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Biophysical studies with AICD-47 reveal unique binding behavior characteristic of an unfolded domain

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ABSTRACT

APP intracellular C-terminal domain (AICD-47), generated upon γ-secretase cleavage of Amyloid precursor's protein (APP), bears the signature of a classical intrinsically unstructured domain (IUD). Comparing the recent crystal structures of AICD-47 peptides bound to its different adaptors such as protein-tyrosine-binding domain-2 (PTB2) of Fe65 and Src homology 2 (SH2) domain of growth factor receptor binding protein 2 (Grb2), the "conformational switching" of AICD-47 becomes evident. In order to understand different binding processes undertaken by this flexible molecule, upon recognizing different interfaces resulting in different 3D conformations, spectroscopic and calorimetric studies have been done. CD spectroscopy has revealed an overall random coil like structure in different pHs while TFE (2′-2′-2′-trifluoro ethanol) and HFIP (Hexa fluoro isopropanol) induced α-helicity to a certain extent. Binding of Tyr phosphorylated AICD-47 (^{P}AICD-47) to Grb2-SH2 domain was carried out by a favorable enthalpic change ($\Delta H = -197.5 \pm 6.2$ kcal mole^{-1} at 25 °C) and an unfavorable entropic contribution ($\Delta S = -631$ cal mol^{-1} deg^{-1} at 25 °C). Alternative conformation of AICD-47 in different biological contexts is another remarkable feature of IUDs which presumably has definitive roles in regulating Alzheimer's disease phenotype.

1. Introduction

Though the classical paradigm of "sequence–structure–function" is valid for most of the proteins, "intrinsically unstructured domains" (IUDs) are exceptions, being devoid of any compact globular folds and being capable of innumerable functional possibilities [1]. IUDs possess a large net charge at neutral pH with low abundance of hydrophobic amino acids [2]. The structural randomness or 'entropy' decreases dramatically upon binding to specific partners where coupled folding and binding allows burial of an exposed surface area even with small interacting domains and generate complexes with high specificity and relatively low affinity, a feature of these proteins [3–5].

Amyloid precursor's protein (APP), a type-I transmembrane protein, with a large extracellular amino-terminal domain and a shorter carboxy-terminal cytosolic tail, is involved in Alzheimer's disease (AD) pathogenesis. Comparing the crystal structures of the Carboxy terminal tail (AICD-47 or APP 649–695, as of APP-695 isoform numbering) peptides bound to its different adaptors like Fe65-PTB2 and Grb2-SH2 domains, a "conformational switching" of AICD-47 is reckoned [6,7]. Both in acidic and alkaline pH, AICD-47 does not possess any tertiary contacts, typical of an IUD, and shows somewhat folded stable conformation with only hydrophobic side chain clusters. The domain is significantly enriched in Glu, Lys, His and other charged amino acids and depleted of hydrophobic or order promoting residues like Trp and Cys. Moreover, the AICD-47 fragment possesses several short eukaryotic linear motifs (ELM), responsible for associating with different binding partners. These transitory conformers are stabilized and rearranged upon binding [8]. The available NMR conformations do point out the inherent flexibility of the molecule, although prove to be inadequate in terms of explaining the molten-globule formation and the underlying thermodynamics [8]. The crystal structures of Grb2-SH2 domain bound peptides have revealed the consensus recognition motif as 'pY-X-N-X' (where 'X' is any amino acid) where the protein–peptide interactions are stabilized by a network of hydrogen bonds mediated by conserved Arg, Ser, Thr with PTR and backbone atoms of peptide. With the exception of AICD-47, where Pro is present at pY + 3 position, all other peptides bound to Grb2-SH2 domain possess Val at the equivalent position [6].

Abbreviations: APP, amyloid precursor's protein; AICD, APP-intracellular C-terminal domain; AD, Alzheimer's disease; SH2, Src homology 2; PTR and pY, phosphorylated tyrosine; IUD, intrinsically unstructured domain; Grb2, growth factor receptor binding protein 2, TFE, 2′-2′-2′-trifluoro ethanol; HFIP, hexa fluoro isopropanol.

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Thermodynamic investigation of the interaction of Shc-derived phosphotyrosine hexapeptide 'Ac-S^{P}YVNVQ-NH2' with Grb2-SH2 reveals both an enthalpically and entropically favorable event, the enthalpic contribution coming primarily from the PTR and Asn (pY + 2) residues and significant entropic contributions arising from the Val (pY + 1) residue [9]. Understanding the binding thermodynamics of AICD-47, therefore, becomes interesting in presence of a hydrophobic Pro, instead of usual Val, located at the core of the binding interface.

Water miscible alcohols like TFE (2′-2′-2′-trifluoro ethanol) and HFIP (hexa fluoro isopropanol) are known to form micelle-like assemblies where hydrophobic groups, buried deep inside, cause diminution of polarity around the polypeptide chain; eventually stabilizing local hydrogen bonds and generating amphiphilic α-helix [10]. The dielectric constant of water-alcohol mixture is closer to protein interior, which could alleviate the interaction of charged groups and might be adequate to build up pre-α-helical conformations having high propensity for helix formation [11,12]. On the other hand, several reports suggest that peptide fragments and proteins differing vastly in their amino acid composition, can be converted into amyloid-like β fibrils and β-sheets in presence of varying amounts of TFE and HFIP [13–20].

The aim of the present study is to understand the thermodynamic basis of binding of unstructured AICD-47 fragment. Addition of TFE or HFIP causes considerable changes into the secondary structural elements of AICD-47, transforming its native random coil like unstructured conformation to α-helical entity. Entropic and enthalpic contributions of Tyr phosphorylated AICD-47 (^{P}AICD-47) upon binding to Grb2-SH2 domain has also been measured to evaluate which thermodynamic parameter drives the complex formation. Understandably, rapid switching of cognate partners in cellular pathways, as evidenced in AICD-47, could contribute to the disease pathogenesis.

2. Materials and methods

2.1. Purification of proteins

Cloning, expression and purification of Grb2-SH2 domain along AICD-47 were described in details before [6]. To obtain Tyr-phosphorylated AICD-47 (^{P}AICD-47), the construct was transformed into pTK-BL21 (DE3) (also known as TKB1, obtained from Stratagene,) strain of *Escherichia coli* acquiring a plasmid which encodes tyrosine kinase Elk (EphB1) gene. The transformation of AICD-47 in TKB1 was performed following the manufactures guideline and grown in LB broth containing appropriate antibiotics at 37 °C. Cells were collected by centrifugation and resuspended in induction medium, containing 1 mM IPTG and 10 mg/ml β-indole acrylic acid, to induce expression of the ephB1 gene. Cells were grown for an additional 3 h at 37 °C, harvested by centrifugation and sonicated in presence of 50 mM Tris–Cl (pH 8.0), 200 mM NaCl, 100 mM sodium orthovanadate and 50 mM sodium fluoride [21]. Like non-phosphorylated AICD-47, the phosphorylated ^{P}AICD-47 was concentrated up to 1 mg/ml in similar buffer and Tyr phosphorylation was confirmed by 4G10 Anti-Phosphotyrosine antibody (Millipore, India).

2.2. Circular dichroism measurement

CD spectra were recorded on a CD spectrometer of BioLogic Science Instruments (France) using a rectangular quartz cell of pathlength of 0.1 mm at 293 K. Measurements were taken at wavelengths between 190 and 280 nm at a scan rate of 3 nm/min. A total of three scans were averaged to obtain each spectrum and they were baseline subtracted for buffer. 10 μM of AICD-47 was taken for each measurement at pH 4.0 (50 mM potassium acetate), pH 6.0 (50 mM Mes) and pH 8.0 (50 mM Tris) with 300 mM NaCl. TFE and HFIP, obtained from Fluka (Sigma Aldrich), were also used as solvents to check their influence on AICD-47 structure. The percentage of helicity was estimated by standard protocol using K2D3 server [22].

2.3. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) measurements were performed with an ITC-200 (Microcal, Northampton, MA, USA) in 20 mM Mes plus 300 mM NaCl, pH 6.5 at two different temperatures: 20 and 25 °C. Titration curves were fit by using the ORIGIN program supplied by the manufacturer. For all experiments, the heat of the dilution for the individual reactions was determined by titration of into the buffer and buffer into Grb2-SH2 domain. The heat of dilution of buffer into Grb2-SH2 domain was appropriately subtracted. The stoichiometry, binding constant (K_B) and enthalpy changes were determined from a fit of the observed heat change as a function of the concentration of ^{P}AICD-47. The corresponding free energy change (ΔG) and entropy change (ΔS) upon ligand binding were calculated from the following relation:

$$-RTlnK_B = \Delta G = \Delta H - T\Delta S \quad (1)$$

R is the gas constant, and T is the absolute temperature. These parameters permit better thermodynamic characterization of the nature of the binding reaction.

As cratic water molecules could also contribute to the binding of unfolded protein molecule, the apparent binding constant (K_B^{app}) was also determined to make out their involvement in overall binding process [23].

$$K_B = (K_B^{app} \times C_w), \text{ where } C_w \text{ is the concentration of water, } 55.5 \text{ mol/L at } 25^{\circ}\text{C}. \quad (2)$$

3. Results

3.1. Circular dichroism of AICD-47 in different conditions

AICD-47, in the absence of any solute, showed considerable unfolded secondary structure in aqueous solution as measured by CD spectroscopy (Fig. 1A). On an average, around 75% of the protein remained as random coil in three different pH values (Table 1). A shift in the spectral minimum was noticed: 200 nm at pH 4.0, 208 nm at pH 6.0 and 197 nm at pH 8.0, respectively, with a linear increase in the spectral intensity in the same order and without any concomitant change in the secondary structure content (Table 1). Addition of TFE caused significant changes in the CD spectra, consistent with reduction of random coil conformation and formation of secondary structures with large contributions from α-helical motif (Fig. 1B). Upon addition of 40% (v/v) TFE, the spectrum became indicative of a helical conformation, with the minimum shifting to 209 nm ($\pi\pi*$ transition) and considerable negative ellipticity being developed at 222 nm ($n\pi*$ transition). Thus for AICD-47, TFE acted as a helix inducing solvent, as expected, and the presence of an isodichroic point (200 nm) where intersections of four spectra were initiated, indicated a simple random coil/helix transition [24].

As an example of the effect of HFIP on an unfolded polypeptide, the HFIP-dependent conformational transition of AICD-47 was also studied (Fig. 1C). The far-UV CD spectra in presence of various concentrations of HFIP showed, similar to that of TFE, that HFIP stabilized the helical conformation and decreased the amount of random coil in AICD-47 as well (Table 1). However, in presence of 20% (v/v) HFIP, AICD-47 showed ~22% β-sheet formation, higher from its conformation at pH 8.0 without any solvent. The addition