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Enzymology with a Spin-Labeled Phospholipase C: Soluble Substrate Binding by ^{31}P NMR from 0.005 to 11.7 T[†]

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ABSTRACT: ^{31}P NMR relaxation studies from 0.005 to 11.7 T are used to monitor water-soluble inositol 1,2-(cyclic) phosphate (cIP) binding to phosphatidylinositol-specific phospholipase C spin-labeled at H82C, a position near the active site of the enzyme, and to determine how activating phosphatidylcholine (PC) molecules affect this interaction. We show that, in the absence of an interface, cIP binding to the protein is not rate-limiting, and that lower activation by PC vesicles as opposed to micelles is likely due to hindered product release. The methodology is general and could be used for determining distances in other weakly binding small molecule ligand−protein interactions.

Phosphatidylinositol-specific phospholipase C (PI-PLC) enzymes catalyze the hydrolysis of phosphoinositides in two steps: an initial phosphotransferase reaction to produce diacylglycerol and inositol 1,2-(cyclic) phosphate (cIP), followed by hydrolysis of the cIP to inositol 1-phosphate. For PI-PLC from *Bacillus thuringiensis*, water-soluble cIP hydrolysis is slow with a K_m of >50 mM (*1*, *2*), but the specific activity is significantly enhanced by the addition of phosphatidylcholine (PC) interfaces, either micelles such as diheptanoyl-PC (diC_7PC) or vesicles of 1-palmitoyl-2-oleoyl-PC (POPC) (*2*, *3*). For example, at 5 mM cIP, the addition of diC_7PC leads to a 40−50-fold increase in specific activity (*4*); POPC SUVs added yield a 3−4-fold increase. While both PC interfaces decrease the K_m, the V_{max} is dramatically different (Figure 1).

These cIP kinetics raise two interesting questions. (i) Does the decreased K_m for PC-enhanced cIP hydrolysis reflect a tighter binding of substrate to the enzyme? (ii) Why is the V_{max} lower with a vesicle activator compared to a micelle surface? To address these, we have used an alternative to standard NMR methods (high-resolution field cycling ^{31}P NMR spectroscopy or fc-P NMR) in which the ^{31}P spins are prepared, and their signals detected, at standard high fields, but their relaxation back toward equilibrium occurs at a lower field, as described in the Supporting Information and elsewhere (*5*−*7*). Resonances for cIP and PC species are well-separated (cIP at 17 ppm and diC_7PC at 0 ppm), at the observation field of 11.7 T, and spin−lattice relaxation rates ($R_1 = 1/T_1$) can be obtained over a wide range of magnetic field strengths (*6*, *7*). The field dependence of R_1, from 0.005 to 11.7 T, can then be analyzed with standard theory (*6*, *7*) to obtain correlation times.

For small phosphorus-containing molecules free in solution, the dipolar contribution to ^{31}P relaxation is small, and the ^{31}P R_1 is dominated by chemical shift anisotropy (CSA) over most of the accessible field range. However, if the small molecule spends part of its time ligated moderately weakly to a larger complex such as an enzyme, then its observed R_1 is the weighted average of its small free R_1 and the much larger R_1 that it would have if bound permanently. The effect is field-dependent and depends on the ligand/protein ratio as well as the proximity of the ^{31}P to the nearby proton dipoles, in either the small molecule or the enzyme, that relax it. A way of enhancing the added effect is to further add an electron spin-label to the protein near a suspected binding site. The much larger magnetic dipole of the electron can have a useful effect on the ^{31}P even with a high ligand/protein ratio.

To explore cIP binding to PI-PLC with fc-P NMR, we used a mutant protein in which a key active site residue [His82, the general acid in the proposed mechanism that protonates the DAG anion initially produced in the phosphotransferase step (*8*)], was altered to cysteine. The H82C enzyme is inactive but should still bind substrate. A nitroxide spin-label (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethiosulfonate) was attached to this cysteine (H82C-SL) as a way of introducing a much larger dipole into the region where the cIP should bind. The field dependence profiles for cIP (5 mM) with spin-labeled PI-PLC (0.5 mg/mL, 14.4 μM) in the absence and presence of diC_7PC (5 mM) micelles are shown in Figure 2A. In the absence of protein, cIP exhibits behavior typical for small phosphate esters:

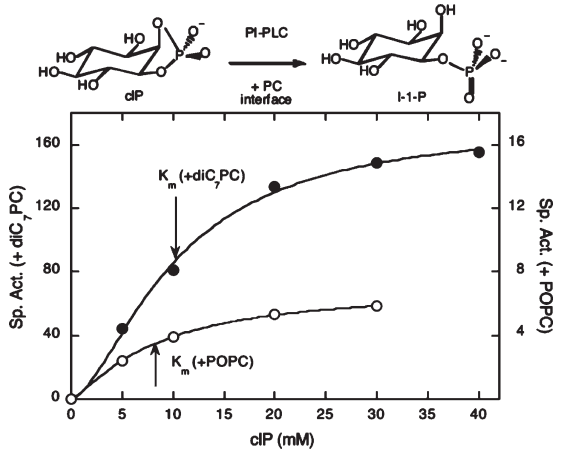

FIGURE 1: Dependence of PI-PLC specific activity, at 25 °C, on cIP concentration with 5 mM diC_7PC micelles (●) or 5 mM POPC SUVs (○) added. Note the 10-fold different specific activity scales for cIP with diC_7PC micelles (left) or POPC SUVs (right).

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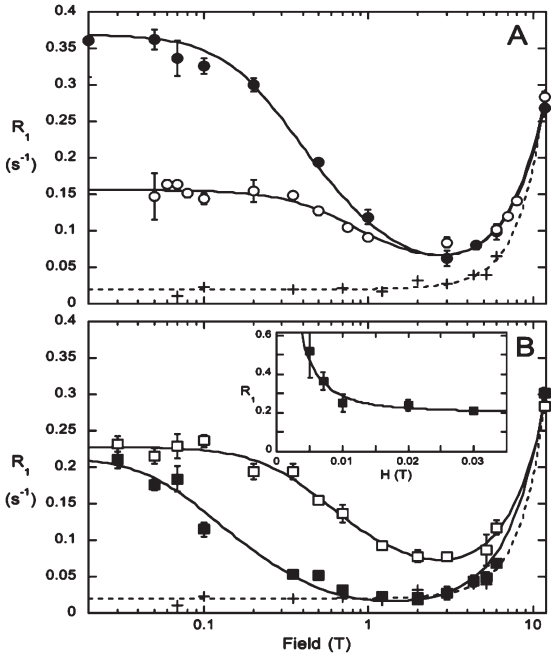

FIGURE 2: (A) Field dependence of 5 mM cIP ^{31}P R_1 in the absence (+) and presence (○) of 0.5 mg/mL H82C-SL, as well as with both H82C-SL and 5 mM diC$_7$PC (●) in 50 mM HEPES (pH 7.5) with 1 mM EDTA. (B) Field dependence of cIP ^{31}P R_1 with 5 mM diC$_6$PC (□) or 5 mM PC SUVs (■) and H82C-SL. For comparison, the relaxation profile for cIP in buffer is shown (+). The inset in panel B shows the very low field profile for cIP with the PC SUVs and H82C-SL present.

the relaxation rate is more or less constant and low below 1 T, with a rise at higher fields due to CSA that follows a square law dependence.

The presence of 14.4 μM non-spin-labeled H82C (with an excess of DTT present) yielded no significant change in cIP relaxation rates, presumably because the fraction of cIP that binds to enzyme is very small and the ^{31}P−^{1}H interaction in the bound state is relatively small as well. However, when the same amount of spin-labeled PI-PLC (H82C-SL) is added to a sample with cIP alone, there is a substantial increase in R_1 below 2 T for cIP [Figure 2A (○)].

While the binding of cIP to protein is weak, it is specific. In a mixture of three different water-soluble phosphates, cIP, glucose 6-phosphate, and diC$_4$PC, in the presence of H82C-SL (Figure S1 of the Supporting Information), the profiles for diC$_4$PC and glucose 6-phosphate were identical with or without the H82C-SL, indicating they did not bind significantly to the enzyme (or if they did were >20 Å from the site of the spin-label). Only the cIP exhibited strong relaxation by the spin-labeled protein.

The part of the ^{31}P relaxation at each field due to the spin-label, ΔR_1, was determined by subtraction of the non-spin-label contribution. The residual relaxation data were analyzed using standard relaxation theory (*5*, *6*), modified for a system in fast exchange on the on-enzyme-turnover time scale:

$$\Delta R_1 = \Delta R_{P-e}(0)/(1 + \omega_P{}^2\tau_c{}^2) \quad (1)$$

where $\Delta R_{P-e}(0)$ is the low-field limit of ΔR_1. We have omitted the usual terms of theory that involve the electron angular frequency from eq 1 since these are negligibly small because of the large magnitude of the electron's frequency compared to ω_P. As expected, the τ_c values so obtained did not depend on total enzyme concentration, E_o, and these are presented in Table 1 for the samples shown in Figure 2. In the absence of the diC$_7$PC micelles, the cIP τ_c increases from 0.019 to 7.1 ns when spin-labeled protein is added. The $\Delta R_{P-e}(0)$ of 0.14 s^{-1} clearly shows that bound cIP must be near the spin-labeled residue. When diC$_7$PC micelles are added, there is a larger effect on $\Delta R_{P-e}(0)$, but τ_c also increases. When POPC SUVs are present, the τ_c for bound cIP is even longer, 24 ns [Figure 2B (■)], and there is a further increase in R_1 below 0.01 T with a correlation time of ~1 μs (inset, Figure 2B). This additional low field rise in R_1 was not observed for cIP in any of the samples with diC$_7$PC micelles as the activator.

Table 1: Parameters Extracted via fc-P NMR for cIP (5 mM) with H82C-SL (14.4 μM) in the Absence and Presence of Different PC Species (5 mM)

PC	ΔR_{P-e} (s^{-1})	τ_c (ns)
–	0.14 ± 0.01	7.1 ± 1.0
diC$_7$PC	0.32 ± 0.01	16.8 ± 1.5
diC$_6$PC	0.21 ± 0.01	9.8 ± 1.2
POPC	0.20 ± 0.02	24.4 ± 5.9

The values of the parameter $\Delta R_{P-e}(0)$ obtained by fitting the data depend on concentrations of total cIP (denoted by $[cIP]_o$) and of total enzyme (E_o):

$$\Delta R_{P-e}(0) = ([H82C\text{-}SL\cdot cIP]/[cIP]_o) \quad (2a)$$

$$\times (S^2\tau_c r_{P-e}{}^{-6})[0.3\mu^2(h/2\pi)^2\gamma_P{}^2\gamma_e{}^2] \quad (2b)$$

Here the term on line 2a is the fraction of cIP bound to the enzyme relative to the total cIP. In term 2b, S^2 is the order parameter of the electron spin−^{31}P dipolar interaction which we take as unity because of the long distance r_{P-e} between the phosphorus of cIP and the electron spin compared to the size of local picosecond motions. We will eventually be interested in the values of r_{P-e} for the different complexes. The last term of term 2b contains standard constants of relaxation theory defined elsewhere (*5*, *6*).

A series of data sets like those depicted in Figure 2A were obtained for several total concentrations of enzyme (E_o) and $[cIP]_o$, the latter chosen so that the R_1 of the ^{31}P of cIP would be within the range permitted by the field-cycling apparatus. We assume that binding of cIP to the enzyme is a simple bimolecular reaction with a binding constant K_d. We define a new parameter v_{P-e} which is equal to $\Delta R_{P-e}(0)$ times $[cIP]_o$, divided by the total enzyme concentration. The new parameter v_{P-e} is then given by eqs 1 and 2 with the first term (eq 2a) replaced (after one familiar step using mass action, and the fact that $[cIP]_o \gg E_o$) by $(1 + K_d/[cIP]_o)^{-1}$ as shown in eq 3:

$$v_{P-e} = (1 + K_d/[cIP]_0)^{-1} \times c \quad (3)$$

where c is comprised of the terms in term 2b. The quantity v_{P-e} is an NMR version of specific activity in enzyme kinetics, and the asymptotic value of v_{P-e} at high concentrations of cIP is an NMR analogue of an enzymatic V_{max}. In Figure 3, v_{P-e} is plotted versus $[cIP]_o$ for the cIP binding to H82C-SL in the presence of either diC$_7$PC micelles or POPC vesicles. The values of K_d for cIP binding to inactive H82C-SL obtained by fitting these curves (25 ± 5 mM with diC$_7$PC and 14 ± 7 mM for POPC SUVs) are roughly twice the K_m values obtained from the kinetics (10 ± 1 mM with diC$_7$PC and 8.2 ± 0.3 mM for POPC SUVs). The asymptotic values of v_{P-e}, which are better determined than the K_d values, differ by a factor of 2 with diC$_7$PC micelles,

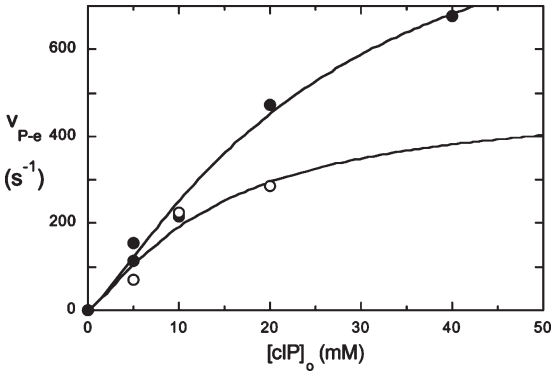

FIGURE 3: Variation of v_{P-e} with total cIP concentration with diC_7PC (●) or POPC SUVs (○) present.

leading to more effective relaxation. Dividing the average τ_c times the constants in term 2b by the asymptote and taking the sixth root, we obtain tentative r_{P-e} distances of 7.6 ± 0.1 Å for cIP bound to H82C-SL with diC_7PC present and 9.2 ± 0.6 Å when POPC SUVs serve as the activating interface. (They are tentative distances because, in this case, we do not know exactly what motion of the spin-label and ^{31}P that τ_c represents.) The two distances are significantly different when assessing the experimental error in the v_{P-e} asymptote in Figure 3 and, more importantly, the difference in τ_c values for the two systems. If we dock cIP into the myo-inositol position in the *Bacillus cereus* PI-PLC crystal structure [Protein Data Bank entry 1PTG (*10*)] and replace His82 with a Cys to which the flexible spin-label is attached, the $^{31}P-$electron distance is predicted to be in the range of 5−9 Å, depending on the orientation of the nitroxide group, consistent with the fc-P NMR analysis.

So what do these results imply about the low activity of PI-PLC hydrolyzing cIP without a PC interface? $\tau_c/\Delta R_{P-e}(0)$ is proportional to r^6 multiplied by the fraction of cIP bound to the enzyme. Since $\tau_c/\Delta R_{P-e}(0)$ in Table 1 is the same in the absence and presence of diC_7PC, the fraction of cIP at the active site must be similar. Therefore, the lower value of K_m in the presence of a PC interface is not the result of increased affinity for the cIP. The PC interface likely promotes a conformational change in the PI-PLC·cIP complex critical for hydrolysis. For "monomeric" diC_6PC activation of PI-PLC, the increased τ_c indicates that the cIP bound to H82C-SL with diC_6PC present forms a complex. The larger τ_c would be consistent with a complex in which more than a single diC_6PC was interacting with the protein to produce a mini-interface.

With respect to the weaker activation by PC SUVs, there are two notable differences in cIP behavior with micelle versus vesicle. (i) The r_{P-e} is longer (by ∼1−2 Å) with POPC SUVs, and (ii) cIP bound to the spin-labeled protein−POPC SUV complex exhibits an increased relaxation rate at very low fields that is not detected for the cIP and protein with diC_7PC micelles. PI-PLC binds tightly to PC SUVs with a K_d in the range of 0.04−0.06 mM (*11*). Displacement of the His82 catalytic residue could cause the poorer cyclophosphohydrolase activity of the enzyme when POPC SUVs serve as the activating interface. Alternatively, the further increase in the relaxation rate for cIP below 0.02 T (Figure 2B, inset) with a correlation time that resembles SUV tumbling rates [∼1 μs (*12*)] indicates that the cIP off rate from the POPC·H82C-SL·cIP complex must be less than 10^6 s^{-1}, an observation that would be consistent with slow release of IP from the POPC·PI-PLC·IP complex. Interestingly, given that the K_d values for cIP binding to H82C-SL with either interface are similar, a shorter off rate with SUVs as activators would mean that the on rate of cIP has been reduced, possibly indicating some occlusion of the active site when the protein is anchored to the vesicle.

Finally, this novel methodology holds promise for identification of binding proximities in other situations. For example, a small amount of protein spin-labeled at a particular site added to a mixture of phosphorus-containing ligands should relax only those ligands that bind near the spin-labeled site, a potentially useful way of identifying non-active-site binding ligands.

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SUPPORTING INFORMATION AVAILABLE

Protein preparation and spin labeling, cIP assays, fc-P NMR details, and data reduction. This material is available free of charge via the Internet at http://pubs.acs.org.

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