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The oligomeric assembly of the phosphodiesterase-5 is a mixture of dimers and tetramers: A putative role in the regulation of function

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ABSTRACT

Background: Phosphodiesterases (PDEs) are a superfamily of evolutionary conserved cyclic nucleotides (cAMP/cGMP) hydrolysing enzymes, components of transduction pathways regulating crucial aspects of cell life. PDE5, one of these families, is the molecular target of several drugs used to treat erectile dysfunction and pulmonary hypertension. Despite its medical relevance, PDE5 macromolecular structure has only been solved for the isolated regulatory and catalytic domains. The definition of the quaternary structure of the full length PDE5 (MmPDE5A1), produced in large amounts in the yeast *Kluyveromyces lactis*, could greatly enhance the knowledge on its assembly/allosteric regulation and the development of new inhibitors for clinical-therapeutic applications.
Methods: Small-angle X-ray scattering (SAXS), analytical ultracentrifugation (AUC), size exclusion chromatography (SEC), native polyacrylamide gel electrophoresis (PAGE) and western blot (WB) were used to assess the assembly of PDE5A1.
Results: The full length MmPDE5A1 isoform is a mixture of dimers and tetramers in solution. We also report data showing that dimers and tetramers also coexist *in vivo* in platelets, blood components naturally containing high levels of PDE5.
Conclusions: This is the first time that structural studies on the full length protein evidenced the assembly of PDE5 in tetramers in addition to the expected dimers.
General Significance: The assembly of PDE5 in tetramers in platelets, beside the dimers, opens the possibility to alternative assembly/allosteric regulation of this enzyme, as component of large signaling complexes, in all cellular districts in which PDE5 is present.

1. Introduction

Cyclic nucleotides (cAMP and cGMP) are the components of evolutionary conserved transduction pathways mediating large number of biological processes. Phosphodiesterases (PDEs) are regulatory components of these pathways ubiquitously distributed in all organisms. PDEs modulate the amplitude and duration of the cAMP/cGMP signal within the cell, catalysing their hydrolysis to AMP and GMP [1]. To date, eleven families of PDE isozymes encoded by 24 distinct genes have been classified in higher eukaryotes according to sequence, substrate specificity, sensitivity to inhibitors and mechanism of regulation [2, 3]. These genes, through alternative splicing and post-translation modifications, encode more than 100 isoforms in the human proteome. PDEs are modular enzymes identified by the presence of a conserved C-terminal catalytic domain and a variable N-terminal regulatory domain embedding the structural determinants for quaternary assembly, post-translational modification, binding of allosteric modulators, subcellular localization and interactions with partner proteins [4–6].

Among these families, PDE5 is cGMP specific and has three isoforms (A1, A2, A3) displaying different expression levels, subcellular localization and tissues specificity [7, 8]. PDE5 has a major role as cGMP-dependent regulator of vascular smooth muscle contraction and it is the molecular target of several well-known drugs used to treat erectile dysfunction and pulmonary hypertension [9]. Recently, the action of

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PDE5 has been extended to learning/memory processes, heart failure, cardio-vascular diseases, human breast and thyroid cancers and many other pathologies [10–16]. Native PDE5, assembled as homodimer [17, 18], contains phosphorylation site(s), separate non-catalytic cGMP allosteric binding sites in the N-terminal part of the protein and the catalytic site for cGMP hydrolysis in the C-terminal part [19, 20]. The N-terminal regulatory region accommodates the c**G**MP, **A**denylyl cyclase, **F**h1A (GAF) -A and -B domains conserved in other PDEs (namely PDE2, PDE6, PDE10 and PDE11). The cGMP binds to the allosteric domain of GAF-A mediating, at the same time, the activation of the catalytic activity and the phosphorylation status of PDE5 [21, 22].

Although the crystal structure of PDE5 has been solved only for the isolated regulatory and catalytic domains [23–26], the quaternary structures of the full length native human platelet PDE5 and bovine rod PDE6 have been solved at 2.8 nm resolution by electron microscopy. These studies revealed a very similar organization of PDE5 and PDE6, as it might be inferred by similarities in their primary sequences [27] (Supplementary Fig. S1).

The possibility to push forward the resolution in the structural organization of the full length PDE5 would further enhance the knowledge on its quaternary assembly and allosteric regulation. Moreover it would lead to the development of new specific inhibitors for clinical and therapeutic applications.

Recently, the entire murine PDE5A isozymes family containing both regulatory and catalytic domains was characterized [8] and produced in large amounts in the yeast *Kluyveromyces lactis* [28]. To gain insight into the quaternary assembly of the full length protein, the purified recombinant PDE5A1 isoform has been subjected to a combination of small-angle X-ray scattering (SAXS) and analytical ultracentrifugation (AUC) sedimentation velocity measurements. These analyses showed the presence of higher molecular weight (MW) oligomers, identified as tetramers, beside the homodimers. To gain better knowledge on these assemblies, we carried out in parallel size exclusion chromatography (SEC) coupled to native gel electrophoresis (PAGE) and western blot (WB) analyses to show that indeed the slower mobility PDE5A1 oligomer is consistent with a tetramer and it is subjected, when fractioned in native PAGE, to the same conformational changes displayed by the dimer in the presence of substrate, inhibitors and thiol compounds [19, 26, 29, 30].

Finally, we have also identified the presence of both tetramers and dimers in rat platelets, blood components containing significant amounts of PDE5A activity using SEC, native PAGE-WB and enzymatic activity.

2. Materials and methods

2.1. Strains, media, culture conditions and vectors

The genotype of the *K. lactis* CBS2359 strain (www.cbs.knaw.nl), the media, the growth conditions, the plasmids and all the other materials and protocols used for the heterologous production, the affinity purification and enzymatic characterization of the recombinant full length MmPDE5A1 enzyme were previously described [28]. Sildenafil was a generous gift from Pfizer. The substrate analog brominated cyclic GMP (Br-cGMP) was purchased from Sigma. A summary of protein production and purification is presented in Fig. 1, Panels A to C.

2.2. PDE activity assay

PDE activity was measured at 30 °C with the two-step method described by [31] using [^{3}H]cGMP (Perkin Elmer, MA, USA). Aliquots of eluted fraction from SEC were incubated in 60 mM HEPES pH 7.2 assay buffer containing 0.1 mM EGTA, 5 mM MgCl2, 0.5 mg/mL bovine serum albumin, 30 μg/mL soybean trypsin inhibitor, in a final volume of 0.15 mL. The reaction was started by adding tritiated substrate at a final concentration of 1 μM [^{3}H]cGMP and stopped by adding 0.1 M HCl.

2.3. Native gel analysis of PDE activity

Native polyacrylamide gel electrophoresis (PAGE) was performed with 5% non-denaturing acrylamide gel with a Tris/glycine pH 8.3 running buffer at 4 °C for 60–80 min under a current of 20 mA in a Bio-Rad Mini-Protean electrophoresis apparatus [32]. Each well was loaded with 1.0 μg of purified recombinant MmPDE5A1, pre-incubated for 3 h at 30 °C with substrate and/or inhibitor/modifier at the concentrations specified in the text or figures, in 5 μL of 50 mM Hepes pH 7.5, 50 mM NaCl, 15 mM $MgCl_2$. The protein bands were revealed by Coomassie-staining.

2.4. 2.3 Solution small-angle X-ray scattering (SAXS) analysis

Solution small-angle X-ray scattering measurements were performed at the BioSAXS beamline BM29 of the European Synchrotron Radiation Facility (ESRF) [33]. The synchrotron was operating in 16-bunch mode giving a beam intensity of 180 mA. Data collection was performed at 12.5 keV with BsxCuBE, exposing each frame for 1 s under 50% beam attenuation, with continuous flow in the capillary. Three dilutions (2.0, 1.0 and 0.5 mg/mL) of each sample were measured at 283 K. The analysed spectra were the result of 7–10 averaged single curves (the average took care to eliminate curves with radiation damage, see Supplementary Table S1). The buffer (50 mM Hepes pH 7.5, 75 mM NaCl, 25 mM $MgCl_2$) was recirculated and measured before and after each protein sample. Data analysis was performed with the ATSAS suite [34]. The scattering images were automatically converted in mono dimensional scattering curves where the intensity (I) is a function of the distance ($Q = 4\ \pi \sin(\theta)/\lambda$). The Guinier analysis at low Q values was performed to determine the radius of gyration (Rg) and the degree of elongation; indirect Fourier transformation of the full scattering curve in reciprocal space was used to calculate the distance distribution function P(r) and the maximal length of the particles in solution. The atomic structure of a nearly full length construct of the closely related protein PDE2A (PDB ID: 3ibj, [6]) was docked into the *ab initio* determined envelopes of dimers and tetramers produced by GASBOR [35], using MultiFoXS program [36] (Supplementary Figs. S5–S6). The quaternary assembly was estimated by using OLIGOMER [37] and MultiFoXS [36]. A summary is presented in Fig. 2 and the 1d solvent subtracted scattering curves are presented in the supplementary Figs. S1 to S3.

2.5. 2.4 Sedimentation velocity analytical ultracentrifugation analysis

Sedimentation velocity experiments were performed at 25,000 rpm with An-50Ti rotor at 20 °C on a Beckman Coulter Proteomelab XLI analytical ultracentrifuge equipped with absorbance optics. MmPDE5A1 was diluted to about 0,8 mg/mL, such as the $A^{280} = 0{,}8$ in 1.2 cm optical path cell in the buffer 50 mM Hepes pH 7.5, 75 mM NaCl, 25 mM $MgCl_2$, 1 mM β-mercaptoethanol. Radial absorbance scans were collected at 280 nm at a spacing of 0.003 cm in a continuous scan mode (50 scans/sample). Sedimentation coefficients were calculated using the program Sedfit [38] and were reduced to water ($S_{20,w}$) according to standard procedures.

2.6. Preparation of whole protein extract from platelets of Rattus norvegicus

Blood sample was collected in 1 volume of anticoagulant buffer (85 mM trisodium citrate dehydrate, 67 mM citric acid monohydrate, 111 mM dextrose) per 6 volumes of rat blood. Blood was centrifuged at 2300 *g*, 10 s per mL of sample, to obtain platelets rich plasma (PRP). PRP was recovered and centrifuged at 2200 *g*, 10 min for 5 mL of collected PRP. Platelets pellet was recovered and lysed with ice cold lysis buffer (20 mM Tris–HCl buffer pH 7.2, 0.2 mM EGTA, 5 mM $MgCl_2$,