N-terminal Tail of Annexin A2: A Switch into Intrinsic Disorder

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Abstract
Dysferlinopathies (e.g. Limb Girdle muscular dystrophy, Myoshi Myopathy) are muscle wasting diseases that develop from reduced levels or absence of the protein dysferlin. Dysferlin is known to be the Ca^{2+} sensor in membrane repair, a process involving a number of proteins, one of which is annexin A2 (A2). The interaction between these two proteins is unclear, but previous studies have shown dysferlin to be marginally stable. Because of this, we proposed that A2 is marginally stable as well in order to maximize the information flow between the two proteins. Specifically, we hypothesized that the N-terminal tail of annexin A2, a region unique to each annexin isoform, acts as a switch into intrinsic disorder where this N-termini destabilizes the core of the protein to allow more potential conformations of the protein. This would enable A2 to respond more acutely to signals from dysferlin as its binding partner. Using calorimetry and spectroscopy, we showed the presence of this disorder region generates a protein with greater plasticity (compared to a protein in the absence of this disordered region), creating a means by which dysferlin and annexin A2 can coordinate in membrane repair and shedding light on how mutations in either protein can lead to miscommunication, and potentially, disease.

Introduction

Calcium ion (Ca^{2+}) is an important signaling molecule for many cells, especially myocytes. If unregulated and too much calcium enters inside, the muscle cell may burst. Influx of these ions may be due to small tears in the muscle cell's plasma membrane, which can be repaired by the protein known as dysferlin. Mutations in this membrane repair protein lead to a category of diseases called dysferlinopathies, a type of muscular dystrophy that results in reduced muscle mass.

Recent work performed on dysferlin has found that the C2A domain is marginally stable [2]. The work of Hilser and colleagues has shown that marginal stability acts to maximize communication within a protein molecule as this allows for the protein to exist in a wide set of possible conformations in response to environmental cues [3]. It seems likely that dysferlin uses this plasticity to propagate information flow intra-molecularly, but also between a multitude of proteins involved in its functionality.

Dysferlin is known to act with a complex of proteins during the membrane resealing process, one of which is annexin A2, which has been found to be up regulated in the muscle tissue of individuals suffering from dysferlinopathies [4]. Despite the discovery that A2 and dysferlin act together during membrane resealing over a decade ago, the significance of their interaction remains unclear. In order to ensure the best information flow between these interacting proteins, we hypothesize that annexin A2 must also be marginally stable. Marginal stability would indicate that annexin A2 is able to exist in a wide set of conformations allowing it to respond acutely to signals from dysferlin. A high number of possible conformations would allow annexin A2 to change its shape to be specific to a given situation, thus information content is higher in a less stable protein than a more rigid protein.

To test the stability of A2, differential scanning calorimetry and circular dichroism were used. Annexin a5 of *Rattus norvegicus* (which shares 98% of its sequence with its human counterpart) was used as a comparison (Fig. 1). These two proteins share highly conserved core domain sequences, but vary in their N-terminal tails. Information on the stability of A2 will give insight into the interactions between annexin A2 and dysferlin in the membrane repair process.

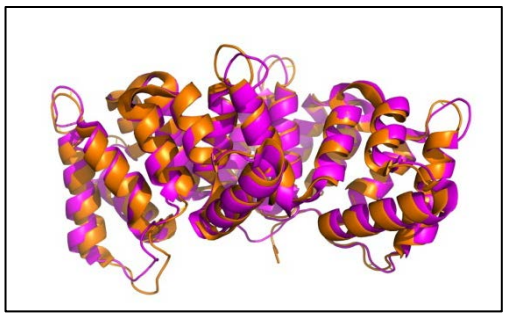

Figure 1. Crystal structure overlay of annexin a5 of Rattus norvegicus (orange) and annexin A2 of Homo sapiens (purple).

Methods

Purification

For human annexin A2, the cDNA was cloned into a p202 vector, and expressed in *E. coli* Bl-21 cells as a MBP 6Xhis tag fusion protein. Cells were grown at 37°C in one liter of LB broth containing ampicillin (0.1mg/mL). Overexpression of A2 was induced by the addition of 1mM isopropyl-β-D-thiogalactopyranoside once the cells reached an optical density between 0.6-0.8 at 600nm. Five hours after induction, cells were harvested by centrifugation. A2 cells were then lysed by sonication in a buffer composed of 20mM 4-(2-hydroxyethyl)-1-piperzineethanesulfonic acid (HEPES), 100mM NaCl, 5 mM EGTA, 1mM phenylmethanesulfonylfluoride and 1mM 2-mercaptoethanol (βME) at pH 8. DNA was digested using benzonase nuclease and $MgCl_2$, and then annexin A2 was then mixed with Ni-NTA column media (Qiagen) for 6 hours. The purified annexin A2 was eluted through an increasing imidazole concentration. Tobacco Etch Virus (TEV), was then added to the eluent to allow cleavage of annexin A2 from the tag. Following cleavage by TEV, protein solution was then

repassed over the nickel column to remove the TEV protease and MBP 6Xhis tag, after which the purity of recombinant annexin A2 was then confirmed through the use of an SDS-PAGE gel.

Differential Scanning Calorimetry

DSC scans were performed using a NanoDSC (TA Instruments, New Castle, DE). All DSC experiments in the absence of Ca^{2+} were done in 1mM EGTA. All scans conducted in the presence of Ca^{2+} were completed in the presence of 5mM Ca^{2+}, while all scans containing lipids were conducted in the presence of 4mM phospholipid as LUVs.

Circular Dichroism

CD experiments were performed on a Jasco CD Spectrometer (Annapolis, MD), at a concentration of 5μM protein for all annexin constructs studied. Spectra collected in the absence of Ca^{2+} were conducted with 500μM EGTA. Data points were collected in 1nm increments and averaged over 5 acquisitions

Results

A2 and a5 share similar core binding domains but differ in their varied N-terminal tails. Knowing that A2 has a longer tail, thus making it a larger protein, one would predict that more interactions would be taking place. This would suggest A2 would be more stable (higher free energy of stability ΔG_{37}). However, the values obtained prove the opposite is true. The free energy of stability of a protein is often interpreted simply as the energy barrier that must be overcome in order to denature the protein. However, the ΔG_{37} can also be thought of as representing the energy barrier that must be overcome in order for a protein to transition from

one state to another. The lower the free energy of stability, the more states are available to the protein in its native ensemble. Typically, a protein's free energy of stability is between 5 and 20 kcal/mol [1]. In the absence of ligand, the a5 isoform falls within this range with a ΔG_{37} of 6.36±0.06 kcal/mol (Table 1). On the other hand, the A2 isoform was found to be significantly less stable with a ΔG_{37} of 3.0±0.1 kcal/mol, just under half of the ΔG_{37} of a5 under the same conditions. The lower stability of A2 indicates that this isoform is more structurally plastic than the a5.

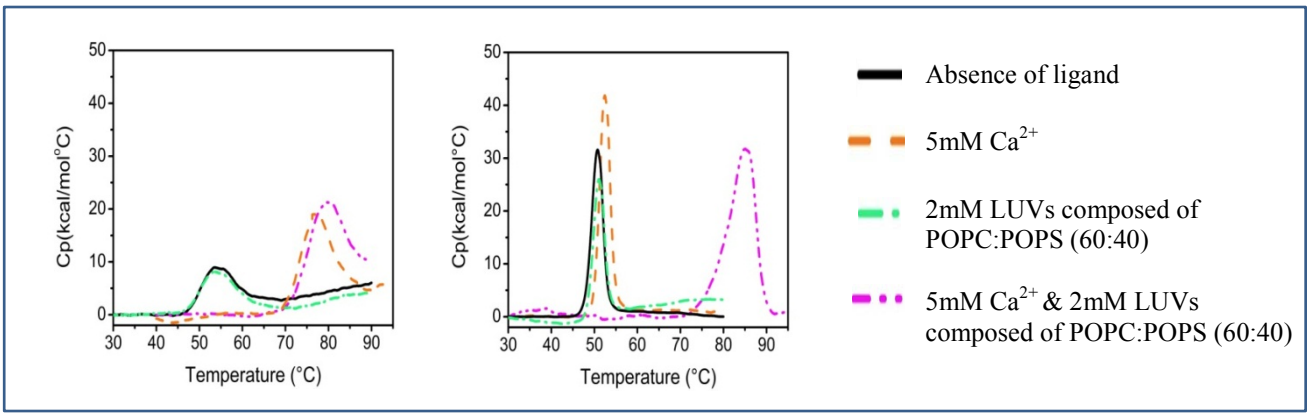

Figure 2. Thermal Denaturation of annexin A2 (left panel) and annexin a5 (right panel) in the presence of various ligands.

Interestingly, greater differences are seen when the proteins are in the presence of their major signaling ion, Ca^{2+}. For A2, a significant increase in the melting temperature (T_M) was found, rising from 53.3±0.3°C in the absence of ligand to 76.3±0.1°C. Coupled with the increase in melting temperature, the A2 isoform also showed an increase in its change in heat capacity (ΔCp) from 2.8±0.5 kcal/molK in the absence of ligand to 5.2±0.6 kcal/molK with Ca^{2+} accompanied by an increase in the enthalpy of denaturation (ΔH). In comparison, a5 showed a modest change in T_M from 50.38±0.04°C in the absence of ligand to 52.44±0.03°C in the presence of Ca^{2+}, and a comparatively minor increase in the ΔH with no significant change in ΔCp. The ΔCp can be thought of as a measure for how buried the hydrophobic amino acids are

within a protein in the folded state. Thus, the increase in the ΔCp of annexin A2 in the presence of Ca^{2+} would suggest that these amino acids are significantly more shielded from solution when in the presence of this ligand. This leads to the conclusion that A2 becomes more structured and ordered when Ca^{2+} is around.

Table 1. Thermodynamic parameters obtained from annexin A2 and a5. All ΔG_{37} values reported represent the free energy of stability at 37°C, values reported represent the average of four denaturations and all error values are at the 95% confidence interval.

	ΔH(kcal/mol)	ΔCp(kcal/molK)	T_M (°C)	ΔG_{37}(kcal/mol)
Annexin A2				
No Ligand	82.7±1.0	2.8±0.5	53.3±0.3	3.0±0.1
Ca^{2+}	129.1±0.1	5.2±0.6	76.3±0.1	2.5±0.1
POPC:POPS (60:40)	78.9±0.4	2.5±0.1	53.16±0.7	2.89±0.04
POPC:POPS (60:40) and Ca^{2+}	131.5±0.5	11.6±0.2	78.7±0.3	-14.04±0.02
Annexin a5				
No Ligand	162.2±0.8	1.5±0.5	50.38±0.04	6.36±0.06
Ca^{2+}	173.5±0.6	1.2±0.4	52.44±0.03	7.8±0.3
POPC:POPS (60:40)	144.8±0.7	1.2±0.4	51.07±0.05	5.9±0.3
POPC:POPS (60:40) and Ca^{2+}	259.5±1	1.4±0.1	83.18±0.01	29.1±0.5

These results are further supported when circular dichroism (CD) spectra are compared. In the absence of ligand (Fig. 3, left panel), it is evident that both proteins have α-helical secondary structures. However, A2 has a much less intense, broader spectrum that a5, consistent with it being less ordered and more flexible than a5. Also, once Ca^{2+} is introduced, A2's CD spectrum showed nearly a 300% increase in the intensity of the minima, while a5 increased approximately 167%. The much larger increase in the α-helical nature for A2 is consistent with a more global ordering for the A2 when in the presence of Ca^{2+}. A greater response to Ca^{2+} would allow this protein to act on changes to its environment more effectively, as well as more acutely.

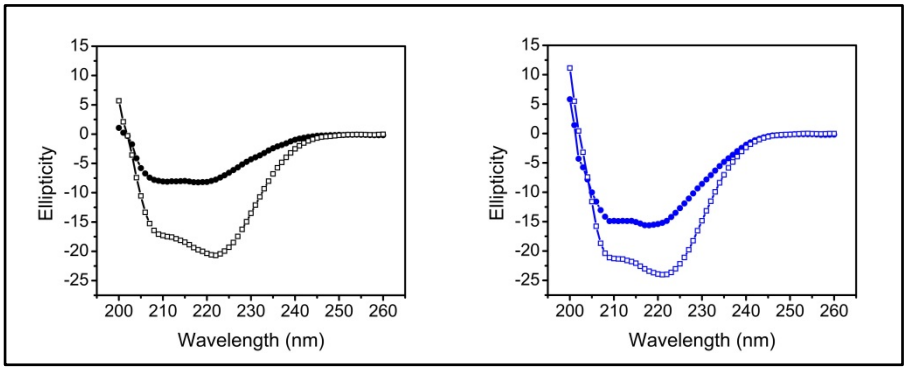

Figure 3. CD spectra in the absence of ligand (left panel) and in the presence of Ca^{2+} (right panel). The solid circles represent annexin A2, whereas the open squares are annexin a5.

Conclusion

It is well known that the annexins primarily differ within their N-terminal domains, which contain a variety of sequences and lengths. This region has often been regarded as a regulatory domain since it contains sites for post translation modification, such as phosphorylation and proteolytic cleavage. However, because this is the only variable region in the isoforms, the N-terminal domains may in fact also act to determine the energetic properties of each isoform. Not only that, but the N-terminal tails may determine the entire function of these different isoforms. From the data presented here, it appears that the N-terminal domain impacts the stability of the folded core in an isoform specific manner, as while A2 is a larger molecule than a5 (suggesting more interactions) it is much less stable and more plastic. Evidence of this heightened plasticity can also be found when the CD spectra of the two isoforms are compared. While both maintain the α-helical fold typical of the annexin core, the broader and less intense spectra obtained for the A2 isoform is consistent with it being less ordered. Since information flow is maximized by weak interactions it appears that A2 has the ability to respond appropriately to signals from dysferlin.

References

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