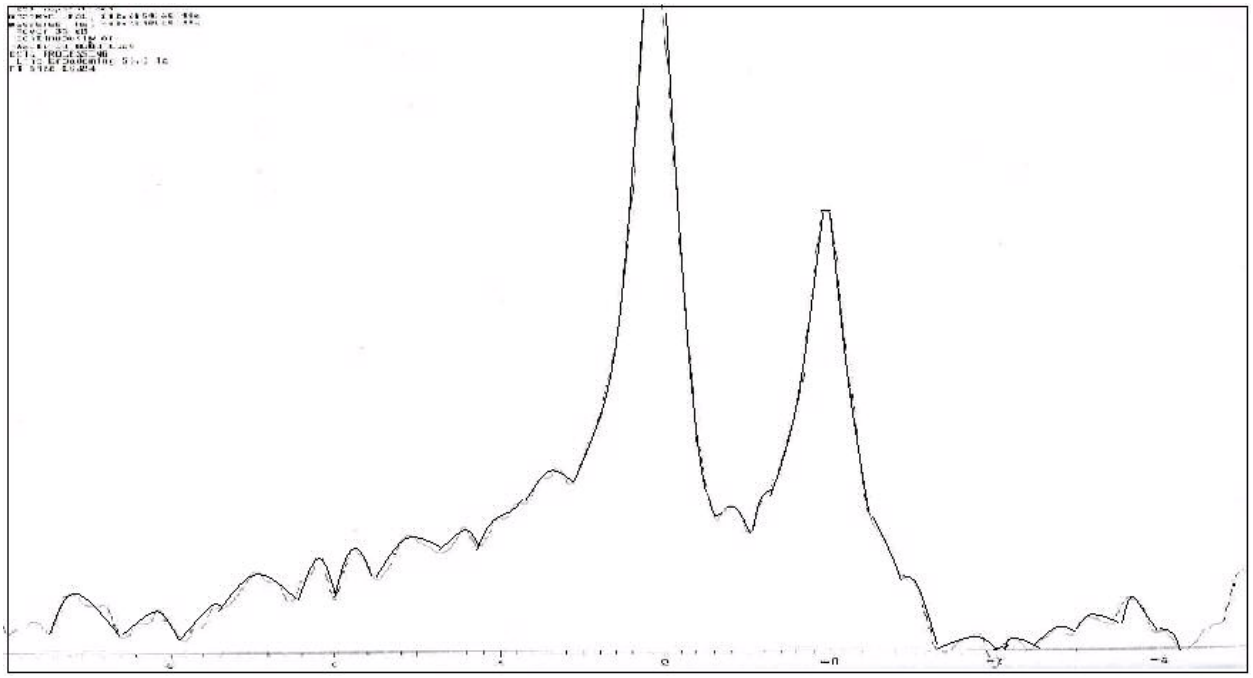

Figure 19: ^{31}P NMR spectra for spin-labeled W242C in 2.5 mM PC/PMe. The central peak is the PMe phosphorus, the peak to the right is the PC phosphorus

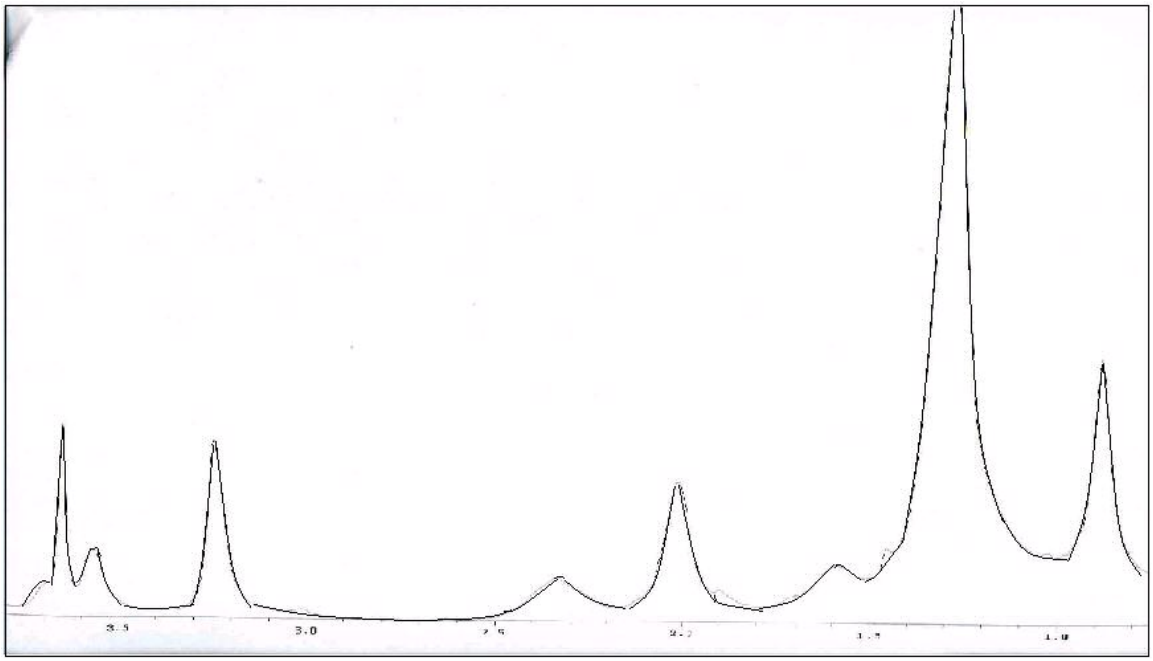

Figure 20: ^{1}H NMR spectra for unlabeled W242C in 3.5 mM PC/PMe. The tallest peak is the $(CH_2)_n$ group and the α-CH_2 group is the peak 0.8 ppm left of this peak. The $–OH_3$ group is the smaller of the two peaks on the far left and the $–N(CH_3)_3$ group is represented by the peak to the right of the $–OCH_3$ group.

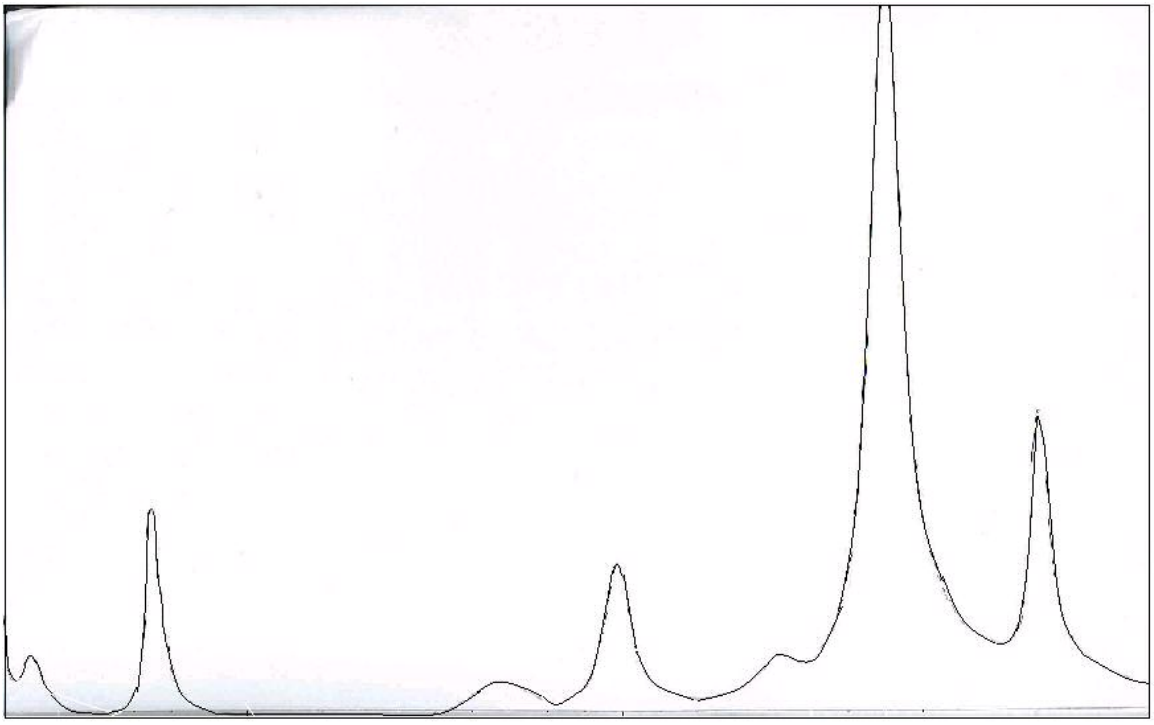

Figure 21: ^{1}H NMR spectra for spin-labeled W242C in 3.5 mM PC/PMe. The tallest peak is the $(CH_2)_n$ group and the α-CH_2 group is the peak 0.8 ppm left of this peak. The $–OH_3$ group is the smaller of the two peaks on the far left and the $–N(CH_3)_3$ group is represented by the peak to the right of the $–OCH_3$ group.

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