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Application of ANS fluorescent probes to identify hydrophobic sites on the surface of DREAM

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ABSTRACT

DREAM (calsenilin or KChIP-3) is a calcium sensor involved in regulation of diverse physiological processes by interactions with multiple intracellular partners including DNA, K_v4 channels, and presenilin, however the detailed mechanism of the recognition of the intracellular partners remains unclear. To identify the surface hydrophobic surfaces on apo and Ca^{2+}DREAM as a possible interaction sites for target proteins and/or specific regulators of DREAM function the binding interactions of 1,8-ANS and 2,6-ANS with DREAM were characterized by fluorescence and docking studies. Emission intensity of ANS–DREAM complexes increases upon Ca^{2+} association which is consistent with an overall decrease in surface polarity. The dissociation constants for ANS binding to apoDREAM and Ca^{2+}DREAM were determined to be 195 ± 20 μM and 62 ± 4 μM, respectively. Fluorescence lifetime measurements indicate that two ANS molecules bind in two independent binding sites on DREAM monomer. One site is near the exiting helix of EF-4 and the second site is located in the hydrophobic crevice between EF-3 and EF-4. 1,8-ANS displacement studies using arachidonic acid demonstrate that the hydrophobic crevice between EF-3 and EF-4 serves as a binding site for fatty acids that modulate functional properties of K_v4 channel:KChIP complexes. Thus, the C-terminal hydrophobic crevice may be involved in DREAM interactions with small hydrophobic ligands as well as other intracellular proteins.

1. Introduction

*D*ownstream *r*egulatory *e*lement *a*ntagonist *m*odulator (DREAM), also known as calsenilin or potassium channel interacting protein 3 (KChIP-3), is a multifunctional calcium binding protein that is involved in regulation of gene expression, apoptosis, and modulation of K_v4 potassium channels [1]. DREAM belongs to the superfamily of EF-hand calcium binding proteins and shares a high sequence homology with other members of the neuronal calcium sensor (NCS) subfamily. Members of the NCS subfamily contain four EF-hand motifs with EF-1 and in some proteins EF-2 is unable to bind Ca^{2+}. In DREAM, EF-3 and EF-4 bind Ca^{2+} with high affinity (K_d ~ 1–10 μM) whereas EF-2 preferentially binds Mg^{2+} [2]. DREAM function and interactions with various target proteins are strongly dependent on its cellular localization. In the nucleus, DREAM functions as an agonist transcriptional modulator of the specific DRE (*d*ownstream *r*egulatory *e*lement) sequence found in prodynorphin and c-fos genes [3–5]. DREAM regulates gene expression in a Ca^{2+} dependent manner with apoDREAM binding to DRE sequence as a tetramer whereas Ca^{2+} bound DREAM forms a stable dimer with a decreased affinity for DRE sequence [4]. In the cytoplasm, DREAM binds to the K_v4 voltage channels through the N-terminus in a calcium independent fashion [6]. In addition, *in vitro* and *in vivo* studies have proposed DREAM involvement in regulating apoptotic processes, accumulation of amyloid β peptides, and N-cadherin processing through interactions with presenilin 1 [7,8] It was recently shown that DREAM forms a heterodimer with calmodulin in a Ca^{2+} independent manner and regulates calmodulin function [9].

The molecular basis of DREAM — target proteins/DNA association and the mechanism of how the Ca^{2+} binding modulates DREAM — target protein/DNA interactions remain unclear. Based on the NMR structure of Ca^{2+} loaded DREAM, Lusin et al. [10] have proposed that solvent exposed basic residues located at the DREAM N-terminal domain may form the DNA binding pocket and that solvent exposed leucine residues stabilizes the Ca^{2+}DREAM dimer. The leucine residue rich domain found close to EF-2 may also mediate DREAM interactions with leucine zipper proteins such as α-CREM [11]. The structure of the Ca^{2+} bound DREAM reveals the presence of a large hydrophobic cavity located between EF-1 and EF-2 which extends into the C-terminal domain. The solvent exposed hydrophobic residues were shown to be involved in DREAM interactions with K_v4 channels and possibly with other intracellular partners. For example, the structure of the KChIP1–$K_v4.3$ T1 complex reveals two interaction sites on the T1 domain [12]. The first interaction site consists of hydrophobic residues forming an α-helix and unstructured loop. Highly conserved residues 7–21 from

Abbreviations: DREAM, downstream regulatory element antagonist modulator; 1,8-ANS, 8-anilinonaphthalene-1-sulfonic acid; 2,6-ANS, 6-anilinonaphthalene-2-sulfonic acid; EF-X, EF-hand X; RET, resonance energy transfer

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T1 domain interact with KChIP1 hydrophobic domain with Trp 19 being in a close contact with the hydrophobic residues from the KChIP1 C-terminus; residues (Tyr134, Ile150, Ile154, and Tyr 155) whereas Trp8 and Phe11 from the T1 domain interact with the hydrophobic residues from the N-terminal domain (Phe60, Val69, Phe74, Ile77, Phe98, Phe111, Ala114, Leu115, Leu118, Trp129) [12]. Site 2 (residues 71 to 90) is in contact with the polar residues on the N-terminal helix 2 from KChIP1 [12]. The functional properties of the KChIP–K_v4 complexes are modulated by the association of physiological and synthetic modulators, such as arachidonic acid [13] and NS5806 [14]. Association of arachidonic acid reduces the time constant for inactivation kinetics of K_v4 A current [13]. The sulfonylurea compound NS5806 was shown to induce current potentiation and slow current decay in Kv4:KChIP complexes [14]. However, the precise mechanism of how the hydrophobic modulators regulate the K current kinetics of Kv4:KChIP complexes remains unknown.

Considering the multifunctional role of DREAM in neuronal cells and its interactions with diverse intracellular targets, we have investigated the structural transitions induced by Ca^{2+} and/or Mg^{2+} binding to DREAM in terms of the identification of hydrophobic binding sites that may be involved in stabilization of DREAM oligomers, specific recognition of intracellular partners, and providing binding sites for small hydrophobic molecules. Using the extrinsic fluorescent probes 8-anilino 1-naphthalene sulfonate (1,8-ANS) and 6-anilino 2-naphthalene sulfonate (2,6-ANS) that are highly sensitive to their immediate environment, two distinct hydrophobic sites with high affinity for ANS probes were identified at the C-terminal domain and characterized in time-resolved fluorescence and docking studies. 1,8-ANS displacement studies show that site 2 located between helix 7, 8, and 10 serves as a binding site for arachidonic acid and possibly for other hydrophobic modulators of K_v4:KChIP complexes. The identification of high affinity hydrophobic sites on DREAM surface may provide a structural insight necessary for the development of small molecules for modulation of DREAM interactions with other proteins.

2. Materials and methods

2.1. Chemicals

1,8-ANS and 2,6-ANS were purchased from Invitrogen and used without further purification. All other reagents were purchased from Sigma-Aldrich.

2.2. Sample preparation

Purification of mouse DREAM recombinant protein (residues 65 to 256) was carried out according to a published protocol [15]. Samples for fluorescence studies were prepared by diluting a DREAM stock solution in 20 mM Tris pH 7.4 and 1 mM DTT. Protein concentration was assessed using $\varepsilon_{280\ nm} = 19{,}000\ M^{-1}\ cm^{-1}$. The concentration of 1,8-ANS and 2,6-ANS stock solutions was calculated using $\varepsilon_{350\ nm} = 4999\ M^{-1}\ cm^{-1}$. The absorption spectra were measured using a single beam UV–Vis spectrophotometer (Cary 50, Varian). To prepare Ca^{2+} and/or $Ca^{2+}Mg^{2+}$ bound DREAM adducts, $CaCl_2$ or $MgCl_2$ solution was added to protein samples to reach a final concentration of 1 mM. Apo or Mg^{2+}DREAM samples were prepared by adding 1 mM EDTA or 1 mM EGTA and 1 mM $MgCl_2$, respectively.

2.3. Steady-state fluorescence

Steady-state emission measurements were carried out on a ChronosFD spectrofluorometer (ISS, Champaign, IL) with bandwidth and slit width for excitation/emission set at 2nm and 0.5 mm, respectively. The emission spectra for 1,8-ANS:DREAM and 2,6-ANS–DREAM complexes were measured using $\lambda_{exc} = 350$ nm and 319 nm, respectively. Titration measurements were carried out at 20 °C and in a 2 mm × 10 mm path length quartz cuvette with excitation along the 2 mm path. Dissociation constants were determined either by titrating a small amount of concentrated ANS or arachidonic acid stock solution into 7 μM DREAM sample or keeping ANS concentration constant and adding aliquots of DREAM stock solution. In order to avoid dilution effects, ANS, or protein was kept in both titrant and titrand at identical concentration. The intensity was corrected for the inner filter effect [16]. The signal from unbound dye was subtracted and the emission intensity was determined by integrating the emission signal. Titration curves were analyzed using the Hill equation [17]:

$$F_{corrected} = \frac{F_{maximum}[DREAM]^n}{Kd_{ss}{}^n + [DREAM]^n} + y_o \quad (1)$$

where, K_{dss} is the dissociation constant, n represents the Hill coefficient, and y_o is an offset due to small uncorrected light scattering.

2.4. Fluorescence lifetime measurements

Frequency-domain fluorescence lifetime and anisotropy decay measurements were performed using a ChronosFD spectrofluorometer. 1,8-ANS and 2,6-ANS were excited with a 305 nm diode or 370 nm laser diode and emission was collected using 400–600 nm band pass filters (Andover). 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP) in ethanol ($\tau = 1.35$ ns) was used as a lifetime reference and polarizers were set at a magic angle configuration [18]. Modulation-phase data were analyzed using GlobalsWE software and the χ^2 parameter was used as a criterion for goodness of fit [16]. Fluorescence intensity decays were analyzed using a multiple-exponential decay model and multiple frequency-domain data from at least three different protein batches were simultaneously fitted using the global analysis approach with the lifetime parameters set as linked variables. The theory behind frequency-domain depolarization has been described elsewhere [19,20]. We implemented a triple associative decay model since multiple correlation lifetimes due to oligomerization equilibrium of DREAM and due to heterogeneous binding of the ANS probes. The validity of the model was judged based on χ^2 values. Frequency-domain depolarization data were analyzed using global analysis between data sets obtained with 305 nm and 370 nm excitation while keeping the rotational time as a linked-variable.

2.5. Calculation of Förster distances and energy transfer

The energy transfer efficiency between Trp donor and 1,8-ANS acceptor was determined according to Eq. (2) [21,22]

$$E = \frac{R_0^6}{R_0^6 + D^6} \quad (2)$$

where E represents the efficiency of energy transfer and D is the distance between the donor and acceptor (D). R_0 describes the critical distance at which rate of energy transfer equals the rate of fluorescence decay can be readily calculated using [23]:

$$R_0^6 = \frac{9000(\ln 10)\kappa^2\Phi_D}{128\pi^5 N n^4}\int_0^\infty F_D(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda \quad (3)$$

where κ is the dipole orientation factor, Φ_D is the quantum yield of the donor, N is the Avogadro's number, n is the index of refraction of water, $F_D(\lambda)$ is the normalized fluorescence emission of the donor and $\varepsilon(\lambda)$ is the extinction coefficient spectra of the acceptor. The upper (κ^2_{max}) and the lower (κ^2_{min}) limit for the orientation factor were determined from measurements of the steady-state and limiting anisotropy as described in Ref. [16].