Ca^{2+} Binding Effects on the C2 Domain Conformation of Human Cytosolic Phospholipase A_2

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Abstract: It has been reported that the cooperative binding of calcium ions indicated a local conformational change of the human cytosolic phospholipase A_2 ($cPLA_2$) C2 domain (Nalefski *et al.*, (1997) *Biochemistry* 36, 12011-12018). However its structural evidence is less known (Malmberg *et al.*, (2003) *Biochemistry* 42, 13227-13240). In this letter, life-time decay and fluorescence quenching techniques were employed to compare the calcium-induced conformational changes. The life-time decay parameters and fluorescence quenching constant changes were small between the apo- and holo-C2 domains when tryptophan residue was excited at 295 nm. In contrast, the quenching constant change was large, from 0.52 M^{-1} for the apo-C2 to 8.8 M^{-1} for the holo-C2 domain, when tyrosine residues were excited at 284 nm. Our results provide new information on amino acid side chain orientation change at calcium binding loop 3, which is necessary for Ca^{2+} binding regulated membrane targeting of human cytosolic phospholipase A_2.

Keywords: C2 domain, Cytosolic phospholipase A_2, Fluorescence quench, Time-resolved fluorescence decay, Conformational change.

INTRODUCTION

C2 domain is a widely distributed signaling motif first described in Ca^{2+}- and lipid-dependent iso-forms of protein kinase C [1, 2], which is composed of an eight-stranded antiparallel β-sandwich. Many of these C2 domains bind to targeted membranes in response to the micromolar Ca^{2+} levels generated during intracellular Ca^{2+} fluxes [3], although exceptions that bind in a Ca^{2+}-independent manner are known [4]. Among them the human cytosolic phospholipase A_2 ($cPLA_2$) C2 domain is one of several classical models being studied extensively. The 85 kDa $cPLA_2$ selectively liberates arachidonic acid from membranes and thus initiates the biosynthesis of potent inflammatory mediators such as prostaglandins, leukotrienes, and platelet-activating factor. Therefore, $cPLA_2$ is considered as an important component of the inflammatory response and potentially an important target for novel anti-inflammatory therapies. The X-ray crystal structure of the full-length $cPLA_2$ enzyme reveals that the C2 domain and enzymatic domain are separated by a flexible linker and are structurally independent [5]. Further biochemical studies have found that the isolated C2 domain retains the ability to bind membranes in a Ca^{2+}-dependent fashion, justifying structural and mechanistic studies of the isolated domain. Structural studies also indicate that the amino acid residue D43 links the two Ca^{2+}, which may explain the reported positive cooperativity during Ca^{2+} binding assays [6, 7]. The cooperative binding property implies a conformational change of the C2 domain at its calcium binding loops induced by Ca^{2+}. Unfortunately, extensive studies support the electrostatic switched model without detectable conformation change of the C2 domain, especially at the calcium binding loops (CBLs). Thus little is known about its mechanism. In this communication, different conformation properties between the apo and holo form of the $cPLA_2$ C2 domains were compared by employing fluorescence life-time measurement and fluorescence quenching of the intrinsic fluorophores. Our results provide new information of amino acid side chain orientation change at CLB 3, which is necessary for Ca^{2+}-binding regulated membrane targeting of human cytosolic phospholipase A_2.

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EXPERIMENTAL PROCEDURES

Protein Purification

The plasmid, pET-28a-$cPLA_2$-C2, containing the fragment 1-138 of human $cPLA_2$ was a kind gift from Dr. Wonhwa Cho (Department of Chemistry, University of Illinois at Chicago). Protein expression and purification was performed essentially as described in detail previously [8]. Protein concentration was estimated by UV absorbance at 280 nm using an extinction coefficient of $A_{280nm}^{0.1\%} = 0.613$ ($mg \cdot ml^{-1} \cdot cm^{-1}$) [9].

Time-Resolved Fluorescence Decay Measurements

The time-resolved tryptophan fluorescence studies were carried out using an Edinburgh Instruments Model 299T differential fluorometer (EI-Model 299T) equipped with temperature controller, a multichannel analyzer (MCA) (5700MCA) and computer (Elonex). The spark lamp was thyratron-triggered, H_2-filled (0.51 bar) and run at 40 kHz. The time calibration was 0.1 ns per channel. Emission was detected through a monochromator (4 nm band pass) by the time-correlated single photon counting technique, using a 5700 MCA with 1024 channels and width 0.2 ns. Response

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files were accumulated until $1.1x10^4$ counts have been registered in the peak channel. The wavelength of excitation and emission were set at 295 nm and 325 nm, respectively. The data stored in a multichannel analyzer were transferred to Edinburgh personal computer MS-DOS ver. 4.01 for analysis. Intensity decay curves were fit to a sum of exponential terms:

$$F(t) = A+\Sigma B_i \cdot \exp(-\tau/\tau_i) \quad (1)$$

where B_i is the amplitude associated with the *i*th decay component having a lifetime of τ_i. The decay parameters were recovered by using non-linear least-squares iterative procedure with the Marquardt algorithm [10]. The validity of the exponential fit was determined from random residual distribution with a χ^2 value close to unity.

Steady-State Fluorescence Measurements

Steady-state fluorescence measurements were carried out on a Shimadzu RF5600 spectrofluorimeter. The fluorescence excitation wavelength of 284 and 295 nm were used to monitor the contributions from both tryptophan and tyrosine or from only tryptophan, respectively. The emission spectra were recorded from 300 nm to 400 nm. The monochromator slit widths were kept at 5 nm in excitation and emission measurements. The Stern-Volmer equation was used to analyze the quenching of the intrinsic fluorescence:

$$F_0/F = 1 + K_{sv}[Q] \quad (2)$$

where F_0 and F were the fluorescence intensities in the absence and presence of quencher, [Q] was the concentration of the quencher, and K_{sv} was the Stern-Volmer quenching constant.

RESULTS

There are four tyrosine residues (Y7, Y16, Y45 and Y96), among which, Y7 and Y16 locate at the N terminal, and are highly flexible with no spatial position determined both in the X-ray crystal [6] and NMR structure of the calcium-bonded forms [7]. The other two, Y45 and Y96, locate in one β-sheet and CBL3, respectively. The spatial distance between Y96 and the calcium ions is about 7.7 Å. There is one tryptophan residue, W71, in the second β-sheet, which is far from the calcium ions (17 Å). When it is excited at 295 nm, it will reflect only the change of the tryptophan residue environment. When it is excited at 284 nm, as used in most reports on this protein [11, 12], both of the tyrosine and tryptophan residues contribute to the detected fluorescence at the maximum emission wavelength (λ=325 nm). The changes of the fluorescence property will reflect the environment changes of both the tyrosine residues and the tryptophan residue.

Time-Resolved Fluorescence Decay Measurement

The time-resolved tryptophan fluorescence decay curve for the apo-protein is shown in Fig. **1**, with excitation at 295 nm and emission at 325 nm. The data were fitted best to a two-exponential model, judged by fitting quality and χ^2 minimization criteria. The fluorescence lifetimes obtained from the exponential analysis were τ_1 = 0.40 ns, and τ_2 = 4.12 ns. The amplitudes of these lifetimes were found to be B_1 = 45.7%, and B_2 = 54.3% respectively. The time-resolved fluorescence decay curve for the holo-protein was similarly obtained and the data were analyzed by the two-exponential method. The fluorescence lifetimes were τ_1 = 0.39 ns, and τ_2 = 4.12 ns, respectively. The amplitudes of them were B_1 = 46.8% and B_2 =53.2%, respectively. These parameters are in fact the same as obtained from apo-protein, and they were summarized in Table **1**. This result indicates that binding of calcium has no obvious structural effect at the local region of tryptophan 71.

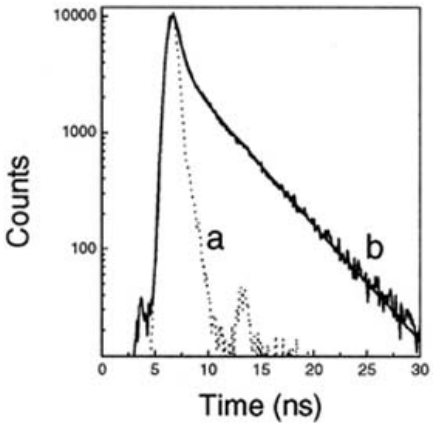

Figure 1. Time-resolved fluorescence intensity decay of the C2 domain of the human cytosolic phospholipase A_2 in apo- form. The protein concentration was 0.3 mg/ml in 10 mM HEPES buffer with 100 mM NaCl at pH 7.4. The sample was excited at 295 nm and emitted at 325 nm, respectively. The sharp peak on the left (line a) is lamp profile. The peak on the right (line b) is sample profile. The data were fitted to a two-exponential function: τ_1 = 0.40 ns, τ_2 = 4.12 ns, B_1 = 45.7%, B_2 = 54.3%. The fitted result was also shown as solid line.

Steady-State Fluorescence Quenching

The quenching of fluorescence requires the molecular contact between the quencher and the fluorophore in either ground or excited states, and can therefore be used to determine the surface accessibility of the intrinsic fluorescence probes by using a quencher. Acrylamide, a neutral and efficient quencher is known to quench the fluorescence probes both exposed to solvent and buried in protein. The excitation wavelength of 295 nm was used to monitor the accessibility change of acrylamide to W71 specially. Fig. **2A** indicates that quenching by acrylamide does not change the position of the emission peak. Thus acrylamide does not appear to cause any structural change in the protein [13]. The nature of the Stern-Volmer plot for acrylamide is linear as showed in Fig. **2B**, implying that the

Table 1. Analysis of the lifetimes of W71 and corresponding fractions of the cPLA2 C2 domain in apo- and holo- form, respectively

Sample	τ_1 ns	B_1	τ_2 ns	B_2
apo	0.40±0.03	45.7%±3.1%	4.12±0.13	54.3%±3.4%
holo	0.39±0.04	46.8%±3.3%	4.12±0.11	53.2%±3.6%

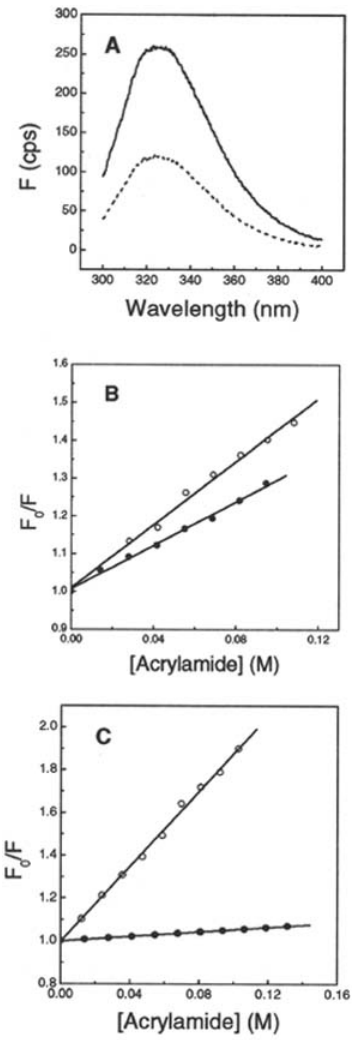

Figure 2. A: Fluorescence spectrum of the human cytosolic phospholipase A_2 C2 domain without (solid line) and with acrylamide (dashed line). B: Stern-Volmer plots for the intrinsic fluorescence quenching of the C2 domain of the human cytosolic phospholipase A_2 by excitation at 295 nm. C: Stern-Volmer plots for the quenching of tyrosine and/or tryptophan residues fluorescence by excitation at 284 nm. The filled circles refer to the apo protein and the open circles to the holo-protein in 2 mM Ca^{2+} buffer. Protein concentrations of the samples were of 10 μg/ml in 10 mM HEPES buffer with 100 mM NaCl, pH 7.4 at room temperature.

fluorescence quenching takes place on a simple collisional mechanism. The quenching constant, K_{sv}, is 2.9 M^{-1}. When 2 mM Ca^{2+} solution was added to the protein sample, the fluorescence intensities of the holo-protein were found to decrease slightly on addition of acrylamide. The Stern-Volmer plot for acrylamide is also linear with a K_{sv} of 4.1 M^{-1}. These data are listed in Table **2**. The smaller change of the K_{sv} on calcium binding indicates a smaller change of the local environment around the tryptophan residue. When excited at 284 nm, the fluorescence intensities of the apo-protein were found to decrease much less on addition of acrylamide. The nature of the Stern-Volmer plot for acrylamide is linear as showed in Fig. **2C**. The quenching constant, K_{sv}, is 0.52 M^{-1}, a very low value, indicating that in the apo form tyrosine and tryptophan residues or only tyrosine residues are buried deeply in a hydrophobic cluster, and almost can't be accessed by acrylamide. When 2 mM calcium ion solution was added to the protein sample, the fluorescence intensities of the holo-protein were found to decrease greatly on addition of acrylamide. The linear Stern-Volmer plot for acrylamide determines a quenching constant, K_{sv}, of 8.8 M^{-1}. The great change of the K_{sv} on calcium binding indicates that a large change occurred at the local environment of tyrosine and tryptophan residues or of only tyrosine residues.

DISCUSSION

Ca^{2+} Has Less Effect on the Local Conformation Near W71.

In both the apo- and holo- forms of the $cPLA_2$ C2 domain, W71 was found to possess much smaller Stern-Volmer quenching constants than free tryptophan (22.0 M^{-1}) [13], indicating that W71 is located in a rather hydrophobic environment, as shown in the X-ray crystal structure. This is consistent with the observation that when this protein is excited at 284 nm or 295 nm, the maximum emission peak is centered at 325 nm, with a blue shift from the unfolded state, 350 nm [3]. The smaller conformation change is further supported by our fluorescence lifetime measurements with one main component around 4.0-5.0 ns and one shorter component [14] for both the apo and holo C2 domains. These results are consistent with previous reports that Ca-binding is triggered by electrostatic interaction without large rearrangement of the C2 domain conformation [15].

Ca^{2+} Could Induce Side Chain Orientation Change at Calcium Binding Loops.

Although the small conformational change near W71 has been reported previously, the local conformational changes at other part of the C2 molecule during calcium binding have been ignored. When the fluorescence excitation is selected at 284 nm, as often used in the reports, only tyrosine residues are excited directly. If there is no tryptophan residue, the energy will be emitted directly with the maximum emission wavelength at 303 nm; if there is tryptophan in the protein, the excited energy in tyrosine will mainly be transferred to the tryptophan residue to excite its electron from ground state to excited state, then the excited tryptophan will transfer back to its ground state by emitting fluorescence,

Table 2. Fluorescence quenching constants of the $cPLA_2$ C2 domain in apo- and holo-forms detected by different intrinsic probes

Sample	Quenching Constant (M^{-1})	
	Tryptophan	Tyrosine
Apo-C2 $cPLA_2$	2.9±0.1	0.52±0.01
Holo-C2 $cPLA_2$	4.1±0.2	8.8±0.2

which could be easily detected. Considering this information, cautious interpretation is required of the fluorescence change of this C2 domain following excitation at 284 nm. As the quenching result suggested a smaller conformation change for W71, the great difference of the acrylamide quenching characteristics to apo- and holo- C2 domain was mainly due to the local conformational change located near the four Tyr residues, when excitation wavelength of 284 nm was employed. Further analysis indicates that the four Tyr residues in apo C2 domain are deeply buried in hydrophobic regions as reflected by the low K_{sv} value, while in the holo form, the Y45 is buried deeply but Y96 is exposed to solution according to X-ray and NMR structures. Thus our results indicate clearly that during Ca^{2+} targeting, the CBL 3 undergoes a conformation change leading to at least the Y96 side chain orientation to a solvent exposure position. This could readily explain the cooperative calcium binding thus highlighting the Ca^{2+}binding triggered membrane targeting mechanism study.

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