Supporting Information for:

ENZYMOLOGY WITH A SPIN-LABELED PHOSPHOLIPASE C: SOLUBLE SUBSTRATE BINDING BY ^{31}P NMR FROM 0.005 TO 11.7 TESLA

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1. PI-PLC preparation and labeling. The *Bacillus thuringiensis* PI-PLC H82C mutant protein, constructed using QuikChange methodology (Stratagene), was overexpressed in *E. coli* and purified as described previously for wild type protein (1). Prior to modification with the spin-labeling reagent, the H81C protein was incubated with 5 mM DTT for 30 min. The DTT was then removed by elution through Micro Bio-spin columns (from Bio-Rad) at a volume of 50 µl enzyme solution per column). The fully reduced H82C mutant protein (typically 3 mg/ml) was modified with S-(2,2,5,5-tetramethyl-1-oxyl-[Δ]3-pyrrolin-3-ylmethyl) methanethiosulfonate maleimide from Toronto Research Chemicals to introduce the spin-label at this position in the active site. The ratio of reagent to Cys ratio was typically 14:1. Excess spin-label was removed by dialysis followed by elution through the Micro Bio-spin columns (Bio-Rad) equilibrated with 10 mM Tris, pH 7.4. Samples for fc-P-NMR were prepared in 50 mM HEPES, pH 7.5, with 1 mM EDTA added to scavenge any paramagnetic cations in solution.

2. PI-PLC cyclophosphodiesterase assay. Inositol 1,2-(cyclic)-phosphate (cIP) was generated from PI-PLC cleavage of PI under low enzyme conditions so that cIP was the only water-soluble product. The cIP was purified as described previously (1). The hydrolysis cIP (from 5 to 40 mM) at 25ºC was measured in the absence or presence of 5 diC_7PC (micelles) or POPC small unilamellar vesicles (SUVs) prepared by sonication. ^{31}P spectra following the hydrolysis of cIP to inositol-1-phosphate (I-1-P) were obtained on a Varian INOVA 500 using parameters described previously (1-3). Specific activities were calculated by measuring the integrated intensity of cIP or I-1-P from the progress curve for less than 20% substrate hydrolyzed. The systematic error in the integrated intensity of cIP in the each spectrum was less than 10%. Note that the plots of specific activity versus cIP concentration are slightly sigmoidal (especially with the diC7PC present). Thus, the curve is fit to a cooperative model for that system. Similar sigmoidal behavior has been observed previously for PI-PLC cleavage of pyrene-PI (4) and cIP hydrolysis in the absence of an activating detergent (2).

3. fc-P-NMR methodology. The ^{31}P field-cycling spin-lattice relaxation rate (R_1) experiments were obtained at 25ºC on a Varian Unity^{plus} 500 spectrometer using a standard 10-mm Varian probe in a custom-built device that moves the sample between the probe and a higher position within, or just above, the magnet, where the magnetic field is between 0.06 and 11.7 T (5,6). To access lower fields (0.005 up to 0.07 T), the sample was shuttled to a region outside and above the magnet and into the middle of a Helmholtz coil, where the current was adjusted to the desired field (7). R_1 at each field strength was measured using 6-8 delay times and analyzing the data with an exponential function to extract $R_1 = 1/T_1$. The samples for field cycling were sealed in a 10 mm tube with a minimum amount of head space to avoid bubble formation as the tube is rapidly shuttled up and down the magnetic bore. Experiments to cover a field range of 0.005 up to 11.7 T typically took a span of 16-24 h (short times at higher cIP concentrations).

4. Specificity of H82C-SL for cIP. The specificity for H82C-SL relaxing only cIP is shown by carrying out a field cycling experiment with three different solutes present (each at 5 mM): cIP, glucose-6-phosphate (G-6-P) and dibutyroyl-PC (diC_4PC). G-6-P does not bind to wild type PI-PLC but is a phosphate monoester with chemical features similar to the inositol ring. DiC_4PC does not activate PI-PLC towards cIP (2), so was not expected to bind to the protein. The field dependence profiles for the diC_4PC and G-6-P with or without the H82C-SL were the same (*Fig. S1*), indicating these solutes did not bind

significantly to the enzyme (or if they did were >20 Å from the site of the spin-label). Only the cIP phosphorus resonance was relaxed by the spin label of H82C-SL. The parameters extracted from the curve ($\Delta R_{P\text{-}e}(0), \tau_c$) were equivalent to those obtained for cIP and H82C-SL alone.

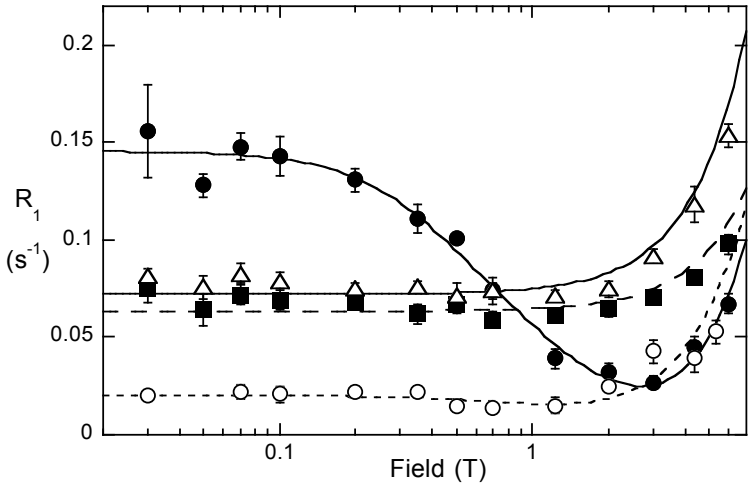

Figure S1. Field dependence of different phosphorylated molecules (5 mM) mixed 0.5 mg/ml H82C-SL PI-PLC: cIP (●), glucose-6-phosphate (■), and diC_4PC (Δ). For comparison the profile for cIP in the absence of spin-labeled protein is also shown (○).

5. Estimation of $\Delta R_{P\text{-}e}$, and τ_c from field dependence data. The spin-lattice relaxation rate, $R_1 = 1/T_1$, for small phosphorus-containing molecules in solution is dominated by the large CSA component at high field. R_1 exhibits a square law dependence since $\omega^2 \tau_c^2 << 1$ as shown in *Fig. S2*, where data for cIP and monomeric dihexanoylphosphatidylcholine (diC_6PC) are shown as a function of field. Since R_1 continues to increase with the square of the field, even at the highest fields observed, we cannot accurately determine a correlation time for small molecules with this method. However, it is fast and likely under 500 ps. The dipolar contribution associated with this correlation time is quite small and a constant, again since $\omega^2 \tau_c^2$ is much less than one.

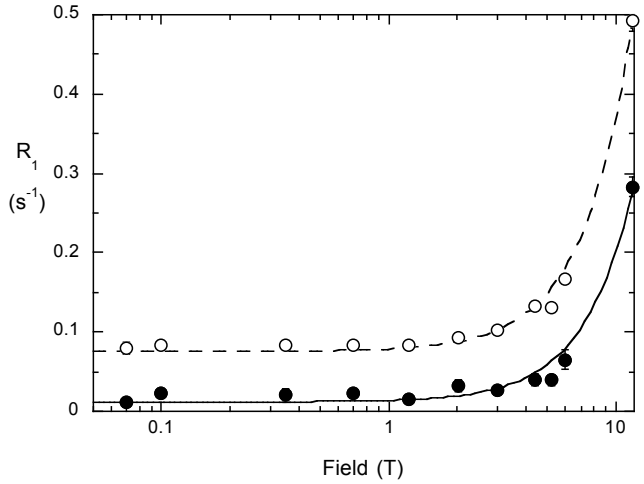

Figure S2. Field dependence of R_1 for 5 mM cIP (●) and 5 mM diC_6PC (○), also monomeric at this concentration.

The R(0) associated with the dipolar relaxation can be well-defined. It varies for each ^{31}P depending on the number and proximity of nearby protons. The $R_c(0)$ value for diC_6PC, 0.076±0.004 s^{-1}, is larger than that for cIP (0.012±0.004 s^{-1}), presumably because there are four protons near the lipid

phosphodiester in diC_6PC and only two for cIP. This profile is the same for cIP when unlabeled H82C is added because it binds weakly.

The specific contribution of the spin-labeled protein to cIP relaxation is provided by subtracting the R_1 contribution for the cIP with non-spin-labeled H82C (*Fig. S2*) from the R_1 values with H82C-SL present. A comparison of the R_1 versus field original profile with the isolated small molecule contribution subtracted is shown in *Fig. S3*. The residual R_1 (noted ΔR_1) can then be fit to the expression:

$$\Delta R_1 = \Delta R_{P\text{-}e}/(1 + \omega^2\tau^2)) + c$$

where $\Delta R_{P\text{-}e}$ is the maximum relaxation enhancement for that fraction of cIP bound to the spin-labeled protein, and τ_c is the correlation time for the bound cIP ^{31}P interaction with the nitroxide. Note that we see a constant residual R_1 at higher fields, *c*, that could reflect a dipolar contribution from a fast motion. For this particular sample, τ_c is 17.9±1.2 ns and $\Delta R_{P\text{-}e}$ = 0.123±0.004 s^{-1}.

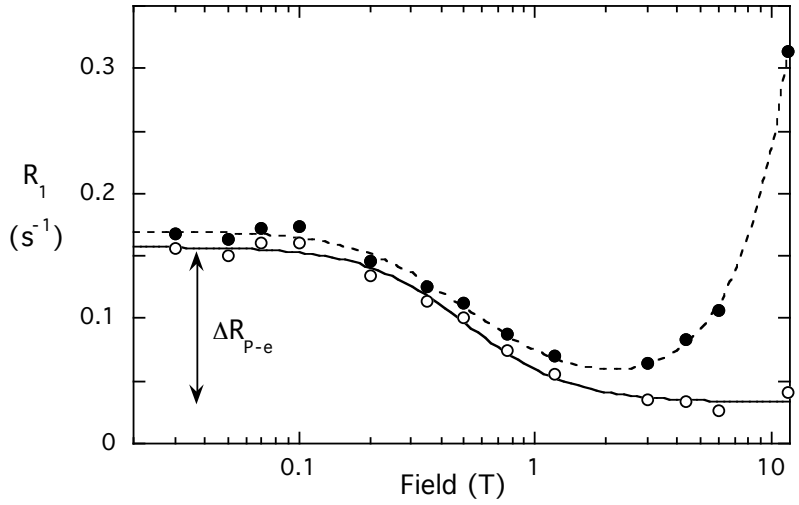

Figure S3. R_1 field dependence profile for 40 mM cIP in the presence of 0.0072 mM H82C-SL and 5 mM diC_7PC before (●) and after (○) subtracting the contribution due to cIP in buffer (or mixed with the same amount of H82C that was not spin-labeled.

In all the field cycling experiments with H82C-SL, there is also an effect of the spin-label on the relaxation rate of the ^{31}P resonance of the diC_7PC micelles or POPC SUVs. However, it is difficult to extract a meaningful distance between the electron of the spin label and the phospholipid headgroups from the changes in R_1. For micelles, there is a monomer/micelle exchange as well as potential changes in micelle size that would complicate obtaining an accurate r_{PH}. For the samples where POPC SUVs are used to activate the enzyme, motions on a nanosecond scale may not reflect specific interactions if the protein is transiently anchored to the surface and likely moving around on it. Therefore, we have focused only on the cIP component that is either free in solution or bound to the PI-PLC.

6. *Model for cIP bound to spin-labeled H82C*. To see if the fc-P-NMR distances between cIP and the spin-label attached to H82C were reasonable, we used Autodock4 and positioned the cIP in the place of myo-inositol in the 1 PTG structure of the *B. cereus* PI-PLC (which has cIP present), then attached the spin-label to the H82C. The distances of the nitroxide atoms to the cIP phosphate are 5-8 Å, values quite consistent with the distance estimated by fc-P-NMR. The size of the nitroxide is not that much larger than the imidazole that normally sits in this position. The attachment of the nitroxide to the protein is flexible and what is shown likely represents a minimum distance. However, it (6.2 Å) is quite consistent with what we measure by fc-P-NMR (7.6 Å with diC_7PC present).

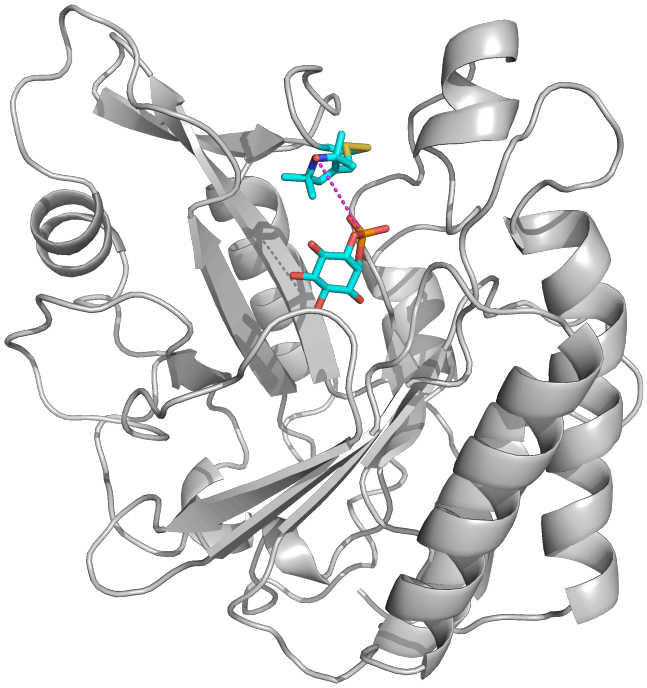

Figure S4. Model of PI-PLC showing the spin label attached to H82C and cIP docked into the active site by aligning cIP with the myo-inositol ligand in the crystal structure of 1PTG. The magenta line indicates the distance between the nitroxide oxygen and cIP phosphorus (6.2 Å for this conformation).

References:

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