



Activation of phospholipase A2 by Hsp70 *in vitro*

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ABSTRACT

We recently suggested a novel mechanism for the activation of phospholipase A2 (PLA2), with a (catalytically) highly active oligomeric state, which subsequently becomes inactivated by conversion into amyloid. This process can be activated by lysophosphatidylcholine which promotes both oligomerization and amyloid activation/inactivation. The heat shock protein 70 (Hsp70), has been demonstrated to be able to revert the conversion of α -synuclein and Alzheimer β -peptide to amyloid fibrils *in vitro*. Accordingly, we would expect Hsp70 to sustain the lifetime of the active state of the enzyme oligomer by attenuating the conversion of the enzyme oligomers into inactive amyloid. Here we show that Hsp70 activates PLA2 *in vitro*, in a manner requiring ATP and Mg^{2+} .

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1. Introduction

Phospholipases A₂ (PLA2) represent a ubiquitous group of enzymes serving a number of functions, from toxicity of several venoms to phospholipid metabolism, digestion, cellular signaling, and antimicrobial activity. These enzymes cleave the *sn*-2 acyl chain of glycerophospholipids to yield lysophospholipids and free fatty acids (FFA), and both their catalytic mechanism and structures are conserved with a high degree of sequence homology between different species, secretory PLA2 in particular [1,2].

The hallmark of lipolytic enzymes, including PLA2, is the so-called interfacial activation: compared to the hydrolysis of monomeric substrates their activity is dramatically enhanced when reacting with phospholipid interfaces [3], such as those present in micelles, monolayers, and bilayers. Three mechanistic models have been proposed to explain this property. The *enzyme* model assumes that a change in PLA2 conformation is induced by the interface [4], whereas

the *substrate* model assigns the activation to the physicochemical properties of the interface [5]. Changes in PLA2 conformation upon interfacial activation were recently demonstrated using Fourier transform infrared spectroscopy, and it was suggested that enzyme conformational alterations, in unison with the physical state of the substrate, would cause the augmented catalytic activity [6,7]. Third model, functional *amyloid oligomer* was recently forwarded by us [8]. De Haas and coworkers originally suggested 'superactivation' of PLA2 to be caused by the formation of PLA2 aggregates at interfaces [9], and this was concluded also by Dennis and coworkers [10]. A critical content of the hydrolytic products lysoPC and FFA, approx. $X = 0.08$ is required for the activation [11] and aggregation of PLA2 in the presence of FFA and lysoPC has been demonstrated [12–14]. We recently characterized this aggregation process in more detail and observed the formation of amyloid-like fibers staining with Congo Red and thioflavin T *in vitro* upon the hydrolysis of DPPC by PLA2 [8]. These fibrils have been recently demonstrated also by atomic force and fluorescence microscopy of PLA2 acting on supported DPPC bilayers [15,16], and more recently by using fluorescence quenching [17].

Protein–peptide interaction, hetero-oligomerization, and fibrillation were observed to accompany also the activation of PLA2 by the antimicrobial peptide temporin B [18]. Incubation of PLA2 with lysoPC *in vitro* results in an inhibition of PLA2 [19] and the formation of fibrils, seen by electron microscopy [20], revealing a dual role of this lipid, promoting both the initial activation and subsequent inhibition of PLA2, resulting in a transient burst in activity.

In order to subject the aforementioned putative mechanism of PLA2 activity control to further testing, we used the molecular chaperone, heat shock protein 70 (Hsp70), which has been demonstrated in several

Abbreviations: C28-O-PHPM, 1-octosanyl-2-(pyren-1-yl)hexanoyl-*sn*-glycero-3-phosphatidylmonomethyl ester; CMC, critical micelle concentration; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; EM, electron microscopy; FFA, free fatty acid; Hsp70, human heat shock protein 70; LUV, large unilamellar vesicles; lysoPC, lysophosphatidylcholine; PAGE, polyacrylamide gel electrophoresis; PLA2, phospholipase A2; SDS, sodium dodecyl sulfate; T_m , main phase transition temperature; ThT, thioflavin T; X, mole fraction

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studies to be able to revert the conversion into amyloid fibrils of proteins such as α -synuclein and A β [21,22], these two proteins representing paradigms for neurodegenerative diseases. More specifically, in the light of the activity control mechanism deciphered in our previous studies, Hsp70 should influence the oligomerization of PLA2, retaining the enzyme longer as a catalytically active oligomer, sustaining activity by attenuating the inactivation by conversion into amyloid. PLA2 catalyzed phospholipid hydrolysis was measured using a pyrene labeled phospholipid substrate, both with and without recombinant human Hsp70.

2. Materials and methods

2.1. Materials

1-Octosanyl-2-(pyren-1-yl) hexanoyl-sn-glycero-3-phosphatidyl-monomethyl ester (C28-O-PHPM) was from Invitrogen (Eugene, OR) and lysoPC (1-hexadecanoly-2-lyso-sn-glycerol-3-phosphocholine) from Avanti (Alabaster, AL). The purities of these lipids were checked by thin layer chromatography on silicic acid coated plates (Merck, Darmstadt, Germany) developed with a chloroform/methanol/water mixture (65:25:4, v/v/v). Examination of the plates after iodine staining revealed no impurities. Bee venom PLA2 was from Sigma and its purity was verified by polyacrylamide gel electrophoresis in the presence of SDS. Hsp70 was expressed and purified as described previously [23]. All other chemicals were of analytical grade and from standard sources. The concentration of the pyrene labeled lipids was determined from absorbance at 342 nm using $42,000\text{ cm}^{-1}$ as the molar extinction coefficient for the pyrene labeled lipid in ethanol. Concentrations of other lipid stock solutions in chloroform were determined gravimetrically with a high-precision electrobalance (Cahn, Cerritos, CA), as described [24]. Freshly deionized filtered water (Milli RO/Milli Q, Millipore Inc., Jeffrey, NH) was used in all experiments. CaCl_2 solution was prepared and filtered through a $0.2\ \mu\text{m}$ filter (Schleicher & Shuell Microscience, Dassel, Germany) prior to use.

2.2. Assay for PLA2

PLA2 activity was determined by the kinetic assay described previously [25–28]. Appropriate amounts of the stock solution of the pyrene-labeled PLA2 substrate C28-O-PHPM were dried under a stream of nitrogen followed by high vacuum for a minimum of 2 h. The lipid residues were subsequently dissolved in ethanol to yield a lipid concentration of $50\ \mu\text{M}$, and aliquots of this ethanolic lipid solution were rapidly injected into the buffer in five $10\ \mu\text{l}$ aliquots with a Hamilton micropipette to obtain a final concentration of $1.25\ \mu\text{M}$ C28-O-PHPM in a total volume of 2 ml. Fluorescence intensities were measured with a Varian Carey spectrofluorometer with an excitation wavelength of 344 nm, and emission at 400 and 480 nm, with both emission and excitation bandpasses set at 5 nm, using magnetically stirred four window quartz cuvettes (with 10 mm path length) at $37\ ^\circ\text{C}$. After 5 min of equilibration the reactions were initiated by adding 4 pmoles of bee venom PLA2. The progress of phospholipid hydrolysis was followed by measuring the pyrene monomer intensity at 400 nm as a function of time. The assay was calibrated by adding known picomolar aliquots of (pyren-1-yl) hexanoate into the reaction mixture in the absence of enzyme while recording pyrene monomer emission intensity. The activity of the enzyme was calculated from the initial velocity of the reaction kinetic curves and converted to $\text{pmoles FFA min}^{-1}\text{ ml}^{-1}$.

When indicated 2 nM of Hsp70 was added to the cuvette containing $1.25\ \mu\text{M}$ C28-O-PHPM 5 min prior to the addition of PLA2. In some experiments (PLA2 final concentration 2 nM) was preincubated for 5 min at $37\ ^\circ\text{C}$ with 0 or $20\ \mu\text{M}$ lysoPC prior to the addition into the fluorescent substrate with or without Hsp70, as

indicated. All reactions were repeated 3–4 times and standard deviations for data points were obtained for the curves using Origin.

3. Results and discussion

PLA2s constitute a large family of structurally related enzymes [2]. Bee venom PLA2 belongs to group III enzymes and has been thoroughly studied. The fluorescent lipid C28-O-PHPM analog is avidly hydrolyzed by the bee venom enzyme, causing an increase in pyrene monomer fluorescence (I_m) and a concomitant decrease in pyrene excimer fluorescence. These changes in fluorescence provide an easy method to monitor the rate and extent of phospholipid hydrolysis [25–27,29]. With the negatively charged phospholipid C28-O-PHPM the hydrolysis starts immediately with no measurable preceding lag (44). This could be due to the negative lipid headgroup charge which has been shown to generically promote the formation of

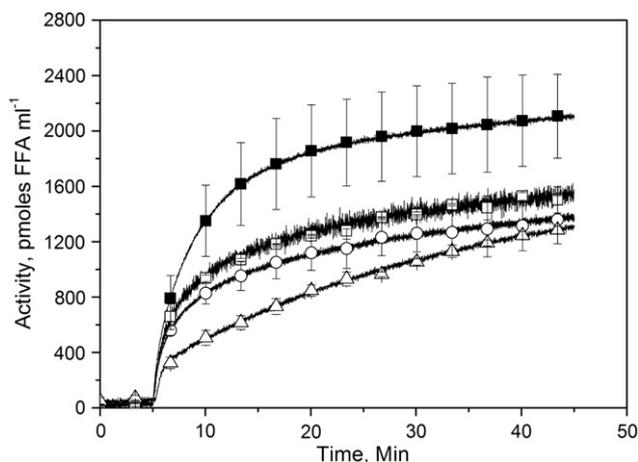


Fig. 1. Hydrolysis as a function of time of $1.2\ \mu\text{M}$ C28-O-PHPM by 2 nM phospholipase-A2 with $2\ \mu\text{M}$ ATP-Mg^{2+} (Δ), or in the absence (\circ) and presence of 2 nM Hsp70 as such (\square) and with $2\ \mu\text{M}$ ATP-Mg^{2+} (\blacksquare) in 2.0 ml of 5 mM HEPES, 0.1 mM EDTA, 1 mM CaCl_2 , pH 7.4 at $37\ ^\circ\text{C}$ with stirring. Each point represents the average of three independent experiments with the error bars giving the standard deviations. The symbols are not individual data points but only one point out of 300, the continuous lines connecting each data point.

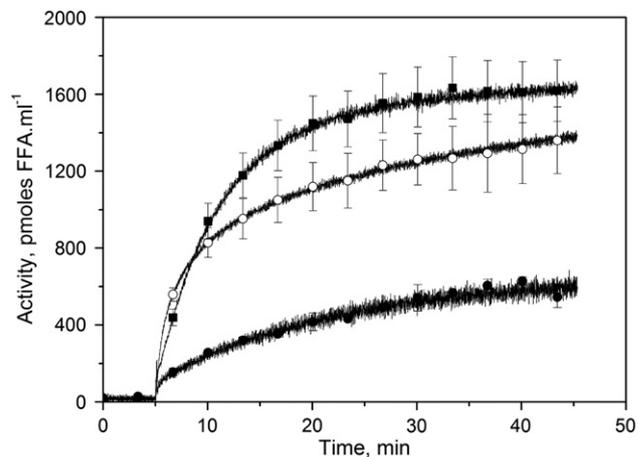


Fig. 2. Hydrolytic activity as a function of time of $1.25\ \mu\text{M}$ C28-O-PHPM by 2 nM PLA2 (\circ), by 2 nM PLA2 preincubated with $20\ \mu\text{M}$ lysoPC prior to adding to the substrate (\bullet), or by 2 nM PLA2 preincubated with $20\ \mu\text{M}$ lysoPC prior to adding the substrate to which 2 nM Hsp70 and $2\ \mu\text{M}$ ATP-Mg^{2+} (\blacksquare) were added. The reactions consist in 2.0 ml of 5 mM HEPES, 0.1 mM EDTA, 1 mM CaCl_2 , pH 7.4 at $37\ ^\circ\text{C}$ with stirring, as described in the legend for Fig. 1.

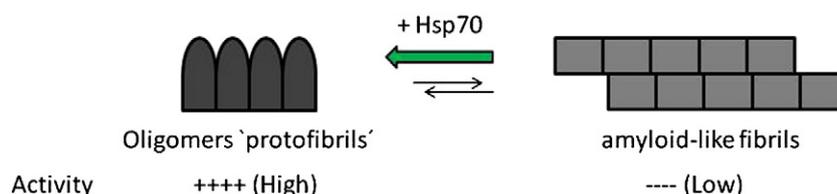


Fig. 3. A schematic model for the activation of PLA2 by Hsp70 in presence of ATP-Mg²⁺ by the attenuation of the conversion of the catalytically active protofibrils into inactive amyloid fibers.

amyloid fibrils [30], as demonstrated for specific peptides, such as e.g. A β [31] and prion protein [32,33].

The following mechanistic sequence was forwarded by us to explain the lag-burst behavior upon hydrolysis of a phospholipid such as DPPC, the interfacial activation of PLA2 and subsequent inhibition of PLA2. More specifically *i*) the soluble enzyme first rapidly binds to the substrate, *ii*) where after there is slow accumulation of the reaction products [18,34,35], *iii*) In the presence of the latter toward the end of the latency period there is a loosening of the PLA2 tertiary structure, caused by the locally enriched products lysoPC and FFA [14], resulting in a transition into a molten globule state [8]. This is accompanied by a fast change in PLA2 conformation, signaled by an increment in the emission from fluorophore labeled PLA2 or from the intrinsic fluorophore Trp [8]. *iv*) After the latency period a burst in the activity of PLA2 takes place. We have suggested this to result from the formation of low molecular weight oligomers, leading to the nucleation of formation of oligomers (amyloid type 'protofibrils'), evident as a pronounced increase in anisotropy [8]. The highly active oligomers produce enhanced fluorescence upon ThT staining and are thus likely to represent amyloid protofibrils. With time the latter develop into *vi*) catalytically inactive amyloid fibers exhibiting the characteristic blue-green fluorescence upon Congo red staining [8], accommodating into a minimum in the folding/aggregation free energy landscape, this process acting as a thermodynamic on-off switch controlling enzyme activity (8).

3.1. Hsp70 sustains PLA2 activity

Hsp70 functions as a chaperone by protecting cells against stress that causes protein denaturation, and also serves a house-keeping role, including dissolution of insoluble protein complexes, folding and refolding of proteins and control of regulatory proteins [36]. Hsp70 requires ATP and Mg²⁺ for activity [36–38] and consists of two major functional domains, the N-terminal ATPase domain which also provides a phospholipid binding site [39,40], and the C-terminal peptide binding domain [41,42]. Hsp70 can prevent amyloid aggregation of proteins by accommodating these in its protein binding site [38], and has been shown to inhibit the assembly of α -synuclein fibrils through a transient interaction mechanism which does not require ATP-mediated refolding [22,43], with the Hsp70 substrate binding domain interacting with partially unfolded α -synuclein intermediates.

In this study we subjected the aforementioned mechanism of PLA2 activity control to further testing using Hsp70. More specifically in the light of the aforementioned Hsp70 can be expected to attenuate the oligomerization of PLA2 and its transformation into amyloid fibers as previously observed with other amyloid forming proteins [44]. Accordingly, we investigated if Hsp70 could sustain the expression of the PLA2 activity. A small increase in the extent of hydrolysis was observed in the presence of Hsp70 without ATP when compared with the reaction of PLA2 alone (Fig. 1), thus suggesting direct interaction between Hsp70 and PLA2 in a manner slightly assisting the hydrolytic action of the latter enzyme. However, when PLA2 was added to the substrate with Hsp70 in the presence of ATP and Mg²⁺ the overall amount of products formed was increased by 30% (Fig. 1). This result

complies with the hypothesis that Hsp70 attenuates, in a manner requiring ATP the conversion of PLA2 into inactive amyloid fibers.

In the second series of experiments we studied if Hsp70 could rescue the activity of PLA2 under conditions where amyloid formation is enhanced by lysoPC induced inhibition of PLA2 [20]. Accordingly, we accelerated amyloid oligomerization of PLA2 by preincubation with lysoPC [20] (Fig. 2). As expected [20] this preincubation of PLA2 with lysoPC reduces the extent of hydrolysis (Fig. 2). However, when Hsp70 is present together with ATP and Mg²⁺ the amount of products is significantly increased compared to the assay of PLA2 alone as well as for PLA2 preincubated with lysoPC (Fig. 2).

The foregoing experiments demonstrate that Hsp70 can rescue the activity of PLA2 in an ATP dependent manner, most likely attenuating the conversion of active protofibrils into inactive amyloid fibers (Fig. 3), resulting in more extensive hydrolysis. Along these lines, Hsp70 may increase the activity of other lipid-activated enzymes by mechanisms similar to that proposed by us for PLA2 [8]. Literature survey suggested one such enzyme to be protein kinase C (PKC) [8]. While for this enzyme the involvement of phosphorylation makes the actual mechanism more complicated than for PLA2, it is of interest that the activation of PKC has been reported to be accompanied by aggregation [45], and another study demonstrated that Hsp70 serves as a stabilizing factor for PKC, prolonging the lifetime of the active state of PKC, sustaining the expression of its catalytic activity [46]. Accordingly, the more general nature of functional amyloid oligomer formation in the control of membrane associated enzymes appears to be feasible and warrants further studies.

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