# A metallomicelle enzyme model for phospholipase C catalysis and inhibition

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**A $Cu^{II}$ metallomicelle mimics phospholipase C enzymes in catalysis and inhibition of transesterification reactions of phosphate diesters.**

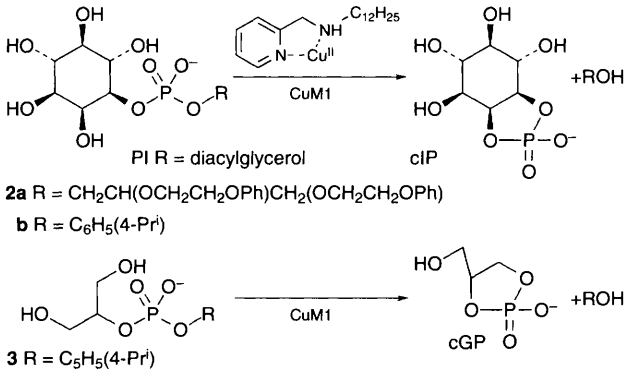
The influence of metal ions on nucleophilic substitution reactions at phosphorus has been studied for many years.[1] Recently, large rate accelerations have been reported for phosphoryl transfer reactions in the presence of metal ion complexes, including lanthanides and metallomicelles.[2–4] Metal-ion catalysis of phosphoryl transfer is a reaction of biological significance, in particular in relation to enzyme catalysis.[5] Enzymes of the phospholipase C (PLC) family catalyse phosphoryl transfer at an interface and the phosphatidylinositol specific enzymes (PI-PLC) have been the subject of considerable contemporary research.[6,7] In order to better understand these enzymes and the role of the interface in phospholipases in general, a copper metallomicelle model has been developed which mimics PI-PLC in catalysis and inhibition.

Bacterial PI-PLC catalyses the formation of *myo*-inositol 1,2-cyclic phosphate (cIP) from transesterification of phosphatidylinositol (PI) substrate, but is poorly active with pure PI unless co-surfactants are present to provide an interfacial environment.[7] Moreover, specific activity towards various substrate analogues is shown to increase 2–10 fold above the substrate CMC.[7] The transesterification reaction involving the 2-hydroxy group as the intramolecular nucleophile is reminiscent of ribonuclease-catalysed cyclizations.[8] Bacterial PI-PLC is specific for substrates containing the D-inositol head-group, but is profoundly inhibited by simple water-soluble polyanions and monoanionic surfactants that do not contain this group.[7] The interface is clearly important in influencing enzyme catalysis and inhibition. Since a Cu-metallomicelle provides a catalyst ($Cu^{2+}$) bound at an interface, such a system is potentially an excellent model.

The use of metal complexes in phosphoryl transfer has been directed more at gaining knowledge of the general principles of enzyme catalysis and obtaining artificial restriction enzymes rather than at the mimicry of specific enzymes. A metallomicellar mimic of PI-PLC cannot involve exchange-inert complexes since the natural enzyme is readily desorbed from the interface. Furthermore, lanthanides are too efficient as catalysts to confine reactivity to the interface. Phosphate triester hydrolysis catalysed by $Cu^{II}$-metallomicelle complexes and $Cu^{II}$ complexes that catalyse cyclization of *vic*-diol phosphate diesters have been described.[2,4a,b] There is no evidence that PI-PLC is a metalloenzyme, but it was reasoned that a metallomicelle might provide an excellent model of PI-PLC and PLCs in general. Gratifyingly, the 1:1 $Cu^{II}$-ligand complex (CuM1)‡ was observed to catalyse the formation of cIP, from natural PI and the PI substrate analogue **2a** at 7.3 < pH < 8.7, Scheme 1.§[9] Both PI and **2a** are good substrates for PI-PLC from *B. cereus*, Fig. 1.[7e]

In order to examine CuM1 in more detail, the reaction of the PI analogue **2b** was monitored by HPLC with product identification by $^{31}P$ NMR (Table 1, Fig. 1). Control experiments at pH 8.0 in *N*-methylmorpholine (NMM) buffer showed low background reaction rates: (i) in the presence of 2-aminomethyl pyridine with or without added $CuBr_2$ no reaction was observed over the timescale of reaction (24 h); (ii) with $CuBr_2$ alone, 9% reaction was observed; (iii) with $CuBr_2$ in the presence of an interface provided by Triton X100, only 1–2% reaction was observed. In contrast, in the presence of CuM1 (2.5–5 mmol $dm^{-3}$) reaction proceeded to completion with pseudo-first order kinetics, Table 1.¶ The only P-containing species observed over the course of reaction by $^{31}P$ NMR were **2b** and the product, cIP, Scheme 1, Fig. 1.

In order to examine the substrate specificity of CuM1, the reactions with dipalmitoylphosphatidylglycerol (PG) and its analogue **3** were examined under similar conditions to those employed for the PI substrates, Scheme 1. Both compounds




**Scheme 1**

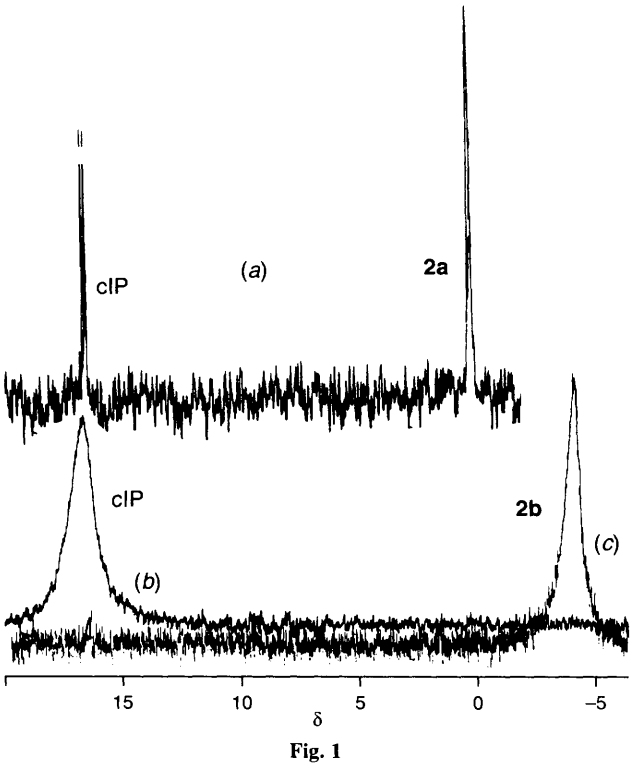



**Fig. 1**

**Table 1** Pseudo-first order rate constants for formation of cIP from **2b** and **3**, $k_{obs} \times 10^5$ s$^{-1}$

| **2b** | pH 7.8 | pH 8.0 | pH 8.2 |
|---|---|---|---|
| [CuM1] = 2.5 mmol dm$^{-3}$ | 5.5 | 7.0 | 8.2 |
| 3.75 mmol dm$^{-3}$ | 7.2 | 9.1 | 9.9 |
| 5.0 mmol dm$^{-3}$ | 9.2 | 10.5 | 11.2 |
| **3** | pH 7.8 | pH 8.0 | pH 8.2 |
| [CuM1] = 2.5 mmol dm$^{-3}$ | 2.1 | 2.7 | 3.5 |
| 3.75 mmol dm$^{-3}$ | 3.3 | 3.9 | 4.5 |
| 5.0 mmol dm$^{-3}$ | 4.6 | 5.1 | 5.7 |

Reactions run in duplicate, substrate (0.5 mmol dm$^{-3}$), NMM (50 mmol dm$^{-3}$), Triton X100 (0.1 mmol dm$^{-3}$), 37 °C. Analysis by quantification of Pr$^i$PhOH product relative to PhOH internal standard using detection at 272 nm after separation of reaction mixture by HPLC on RP C18 column using MeOH/water gradient.

were converted to the cyclic phosphate as sole P-containing product.‖ The rate of reaction of the glycerol ester **3** is slightly greater than that of the inositol ester **2b** in the presence of CuM1 taking account of statistical bias, Table 1. The favourable *cis*-orientation of the intramolecular nucleophile in **2b** is apparently redundant in the ternary complex of CuM1 with substrate.**[10] Reactions of **2a** and **3** are not first order in hydroxide in the pH region studied consistent with previous arguments concerning the low p$K_a$ of ROH complexed to Cu$^{II}$ and within the Stern layer of a micelle.[4a] Reaction is first order in CuM1 for the slowest reaction, with **2a** at pH 7.8, but a plateauing effect is seen with the faster reactions. The cumulative observations are consistent with: (1) binding of the 4-iso-propylphenyl ester substrates into the micellar interface; and (2) Lewis acid catalysis by allowing reaction of the internal alkoxide nucleophile at neutral pH within the ternary complex with CuM1.

Phytic acid and Aerosol OT (a sulfonate detergent) are examples of water-soluble, polyanion and monoanionic surfactant PI-PLC inhibitors, respectively.[7e] Both compounds also inhibited metallomicelle catalysed breakdown of the PI analogue **2b**. No reaction was observed at pH 8.0, 37 °C, in NMM buffer, after 24 h, at concentrations of inhibitor equimolar with CuM1 and **2b** (1.4 mmol dm$^{-3}$). This inhibition is not surprising: phytic acid as an excellent metal chelator would effectively remove Cu$^{II}$ from the interface;[11] and Aerosol OT incorporated into the interface would be expected to compete with substrate for binding to $Cu^{2+}$.

The simple CuM1 metallomicellar system provides an excellent model system for further examining interfacial activation and inhibition associated with PLC enzymes. The enzyme-catalysed transesterification reaction is mimicked, yielding cyclic phosphate product. Most importantly, the reaction is confined almost entirely to the interface, since in the absence of the amphiphilic diamine ligand only 1–2% reaction is observed. Furthermore, inhibition is observed by both major classes of PI-PLC inhibitors.

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## Footnotes

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‡ CuM1 was isolated and characterized as the 1 : 1 complex (CuM1Br$_2$) by elemental analysis. The pyridyl ligand has been described previously.[4b] CuM1 is soluble in aqueous solution in a variety of buffer systems. Precipitation occurs at higher pH, higher CuM1 concentrations and with prolonged standing, but is mitigated by cosurfactants such as Tritton X100 and CTAB.

§ PI analogues **2a,b** were synthesized by adaptation of literature procedures using P$^{III}$ methodology and fully characterized as homogeneous by spectroscopic and HPLC analysis.[7e,8] Analogue **2b** (12 mmol dm$^{-3}$; $^{31}$P δ ≈ −3.9) was converted to cIP ($^{31}$P δ ≈ 16.7) after overnight incubation at pH 8.0, 37 °C in NMM buffer by CuM1 (10 mmol dm$^{-3}$). Natural PI (8.3 mmol dm$^{-3}$; from Serdary, Ontario) was converted approx. 70% to cIP after 5 d incubation with monitoring by $^{31}$P NMR. Analogue **2a** (0.5 mmol dm$^{-3}$; $^{31}$P δ ≈ 0.3) was converted approx. 20% to cIP after 18 h in NMM buffer, pH 8.0, 37 °C, by CuM1 (5 mmol dm$^{-3}$).

¶ The half-life for hydrolysis of **3** (0.5 mmol dm$^{-3}$) in NMM buffer (50 mmol dm$^{-3}$, pH 8.2) at 37 °C is approx. 42 d. The rate enhancement owing to catalysis by CuM1 is thus ≥300, Table 1.

‖ PG analogue **3** was synthesized by adaptation of literature procedures using P$^{III}$ methodology and the final product fully characterized as homogeneous by spectroscopic and HPLC analysis.[8] Analogue **3** (13 mmol dm$^{-3}$; $^{31}$P δ ≈ −3.7) was converted to cyclic product ($^{31}$P δ ≈ 19.1) after overnight incubation at pH 8.0, 37 °C in NMM buffer by CuM1 (10 mmol dm$^{-3}$). Natural PG [7.3 mmol dm$^{-3}$; from Serdary; in the presence of Triton X100 (4 μl ml$^{-1}$)] was converted approx. 40% to cyclic product after 5 d incubation with monitoring by $^{31}$P NMR.

** Evidence for this preference is seen in the study of the alkaline hydrolysis of the *cis*- and *trans*-isomers of glycerol 1-(2-hydroxycyclohexyl phosphate).[10]

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