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Fluorometric detection of protein-ligand engagement: The case of phosphodiesterase5

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ABSTRACT

Phosphodiesterases (PDEs) regulate the intracellular levels of cAMP and cGMP. The great clinical success of the PDE5 inhibitors, Sildenafil (Viagra), Vardenafil (Levitra) and Tadalafil (Cialis) has led to an increasing interest for this class of enzymes. Recent studies have shown a correlation between tumor growth and PDE5 overexpression, making PDE5-selective inhibitors promising candidates for cancer treatment. The search for such inhibitors rests today on radioactive assays. In this work, we exploit the conserved catalytic domain of the enzyme and propose a faster and safer method for detecting the binding of ligands and evaluate their affinities. The new approach takes advantage of Förster Resonance Energy Transfer (FRET) between, as the donor, a fluorescein-like diarsenical probe able to covalently bind a tetracysteine motif fused to the recombinant PDE5 catalytic domain and, as the acceptor, a rhodamine probe covalently bound to the pseudosubstrate cGMPS. The FRET efficiency decreases when a competitive ligand binds the PDE5 catalytic site and displaces the cGMPS-rhodamine conjugate. We have structurally investigated the PDE5/cGMPS-rhodamine complex by molecular modelling and have used the FRET signal to quantitatively characterize its binding equilibrium. Competitive displacement experiments were carried out with tadalafil and cGMPS. An adaptation of the competitive-displacement equilibrium model yielded the affinities for PDE5 of the incoming ligands, nano- and micromolar, respectively.

1. Introduction

Phosphodiesterase enzymes (PDEs) are phosphohydrolases that selectively hydrolyse ubiquitous second messengers (cGMP and cAMP) thereby regulating their signaling pathways and downstream biological effects [1–3]. PDEs accomplish different biological roles, including smooth muscle relaxation and blood pressure regulation, platelet aggregation and disaggregation, ion channel conductance and neurotransmission, cell growth, apoptosis and cellular mobility and contractility [2]. PDEs have been exploited pharmacologically for more than half a century and some of the most successful drugs worldwide like Sildenafil (Viagra), Vardenafil (Levitra) and Cialis (tadalafil) affect PDE5 function. Therefore, these drugs are subjected to extensive mechanistic investigations and clinical trials [6]. PDEs comprise a 21-gene super family categorized into 11 families. Among them, type 5 phosphodiesterase (PDE5), of which three isoforms (PDE5A1, PDE5A2 and PDE5A3) are known, is responsible for cGMP hydrolysis [1–3]. PDE proteins, with a few exceptions, contain a highly conserved catalytic domain, but differ in the regulatory domain at the N-terminus [3]. Recently, functional variants of PDE genes have been suggested to play a role in predisposition to tumors [1,4–7]. These activities would strongly benefit from the development of fast, sensitive and reliable tools for rapidly screening potential PDE5 inhibitors. Herein,

Abbreviations: PDE5, Phosphodiesterase 5; PDE5C, Phosphodiesterase 5 catalytic domain; PDE5C-TC, phosphodiesterase 5 catalytic domain modified with a tetracysteine tag; PDE5C-TC-FlAsH, complex obtained by conjugation of the FlAsH fluorophore to Phosphodiesterase 5 Catalytic domain modified with a tetracystein tag; FlAsH-EDT_2, 4′5′-Bis(1,3,2-dithiarsolan-2-yl)-fluorescein; cGMPS, 2′-(6-Aminohexylcarbamoyl)guanosine-3′,5′-cyclic monophosphorothioate.

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we demonstrate that the tetracysteine-tag technology applied to the PDE5 catalytic domain (PDE5C, coding region Glu536-Gln860) is a promising approach in this respect. This method exploits the binding of a fluorescent probe, the fluorescein diarsenical hairpin binder (FlAsH), to a short peptide sequence which includes four cysteine residues, namely Cys-Cys-Xaa-Yaa-Cys-Cys (CCXYCC, in which X and Y denote any amino acid) to be engineered on the target protein [8,9]. Site-selective labelling of proteins in living cells can be achieved with this method that has been applied to address a variety of issues involving detection of protein-protein interactions [10–12]. This molecular technology has also been recently employed to detect and quantitatively characterize the engagement of a small-molecule inhibitor with its target enzyme in a cell lysate [13] but, to our knowledge, it has never be applied to purified enzyme. We have expressed the catalytic domain of the PDE5A2 (identifier: **O76074-2**) with a genetically encoded 6xHis tag at the N-terminus and the 6-amino-acid motif CCPGCC (TC) at the C terminus. This tetra-cysteine motif proved able to covalently bind the biarsenical fluorescein probe (FlAsH-Fig. 1) [9,11]. The complex between the fluorescent protein and the non hydrolysable pseudosubstrate cGMPS, tagged with rhodamine, was obtained and used for displacement studies by the known PDE5 inhibitor tadalafil and by cGMPS. A Förster resonance energy transfer (FRET)-based determination produced the binding constant for the fluorescent cGMPS-rhodamine pseudosubstrate. Similarly, in experiments where either tadalafil or cGMPS were added to PDE5C-TC-FlAsH/cGMPS-rhodamine complex solutions, the observed decrease in the efficiency of FRET between FlAsH and rhodamine enabled us to prove the occurrence of ligand displacement as well as estimate the affinity constant of the two ligands for the PDE5 catalytic site. In addition, we derived structural information on the binding mode of the pseudosubstrate by molecular docking calculations of the cGMPS-rhodamine at the modelled PDE5A2 catalytic site (PDE5C). Because the presently available X-ray diffraction structures for PDEs are limited to the catalytic domains, the homology model of the related human PDE5A2 isoform created by us was limited to this portion of the protein.

2. Materials and methods

2.1. Materials

Molecular Biology Reagents: Primers for the cloning of the PDE5A2 catalytic domain were purchased from Eurofins Genomics S.r.l (Italy). The plasmid pLATE 52, IPTG (β-D-thiogalactopyranoside), β-mercaptoethanol and phenylmethylsulfonyl fluoride (PMSF) were all purchased from Thermo Fisher Scientific (Waltham, MA, USA). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Sigma-Aldrich (St. Louis, MO, USA)

Ammonium bicarbonate (AMBIC), dithiothreitol (DTT), iodoacetamide (IAA) were all purchased by Sigma-Aldrich. FlAsH $-EDT_2$ was from Santa Cruz Biotechnology (Dallas, TX, USA).

2′-(6-Aminohexylcarbamoyl)guanosine-3′,5′-cyclic monophosphorothioate (RP-2′-AHC-cGMP) and the sulforhodamine B-X succinimidyl ester (Rhodamine Red-X^{TM}) were purchased from BioLog (Bremen, Germany) and Thermo Fisher Scientific, respectively. Tadalafil was purchased from Sigma-Aldrich.

The solvents Trifluoroacetic acid (TFA), Acetonitrile (CH_3CN) and N,N-Diisopropylethylamine (DIPEA), were purchased from Sigma-Aldrich.

2.2. Production of recombinant PDE5C-TC

The wild type PDE5 catalytic domain (PDE5C), Glu536-Gln860 coding region, was constructed from the full length human PDE5A2 cDNA (kindly provided by Federica Barbagallo of the "La Sapienza" University, Rome) as the template. The gene fragment coding for PDE5C was amplified by PCR (Polymerase Chain Reaction) using the following primers:

FW 5′-GGTTGGGAATTGCAAGAAACAAGAGAGCTACAGTCG-3′;

RV 5′-GGAGATGGGAAGTCATTAGTTCCGCTTGGCCTGGCCG-3′.

The DNA fragment was then subcloned into the His-tagged expression vector pLATE 52. The positive clones were sequenced and then transformed into *E. coli* BL21 cells (Strata-

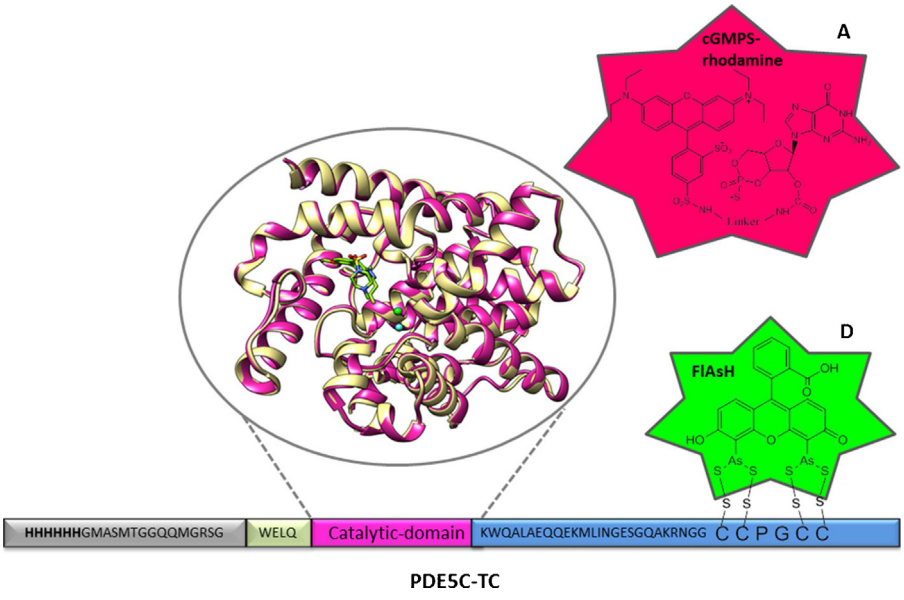

Fig. 1. Schematic outlook of the main structural features of the proposed technology. Bottom: details of the PDE5C-TC a.a. construct with, in grey, the sequence genetically encoding the 6xHis tag added for the purification of the protein and, in blue, the sequence genetically encoding the tetracysteine tag added at the C-terminus. The WELQ sequence (light green) is necessary for a further proteolytic cleavage of the 6xHis tag. Zoomed section: superimposition of the derived PDE5A2 model (magenta) on the PDE5 coordinates (light yellow). The co-crystallyzed inhibitor Sildenafil is depicted in stick mode (*C* atom; green). Mg^{2+} and Zn^{2+} metal ions are shown in cyan and green, respectively, as space filling. **A, Acceptor**: The synthesized pseudosubstrate (cGMPS)-rhodamine (the FRET acceptor) conjugate. **D, Donor**: Binding mode of the biarsenical FlAsH probe to the tetracysteine tag. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)