Analytical Biochemistry 387 (2009) 64–70

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

An alternative assay to discover potential calmodulin inhibitors using a human fluorophore-labeled CaM protein

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ARTICLE INFO

Article history:
Received 29 September 2008
Available online 13 January 2009

Keywords:
Calmodulin
Fluorophore-labeled CaM protein
Fluorescence assay
CaM inhibitors

ABSTRACT

This article describes the development of a new fluorescent-engineered *human* calmodulin, *h*CaM M124C–mBBr, useful in the identification of potential calmodulin (CaM) inhibitors. An *h*CaM mutant containing a unique cysteine residue at position 124 on the protein was expressed, purified, and chemically modified with the fluorophore monobromobimane (mBBr). The fluorophore-labeled protein exhibited stability and functionality to the activation of calmodulin-sensitive *c*AMP phosphodiesterase (PDE1) similar to wild-type *h*CaM. The *h*CaM M124C–mBBr is highly sensitive to detecting inhibitor interaction given that it showed a quantum efficiency of 0.494, approximately 20 times more than the value for wild-type *h*CaM, and a large spectral change (~80% quenching) when the protein is in the presence of saturating inhibitor concentrations. Two natural products previously shown to act as CaM inhibitors, malbrancheamide (**1**) and tajixanthone hydrate (**2**), and the well-known CaM inhibitor chlorpromazine (**CPZ**) were found to quench the *h*CaM M124C–mBBr fluorescence, and the IC_{50} values were comparable to those obtained for the wild-type protein. These results support the use of *h*CaM M124C–mBBr as a fluorescence biosensor and a powerful analytical tool in the high-throughput screening demanded by the pharmaceutical and biotechnology industries.

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Calmodulin (CaM)[1] is a small Ca^{2+}-modulated protein of 148 amino acids (16,706 Da) considered as the primary transducer of Ca^{2+}-mediated signals in eukaryotes. It has four motifs called EF hands (each bind a single Ca^{2+} ion) that are composed of two α-helices linked by a 12-residue loop. CaM amino acid sequence is highly conserved in animal and plants, although the latter organisms express several CaM isoforms.

CaM acts on many cellular targets, including soluble enzymes, ion channels, and primary pumps, resulting in a variety of essential downstream cellular effects [1–6]. Accordingly, this protein influences a number of important physiological processes representing an important drug target [7]. Indeed, many CaM inhibitors are well-known antipsychotic smooth muscle relaxants, antitumoral and α-adrenergic blocking agents, among others. The interaction of CaM with its physiological targets depends on the exposure of two hydrophobic pockets (Fig. 1) following the conformational change elicited by Ca^{2+} binding to the protein.

Many compounds, including drugs, pesticides, and research tools, interact with CaM at the same hydrophobic sites also provoking conformational changes in the protein. Many of these substances behave as CaM antagonists, the best-known structural examples of these interactions are the antipsychotic analogs of trifluoroperazine (TFP) (Fig. 1) [8]. Such interactions can be detected using several analytical methods, including affinity chromatography, ultraviolet (UV), circular dichroism (CD) spectroscopy [9], gel electrophoresis [10,11], nuclear magnetic resonance (NMR) [12,13], X-ray diffraction [8,14–16], functional enzymatic assays [17–19], and fluorescence-based technologies such as fluorescence resonance energy [20–23]. The fluorescence-based methods are highly specific, low cost, selective, and they have rapid reaction time, although sometimes the preparation of the sensing element can be laborious. Among the fluorescence-based methods, the use of site-selective fluorescently labeled CaM has become popular

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[1] *Abbreviations used:* CaM, calmodulin; TFP, trifluoroperazine; UV, ultraviolet; CD, circular dichroism; NMR, nuclear magnetic resonance; *h*CaM, *human* CaM; mBBr, monobromobimane; *h*CaM M124C–mBBr, *h*CaM labeled with mBBr at position 124; PDE1, calmodulin-sensitive cAMP phosphodiesterase; **1**, malbrancheamide; **2**, tajixanthone hydrate; **CPZ**, chlorpromazine; PCR, polymerase chain reaction; cDNA, complementary DNA; LB, Luria–Bertani; OD, optical density; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; DTNB, 5-5'-dithiobis-(2-nitrobenzoic) acid; TNB, 2-nitrobenzoate; BSA, bovine serum albumin; ACN, acetonitrile; SEM, standard error of the mean.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.01.002

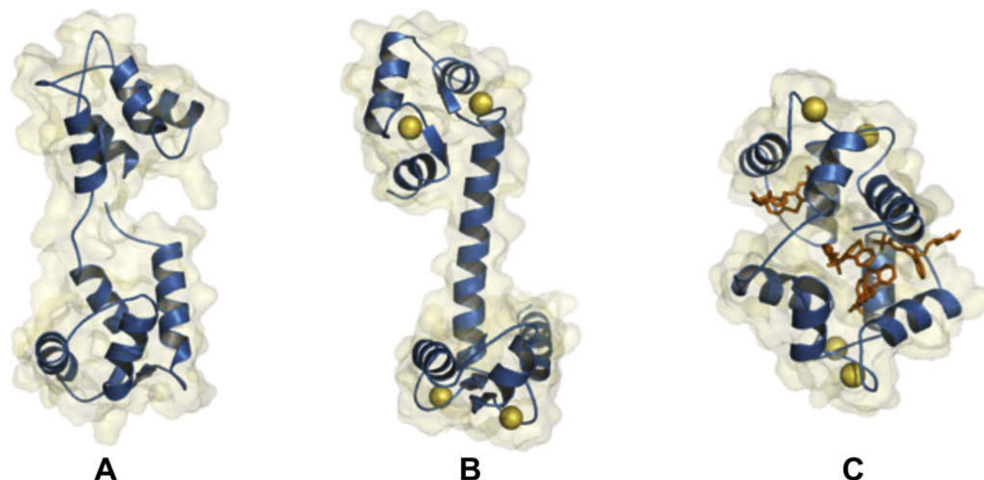

Fig. 1. Three-dimensional structures of CaM in its different conformations: (A) calcium free (Protein Data Bank [PDB] code: 1CFD); (B) with calcium (PDB code: 1CLL); (C) with TFP (PDB code: 1LIN). The structures were drawn using the PyMOL program [38].

recently. In addition, these methods have been demonstrated to be a powerful biosensing system for screening certain classes of drugs such as tricyclic antidepressants [24]. One of the most widely used procedures involves the covalent attachment to CaM of a thiol-reactive fluorophore strategically located using site-directed cysteine mutagenesis. With such a molecular probe, it is possible to correlate the conformational changes with ligand binding by the changes in the emission properties of the labeled proteins.

In previous investigations, it has been determined that the attachment of different fluorophores at the cysteine residue located at position 109 produced better extrinsic fluoresce enhancement on ligand binding [25]. Here we describe the development of a fluorescence-based assay useful for detecting potential CaM inhibitors using a fluorescent *human* CaM (*h*CaM). The protein was engineered by rational design, replacing Met124 by cysteine using site-directed mutagenesis; the resulting protein, *h*CaM M124C, was purified by hydrophobic exchange chromatography and monobromobimane (mBBr) was attached covalently to Cys124 as fluorescent probe. The fluorophore mBBr was selected because of its high sensitivity. Such sensitivity has been successfully exploited in the elucidation of the secondary structure of T4 lysozyme [26]. The stability and functionality of *h*CaM M124C–mBBr (*h*CaM labeled with mBBr at position 124) were determined by CD measurement and functional enzymatic assay using calmodulin-sensitive *c*AMP phosphodiesterase (PDE1) as a monitor enzyme. Furthermore, the usefulness of *h*CaM M124C–mBBr was demonstrated by testing the ability of the known CaM inhibitors malbrancheamide (**1**), tajixanthone hydrate (**2**) [27,28], and chlorpromazine (**CPZ**) (Fig. 2) to quench the fluorescence of the engineered protein and to inhibit CaM–PDE1 complex.

Materials and methods

Reagents

CALM1 (human phosphorylase kinase, delta) gene was purchased from Origene Technology (Rockville, MD, USA). pGEM-T Easy Vector System I was purchased from Promega (Madison, WI, USA). pET12b vector was obtained from Novagen (Darmstadt, Germany). Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA, USA). *Nde*I and *Bam*HI were obtained from New England Biolabs (Ipswich, MA, USA). Primers for polymerase chain reaction (PCR) mutagenesis and *Escherichia coli* BL21-AI One Shot were purchased from Invitrogen (Carlsbad, CA, USA). mBBr was purchased from Toronto Chemical Research (Toronto, Canada). All other reagents were of analytical reagent grade and were purchased from Sigma (St. Louis, MO, USA).

Subclonig of gene encoding for hCaM and site-directed mutagenesis

CALM1 gene encoding *h*CaM was amplified using PCR from the complementary DNA (cDNA) clone pCMV6-XL5 (Origene Technology) along with the primers (5′-CATATGGCTGATCAGCTGACCG-3′ and 5′-CCTAGGAGTAAAACGTCAGTAGT AGAC-3′) to insert restriction sites *Nde*I and *Bam*HI at the start and end of the gene. Amplified products were cloned into a vector (pGEM-T Easy Vector System I) and further subcloned into the protein expression vector pET12b Novagen (EMD Chemicals, Darmstadt, Germany). Single amino acid substitutions were generated by overlapping PCR mutagenesis using the Quick Change Kit (Stratagene). All clones and mutations were confirmed by nucleotide sequencing with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). In all cases, the single methionine at position 124 in the wild-type sequences was replaced by cysteine to facilitate conjugation of the reporter group to this thiol. Plasmids were transformed into *E. coli* BL21-AI One Shot following the specifications of the kit's manufacturer.

Protein purification of hCaM and the mutant hCaM M124C

A single colony of *E. coli* strain BL21-AI/pET12b was grown in Luria–Bertani (LB) medium containing 100 mg/ml of ampicillin overnight with shaking at 37 °C and was inoculated into 500 ml of LB medium containing 100 mg/ml of ampicillin until the optical density (OD) of the culture at 550 nm reached between 0.8 and 1.0. Expression was induced by the addition of L-(+)-arabinose (0.2%, w/v) overnight with shaking at 37 °C. The cells were harvested by centrifugation (10 min, 4000*g*), resuspended in 50 mM Tris–HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 200 mg/ml of egg white lysozyme at pH 7.5 and were chilled on ice for 30 min. Resuspended cells were lysed by sonication, and cellular debris was removed by centrifugation for 15 min at 15,000*g*. The supernatant was collected, and $CaCl_2$ and NaCl were added to final concentrations of 5 and 500 mM, respectively. The protein was purified using a Phenyl Sepharose CL-4B chromatographic column. Briefly, the supernatant was applied to the column preequilibrated with 50 mM Tris–HCl, 0.5 mM DTT, 0.1 mM $CaCl_2$, and 500 mM NaCl at pH 7.5. The col-

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