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Metal ions and phosphatidylinositol 4,5-bisphosphate as interacting effectors of α-type plant phospholipase D

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ABSTRACT

Plant phospholipases D (PLD) are typically characterized by a C2 domain with at least two Ca^{2+} binding sites. *In vitro*, the predominantly expressed α-type PLDs need 20–100 mM $CaCl_2$ for optimum activity, whereas the essential activator of β- or γ-type PLDs, phosphatidylinositol 4,5-bisphosphate (PIP_2), plays a secondary role. In the present paper, we have studied the interplay between PIP_2 and metal ion activation of the well-known α-type PLD from cabbage (PLDα). With mixed micelles containing phosphatidyl-*p*-nitrophenol as substrate, PIP_2-concentrations in the nanomolar range are able to activate the enzyme in addition to the essential Ca^{2+} activation. Mg^{2+} ions are able to replace Ca^{2+} ions but they do not activate PLDα. Rather, they abolish the activation of the enzyme by Ca^{2+} ions in the absence, but not in the presence, of PIP_2. The presence of PIP_2 causes a shift in the pH optimum of PLDα activity to the acidic range. Employing fluorescence measurements and replacing Ca^{2+} by Tb^{3+} ions, confirmed the presence of two metal ion-binding sites, in which the one of lower affinity proved crucial for PLD activation.

Moreover, we have generated a homology model of the C2 domain of this enzyme, which was used for Molecular Dynamics (MD) simulations and docking studies. As is common for C2 domains, it shows two antiparallel β-sheets consisting of four β-strands each and loop regions that harbor two Ca^{2+} binding sites. Based on the findings of the MD simulation, one of the bound Ca^{2+} ions is coordinated by five amino acid residues. The second Ca^{2+} ion induces a loop movement upon its binding to three amino acid residues. Docking studies with PIP_2 reveal, in addition to the previously postulated PIP_2-binding site in the middle of the β-sheet structure, another PIP_2-binding site near the two Ca^{2+} ions, which is in accordance with the experimental interplay of PIP_2, Ca^{2+} and Mg^{2+} ions.

1. Introduction

Phospholipase D (PLD; EC 3.1.4.4) is a phosphodiesterase occurring in animals, plants and microorganisms (Selvy et al., 2011). PLD hydrolyzes glycerophospholipids such as phosphatidylcholine (PC) at the terminal phosphodiester bond, producing the second messenger phosphatidic acid (PA). In plants, PLD and PA play manifold roles in growth, senescence, and stress responses (Bargmann and Munnik, 2006; Wang, 2005; Testerink and Munnik, 2011). Moreover, the enzyme is capable of exchanging the alcohol in the polar head group and thus modifies phospholipids. While the significance of this second reaction in nature is not yet clear, it is widely exploited in biotechnology for the synthesis of rarely occurring or artificial phospholipids (Ulbrich-Hofmann et al., 2005; De Maria et al., 2007; Damnjanovic and Iwasaki, 2013).

In recent decades, mammalian and plant PLDs have been extensively discussed in the light of their involvement in the network of interacting signalling pathways (Hong et al., 2010; Jang et al., 2012). In addition to PA as reaction product, Ca^{2+} ions and phosphatidyl-inositol 4,5-bisphosphate (PIP_2) belong to the most prominent signalling molecules with influences on PLD activity

Abbreviations: *diac*-PIP_2, 1,2-diacetyl-glycero-3-phosphoinositol-4,5-bisphosphate; MD, Molecular Dynamics; MES, 2-(N-morpholino)ethanesulfonic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PIP_2, phosphatidylinositol 4,5-bisphosphate; P*p*NP, phosphatidyl-*p*-nitrophenol; PLD, phospholipases D; PLDα, α-type PLD; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulphate.

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(Mansfeld and Ulbrich-Hofmann, 2009). In plants, a substantial number of isoenzymes, which differ in their regulatory mechanisms, complicate the overall assessment of PLD function. Thus, 12 PLD isoenzymes were found in *Arabidopsis thaliana*. Based on gene architecture, sequence similarity, domain structure, and biochemical properties, they have been classified into six types: PLDα (3), β (2), γ (3), δ, ε and ζ (2) (Qin and Wang, 2002), where the numbers in parentheses give the number of the corresponding isoenzymes. As many as 17 or 18 PLD isoenzymes have been identified in rice (Li et al., 2007) or soybean (Zhao et al., 2012). In white cabbage two α-type PLDs (Schäffner et al., 2002) and one or two γ-type PLDs (Novotná et al., 2003) have been described hitherto. Based on the cabbage genome, as far as sequenced (BolBase, www.ocri-genomics.org), nine isoenzymes (PLDα (2), β (2), γ (1), δ (1), ε (1) and ζ(2)) can be deduced.

All PLDs that are homologous to each other belong to the PLD superfamily (Ponting and Kerr, 1996; Liscovitch et al., 2000), which additionally contains some other proteins such as cardiolipin synthases or phosphatidylserine synthases. Two conserved regions are a common feature of all these PLDs, the so-called HKD motifs, which form the catalytic site of the enzyme. In contrast to microbial PLDs, which are best studied from several *Streptomyces* species (Uesugi and Hatanaka, 2009), mammalian and plant PLDs are characterized by one or two regulatory domains. While mammalian PLDs typically contain PH and PX domains (Selvy et al., 2011), most plant PLD isoenzymes are distinguished by the C2 domain. Ten of the 12 PLD isoenzymes from *A. thaliana* (α-, β-, γ-, δ-, ε-types) contain one C2 domain at their N-terminus and are designated as Ca^{2+}-dependent or C2-PLDs, whereas the two PLDs of the ζ-type (PLDζ1 and PLDζ2) contain N-terminal PX and PH domains like the mammalian PLDs and are designated as Ca^{2+}-independent or PH/PX-PLDs.

The C2 domain contains *ca.* 130 amino acids that are organized as 8 antiparallel β-strands, connected by loop regions (Nalefski and Falke, 1996; Hurley and Misra, 2000). Generally, there is a large number of proteins containing C2 domains, most of which are involved in membrane-associated processes (Cho and Stahelin, 2006). A variety of different ligands such as Ca^{2+} ions, phospholipids, inositol polyphosphates, and intracellular proteins are known to regulate C2 domain-containing proteins (Rizo and Südhof, 1998; Corbalan-Garcia and Gómez-Fernández, 2014). Both Ca^{2+}-binding loops and PIP_2-binding regions have been identified in the C2 domains of plant PLDs (Selvy et al., 2011).

The predominant PLD isoenzyme found in leaves, seeds and other plant tissues is the α-type. This form is proposed to have catabolic functions in the degradation of membranes, whereas the other forms are strictly involved in cell signalling and are therefore regulated more stringently by PIP_2 and other molecules. This opinion, however, seems to be over-simplified, as several α-type PLDs contain PIP_2- as well as Ca^{2+}-binding sites, as shown for the C2-domains of PLDα from *Arabidopsis thaliana* (Zheng et al., 2000) and tomato (Tiwari and Paliyath, 2011). Moreover, Ca^{2+}-binding plays an enigmatic role, because non-physiologically high concentrations (20–100 mM) of this metal ion are necessary for optimal activity *in vitro* (Heller, 1978; Mansfeld and Ulbrich-Hofmann, 2009). Because such high Ca^{2+} ion concentrations are not realistic *in vivo*, the activation of PLDα is presumably the result of one or several additional influences. Thus, it was shown for PLDα from castor bean, for example, that PIP_2 has an activating influence at Ca^{2+} concentrations that are suboptimal in the absence of PIP_2 (Pappan and Wang, 1999). Similar effects were observed for PLDα from Indian mustard seeds (Khatoon et al., 2015).

In this paper, the effect of PIP_2 on the well-known α-type PLD isoenzyme 2 from cabbage (abbreviated to PLDα), the interplay of this effect with the presence of metal ions, and the competition between different metal ions such as Tb^{3+} and Mg^{2+} ions with Ca^{2+} ions have been analysed in a micellar system containing phosphatidyl-*p*-nitrophenol (P*p*NP) as substrate, Triton X-100 and sodium dodecyl sulphate (SDS). A three-dimensional model of the C2 domain of PLDα containing two Ca^{2+} -binding sites has been constructed by homology modelling and Molecular Dynamics (MD) studies. Putative PIP_2-binding regions and their relation to the Ca^{2+}-binding sites were suggested from molecular-docking studies.

2. Results and discussion

2.1. The binding of metal ions to PLDα and its activation in the absence of PIP_2

The unusually high requirement for Ca^{2+} ions (>20 mM) of α-type PLDs to attain maximal activity has been described for α-type PLDs from several sources, such as *A. thaliana* (Qin et al., 1997), white cabbage (Schäffner et al., 2002), poppy seedlings (Lerchner et al., 2005), sunflower seeds (Abdelkafi and Abousalham, 2011) or mustard seeds (Khatoon et al., 2015). In most cases Ca^{2+} ions cannot be replaced by any other ions such as Mg^{2+}, Sr^{2+} or Ba^{2+}. Interestingly, we have recently found for PLDα from cabbage that lanthanide ions such as Tb^{3+} ions are able to substitute for Ca^{2+} ions (Dressler et al., 2014). These ions effect their maximum activation at much lower concentration, however, achieve only 36% of the activity, compared to the activation by Ca^{2+} ions. Because of their high efficiency, Tb^{3+} ions (6 mM) are able to replace Ca^{2+} ions in PLDα after pre-incubation with 100 mM $CaCl_2$, whereas Ca^{2+} ions (100 mM) are not able to replace Tb^{3+} ions after pre-incubation with 6 mM $TbCl_3$. Here, we continue the competition experiments by showing that the replacement or non-replacement of these ions can also be observed by fluorescence measurements in the absence of substrate (Fig. 1). The fluorescence of Tb^{3+} ions (6 mM) in the presence of PLDα was nearly unchanged if Ca^{2+} ions up to 100 mM were added (Fig. 1A), whereas the fluorescence increased as a function of the Tb^{3+} ion concentration if the enzyme was pre-incubated with 100 mM $CaCl_2$ (Fig. 1B). From the data shown in Fig. 1B, two dissociation constants could be calculated according to section 4.6 (K_{D1} = 0.11 ± 0.01 mM; K_{D2} = 32.1 ± 0.93 mM; R^2 = 0.952), which reflect two binding events with very different affinities. These constants are in the same range as the dissociation constants (0.11 and 10.2 mM) previously determined for Ca^{2+} ion-binding based on data from near-UV CD spectroscopy (Stumpe et al., 2007).

From the fluorescence measurements with lanthanide ions, which are only able to substitute for Ca^{2+} ions with respect to enzyme activation, it can be concluded that these metal ions bind to PLDα via two binding sites with very different affinities, where the occupation of the binding site with higher affinity has almost no influence on the activity of the enzyme, as concluded from the results of PLD activation by lanthanides (Dressler et al., 2014). Only the occupation of the binding site with low affinity induces significant activity. Even if the binding data obtained in the absence of substrate by measuring the fluorescence of Tb^{3+} ions (Dressler et al., 2014 and Fig. 1B) do not allow any statement to be made regarding the cooperativity between the two binding events, all activation data strongly suggest a cooperative interaction between the two binding sites (Dressler et al., 2014). Clearly, the occupation of the first metal ion-binding site by Ca^{2+} or Tb^{3+} promotes the conformational change induced by occupation of the second binding site and is necessary for the development of full activity. Alternative mechanisms, e.g. the involvement of the second Ca^{2+} ion in the catalytic mechanism, could be excluded (Dressler et al., 2014).

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