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Mutation of Two Active-Site Residues Converts a Phosphatidylinositol-Specific Phospholipase C to a Glucose Phosphatase

Jianwen Feng, Kimberly Stieglitz, and Mary F. Roberts*

Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02467

Received September 15, 2003; E-mail: mary.roberts@bc.edu

Phosphatidylinositol-specific phospholipase C (PI-PLC) and glucose phosphatases carry out very different cleavage reactions. PI-PLC enzymes are very specific and carry out PI cleavage in two steps; there is no covalent intermediate.[1] Two histidines, His32 and His82, have been postulated to act as general base and general acid, respectively.[2] There is an initial intramolecular attack of the inositol 2-OH on the phosphorus of PI to generate a cyclic intermediate, cyclic-inositol-1,2-phosphate (cIP), followed by attack of an appropriately activated water molecule on the cIP to generate inositol-1-phosphate (I-1-P).[2] Arg69 is a key residue positioned in the active site to stabilize the negatively charged pentacovalent transition state;[3] it also weakly interacts with the 2-OH group of myo-inositol.[4] The active site of the enzyme has a large number of negatively charged residues that form hydrogen bonds with the substrate inositol hydroxyl groups.[4] None of the PI-PLC enzymes studied have been shown to generate inorganic phosphate (Pi) from phospholipid substrates or from water-soluble cIP (or I-1-P). The glucose phosphatases belong to the class of histidine acid phosphatases that use a histidine imidazole nitrogen as a nucleophile to produce glucose and a phosphoenzyme intermediate that is subsequently hydrolyzed to inorganic phosphate, Pi.[5,6] Critical conserved catalytic residues in glucose phosphatases include a His, Arg, and Asp or Glu.[7−11] In particular, the acidic residue in the active site is proposed to participate in protonation of the leaving group.

Minor changes in the PI-PLC active site have already been shown to alter the specificity for substrate binding. Tsai and co-workers have converted the bacterial PI-PLC into a metal-dependent enzyme by introducing an aspartate into the Arg69 site.[12] The very low specific activity of R69D could be improved by introducing calcium. However, R69D still exhibits specificity for cleavage of phosphodiesters (i.e., no phosphatase activity). Lys115 is a residue in the active site that interacts with His32. We have changed this to a glutamate residue, a change that could alter the pK_a of the histidine and its interaction with phosphate esters. In particular, the double mutant, R69D/K115E, was able to generate Pi from PI, suggesting it possesses phosphatase activity (Figure 1).

Neither recombinant wild-type PI-PLC (rPI-PLC), R69D, nor K115E alone exhibited any production of Pi from PI or cIP. The rate of generating Pi from PI solubilized in Triton X-100 micelles was almost 10-fold lower than the already low rate for PI cleavage to cIP (Table 1) but comparable to the rate of cIP hydrolysis by this double mutant. Pi was also produced after incubating R69D/K115E with cIP and L-I-1-P as well as D-I-1-P, indicating a loss of specificity in substrate binding. The new phosphatase activity of R69D/K115E (unlike rPI-PLC phoshodiesterase activity) was inhibited by Pi (1.5 mM K_i).

Relaxed specificity for inositol phosphate isomers was also accompanied by a new ability to bind and hydrolyze sugar phosphates. R69D/K115E PI-PLC hydrolyzed glucose-6-P (G-6-P), fructose-6-P, fructose-1,6-bisphosphate, and mannose-6-P with similar rates (Table 2) that were much higher than those toward inositol phosphate (370-fold increase when compared to D-I-1-P). Substitutions on the glucose ring had little effect on this phosphatase activity.

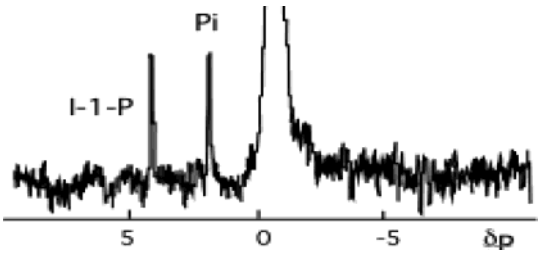

Figure 1. ^{31}P NMR spectrum showing R69D/K115E cleavage of PI (large peak) to I-1-P and Pi.

Table 1. Specific Activities for Cleavage of Inositol Esters by R69D/K115E[a]

	specific activity (μmol min^{-1} mg^{-1})	
ksubstrate (μM)	phosphodiesterase	phosphatase
PI/diC$_7$PC (4:16)	0.093	0.0007
PI/TX-100 (8:16)	0.024	0.0022
cIP (10)	0.007	0.0040
D-I-1-P (5)		0.0018
D-I-1-P (5)		0.0008

[a] Reaction carried out in 50 mM HEPES, pH 7.5, at 37 °C. Errors in the specific activities were ±25%. For comparison, the specific activities for cleavage of PI/TX-100 to cIP and cIP hydrolysis were 380 and 8.4 μmol min^{-1} mg^{-1} for rPI-PLC.

Table 2. Phosphatase Activity of R69D/K115E toward Various Phosphomonoesters[a]

substrate	amount (mM)	specific activity (μmol min^{-1} mg^{-1})
pNPP	5	9.24
glucose-6-P	10	0.67
2-deoxy-glucose-6-P	10	0.23
D-glucosamine-6-P	10	0.70
glucose-1-P	10	0.68
D-mannose-6-P	10	0.32
fructose-6-P	10	0.68
fructose-1,6-P$_2$	10	1.1 (F-6-P); 0.9 (Pi) [b]
3-phosphoglycerol	10	0.0
2-phosphoglycerol	10	0.0
pyrophosphate	10	0.0

[a] Assays were carried out in 50 mM HEPES, pH 7.5, 25 °C, using the concentration of phosphate ester listed and analysis by ^{31}P NMR (except for pNPP hydrolysis, which was followed colorimetrically). [b] Fructose-6-P was produced along with Pi; no F-1-P was observed.

p-Nitrophenyl phosphate (pNPP), a substrate for many phosphatases, was an excellent substrate for R69D/K115E, while glycerol phosphates and pyrophosphate were not substrates (although pyrophosphate, like Pi, could inhibit pNPP hydrolysis). Phosphatase substrate specificity of R69D/K115E PI-PLC was similar to that of glucose-6-phosphatase.

Using pNPP as a substrate, we found that R69D/K115E exhibited a broad pH profile with an optimum pH ∼7−7.5. Ca^{2+} did not

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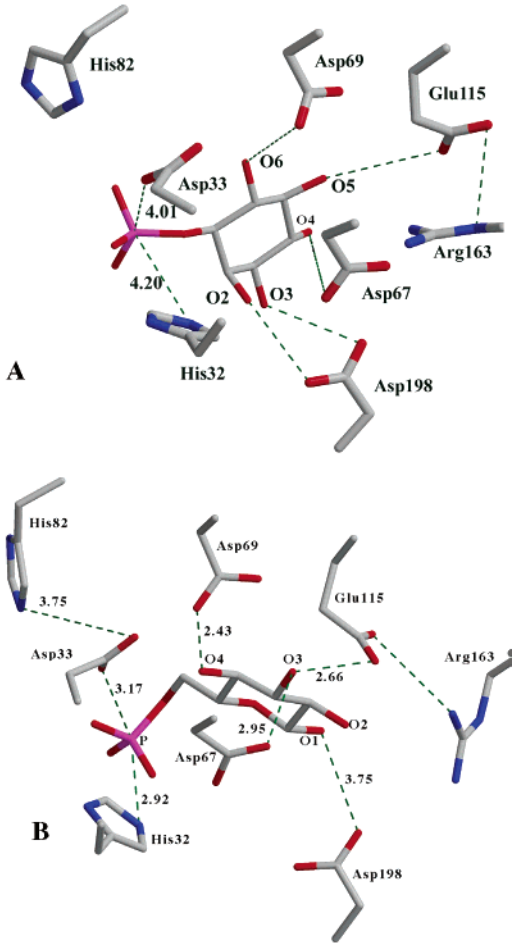

Figure 2. Energy-minimized active site of R69D/K115E with (A) I-1-P or (B) G-6-P bound. Dotted lines indicate distances ≥4 Å unless otherwise indicated.

enhance this phosphatase activity but inhibited it. At pH 7.5, the K_m for pNPP was 2.8 ± 0.4 mM and the V_{max} was 14 ± 1 μmol min^{-1} mg^{-1}. For G-6-P, the K_m was 0.67 ± 0.05 mM and the V_{max} was 0.71 ± 0.02 μmol min^{-1} mg^{-1}. The K_m for G-6-P was much lower than the K_m of rPI-PLC for cIP,[13] suggesting that the active site has been rearranged to optimize binding of the sugar ring structure. The lower V_{max} with G-6-P presumably reflects the fact that pNP is a better leaving group.

DiC_7PC (10 mM), which binds at the top of the PI-PLC $(\beta\alpha)_8$-barrel and is a good activator of PI cleavage and cIP hydrolysis,[13,14] inhibited R69D/K115E PI-PLC-catalyzed hydrolysis of 2 mM pNPP ~2-fold. Other detergents (e.g., TX-100 and deoxycholate) inhibited pNPP hydrolysis to the same extent, suggesting that amphiphiles binding to the interfacial site inhibit interaction of the water-soluble sugar phosphates at the active site. PI, solubilized in TX-100, caused little inhibition of R69D/K115E hydroysis of pNPP above that of the detergent alone. Myo-inositol (50 mM) had no effect on the phosphatase activity of R69D/K115E, whereas this concentration reduced rPI-PLC hydrolysis of cIP to <10%.[15] Vanadate (0.05 mM), a potent inhibitor of histidine acid phosphatases, had no effect on rPI-PLC cleavage of PI or hydrolysis of cIP. However, this concentration (and lower) of vanadate dramatically inhibited R69D/K115E hydrolysis of pNPP, with a K_i ~0.7 μM (assuming competitive inhibition). Vanadate, a phosphate analogue capable of expanding its coordination geometry, could form a pentavalent complex with an active site residue mimicking a transition state.

Insight into the altered chemistry and specificity of R69D/K115E was provided by modeling based on the wild-type PI-PLC crystal structure and docking the two phosphate substrates. The model of *Bacillus cereus* PI-PLC (1PTD) with waters removed together with an I-1-P ligand was imported into Quanta2000 and minimized using the CHARMM minimization protocol of conjugate gradient.[16] In the exported minimized structure, stripped of ligand and loaded into O,[17] Arg69 was replaced by an aspartate residue and Lys115 replaced by a glutamate and the geometry refined. Hydrated I-1-P (or G-6-P) was added and the model minimized. While there are side chains poised around the inositol hydroxyl groups in the model of R69D/K1115E (Figure 2A), most of these are at fairly long hydrogen-bonding distances (~4 Å), except for the 6-OH with Asp69 and the 2-OH with Asp198. Similarly, a likely hydrogen bond between Glu115 and the 5-OH group was ~4.0 Å. The inositol ring in this model is not poised above Tyr200, as it is in the native PI-PLC crystal structure, but at an angle. More tellingly, the phosphate group between the two histidines is ≥4 Å from His32 and Asp33 and much farther away from His82 (>5.4 Å). The model of the mutant with G-6-P docked (Figure 2B) has many of the ring hydroxyl groups within good hydrogen-bonding distance of active residues, including the mutated Asp69 and Glu115. The different orientation of the glucose versus the inositol ring that is adopted to optimize hydrogen-bonding interactions is also consistent with the broader specificity of the phosphatase reaction of R69D/K115E. However, more striking is the observation that G-6-P phosphate is positioned much closer to His32 (2.9 Å), with Asp33 also close to the phosphate (3.2 Å). The positioning of the phosphate of G-6-P in this modified active site suggests that in R69D/K115E, His32 could act as a nucleophile in the phosphatase reaction with little participation from His82. Consistent with this proposal, H32A/R69D/K115E has no detectable activity while R69D/H82A/K115E has a specific activity toward G-6-P of 0.03 μmol min^{-1} mg^{-1}, only 22-fold lower than that of the double mutant. Regardless of the detailed mechanism, this is a unique example of changing the chemistry of an enzyme with minimal mutations.

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References

(1) Hondal, R. J.; Zhao, Z.; Tsai, M. D.; et al. *Biochemistry* **1998**, *37*, 4568−4580.
(2) Heinz, D. W.; Ryan, M.; Bullock, T. L.; Griffith, O. H. *EMBO J.* **1995**, *14*, 3855−3863.
(3) Mihai, C.; Kravchuk, A. V.; Tsai, M. D.; Bruzik, K. S. *J. Am. Chem. Soc.* **2003**, *125*, 3236−3242.
(4) Gassler, C. S.; Ryan, M.; Liu, T.; Griffith, O. H.; Heinz, D. W. *J. Biol. Chem.* **1997**, *36*, 12802−12813.
(5) Van Etten, R. L. *Ann. N.Y. Acad. Sci.* **1982**, *390*, 27−51.
(6) Vincent, J. B.; Crowder, M. W.; Averill, B. A. *Trends Biochem. Sci.* **1992**, *17*, 105−110.
(7) Lim, D.; Golovan, S.; Forsberg, C. W.; Jia, Z. *Nat. Struct. Biol.* **2000**, *7*, 108−113.
(8) Kostrewa, D.; Grüninger-Leitch, F.; D'Arcy, A.; Broger, C.; Mitchell, D.; van Loon, A. P. G. M. *Nat. Struct. Biol.* **1997**, *4*, 185−190.
(9) Kostrewa, D.; Wyss, M.; D'arcy, A.; van Loon, A. P. G. M. *J. Mol. Biol.* **1999**, *288*, 965−974.
(10) Schneider, G.; Lindqvist, Y.; Vihko, P. *EMBO J.* **1993**, *12*, 2609−2615.
(11) Lee, C. D.; Cottrill, M. A.; Forsberg, C. W.; Jia, Z. *J. Biol. Chem.* **2003**, *278*, 31412−31418.
(12) Kravchuk, A. V.; Zhao, L.; Bruzij, K. S.; Tsai, M. D. *Biochemistry* **2003**, *42*, 2422−2430.
(13) Zhou, C.; Wu, Y.; Roberts, M. F. *Biochemistry* **1997**, *36*, 347−355.
(14) Zhou, C.; Qian, X.; Roberts, M. F. *Biochemistry* **1997**, *36*, 10098−10097.
(15) Feng, J.; Wehbi, H.; Roberts, M. F. *J. Biol. Chem.* **2002**, *277*, 19867−19875.
(16) *QUANTA Generating and Displaying Molecules*; Molecular Simulations Inc.: San Diego, 1997.
(17) Jones, T. A.; Zou, J.-Y.; Cowan, S. W.; Kjeldgaard, M. *Acta Crystallogr.* **1991**, *A47*, 110−119.

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