NOVEL PERSPECTIVES IN THE STRUCTURAL BIOLOGY OF S100 PROTEINS

PhD thesis

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INTRODUCTION

S100A4 is a member of the vertebrate specific S100 protein family, which belongs to the EF-hand Ca^{2+}-binding protein superfamily comprising calmodulin as well. The S100 proteins do not possess enzymatic activity, they act by modulating the function of their interaction partners. They have versatile roles, e.g. they play a role in the regulation of the cell cycle, affect the cytokinesis and differentiation, and have a role in cell adhesion and migration. They generally form homodimers, but monomeric and heterodimeric S100 proteins are also known, One subunit contains two EF-hands. Upon Ca^{2+}-binding a large conformational change occurs resulting in the formation of two symmetric hydrophobic binding clefts. One S100 dimer generally binds two amphipatic α-helices in a symmetric manner. S100A4, also known as metastasin is one of the most intensely investigated member of the S100 family. Numerous pieces of experimental and clinical evidence support its role in diseases such as tumor metastasis, rheumatoid arthritis, and fibrosis. All of these pathological conditions correlate with the biological process of epithelial-mesenchymal transition (EMT). During EMT the epithelial cells lose their cell-cell adhesions, detach from the basal lamina, and gain invasive properties to become mesenchymal stem cells. S100A4 contributes to EMT via both intra- and extracellular protein-protein interactions. Outside the cell it facilitates the t-PA mediated plasminogen activation leading to the activation of matrix metalloproteases (MMPs), while binding to cell surface receptors (e.g. RAGE) promotes MMP expression. In the cytoplasm it binds to non-muscle myosin IIA (NMIIA) causing the myosin filaments to disassemble which results in augmented cell migration. Binding to the tumor supressor p53 may regulate the cell cycle and apoptosis.

Non-muscle myosins are actin binding motor proteins possessing ATPase activity. Organized in bipolar filaments, they crosslink the actin filaments. As a consequence they play a crucial role in cell adhesions and cell motility, cytokinesis and cell polarization. Three different NMII heavy chain isoforms were described in humans. While NMIIA localizes mainly in the leading edge of migrating cells, NMIIB can be found in the trailing edge and shows perinuclear localization. Regarding their enzymatic properties, the duty ratio of NMIIB is 4-8-fold higher than that of NMIIA, meaning that NMIIB spends significantly more time in strongly actin-bound states than NMIIA. This feature of NMIIB is proposed to support functions which require prolonged tension maintenance with low ATP consumption (e.g. cytokinesis). There has been little characterization of NMIIC in terms of its cellular distribution and function.

According to the literature, S100A4 interacts selectively with NMIIA resulting in NMIIA filament disassembly and the inhibition of filament formation. The experiments with cultured tumor cell lines showed that this interaction leads to increased cell migration and gain of metastatic phenotype. The S100A4 binding-site was mapped to the C-terminus of the coiled-coil region of NMIIA. This region is adjacent to the assembly competence domain (ACD), which is essential to filament formation. Although the physiological-pathological role of S100A4 – NMIIA interaction has been well characterized, the structural basis of S100A4-induced NMIIA filament disassembly was quiet obscure. Neither the strength, nor the stoichiometry of the interaction was established unambiguously, hence the structural details of this functionally well-known interaction remained hidden so far.

AIMS AND METHODS

My PhD work consists mainly of two subprojects. My primary goal was to reveal the mechanism of S100A4-dependent NMIIA filament disassembly using versatile techniques. To achieve this goal my aims were:

1. to map the S100A4 binding-site on NMIIA,
2. to determine the biophysical parameters of the interaction, such as affinity, stoichiometry and kinetics, using isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR),
3. to determine the 3D structure of S100A4 – NMIIA complex with X-ray crystallography,
4. to characterize the interaction of S100A4 with dimeric NMIIA fragments (including their affinity, stoichiometry, kinetics and secondary structural changes) using ITC, stopped-flow fluorescence spectroscopy and circular dichroism (CD) spectroscopy,
5. to investigate the interaction of S100A4 with NMIIA filaments with turbidometry,
6. to revise the role of the random coil S100A4 C-terminal region in NMIIA binding with ITC, fluorescence polarization measurements and turbidometry.

Secondly, I investigated the structural basis of the isoform selectivity of S100A4 − NMII interactions. Namely, my aim was to point out amino acid position(s) or regions in the S100A4 binding site of NMIIs which negatively affect the binding of NMIIB to S100A4. To determine the reasons underlying the selectivity of NMII isoforms I wished:

1. to point out regions and amino acid positions using NMII chimeras and combinatorial paralog scanning via phage display, respectively,
2. to test chimeras and mutants(designed based on the results of the paralog scanning) in monomeric and dimeric NMII fragments using SPR, stopped-flow fluorescence spectroscopy and turbidometry.

RESULTS

1. I characterized the interaction of monomeric, partially overlapping NMIIA heavy chain fragments with S100A4 using ITC. My results revealed that the S100A4 binding site of NMMIIA is unusually large (45 amino acids) and binds to S100A4 with unprecedented affinity (< nM) and stoichiometry (one NMIIA chain to an S100A4 dimer).
2. We crystallized the 45-residue long NMIIA fragment in complex with S100A4. The first 3D structure of S100A4 complexed with a protein target revealed a unique interaction mode in the S100 family, as the myosin chain is wrapped around the S100A4 dimer forming an asymmetric interaction.
3. The stability of the NMIIA coiled-coil decreases upon S100A4-binding, which probably affects the ACD.
4. I demonstrated that the complete disassembly of the NMIIA filament requires 1 S100A4 dimer / 1 myosin heavy chain.
5. The random coil S100A4 C-terminus does not contribute to the interaction with NMIIA.
6. We revealed that the S100A4 C-terminal region binds to the "empty" EF-hands modulating the Ca^{2+}-binding of S100A4.
7. I discovered that NMIIC binds to S100A4 with similar affinity than NMIIA, while NMIIB shows a 300-fold lower affinity to S100A4 compared to the two other isoforms.
8. A single amino acid substitution in the helical binding region of NMIIs has a key role in the isoform selectivity. In this position NMIIB has a residue that causes a 60-fold decrease in binding affinity compared to NMIIA and NMIIC.
9. Shuffling the non-helical tailpiece regions of NMIIA and NMIIB revealed that the association rate constants of the coiled-coil forming NMII fragments comprising the NMIIA tail is significantly higher than that of the NMIIB tail.
10. Thermal denaturation experiments showed that NMIIB coiled-coil is significantly more stable than NMIIA which may contribute to the observed differences in the association rates.
11. Based on these results we presented the first structural model of S100A4-induced NMIIA filament disassembly.

DISCUSSION

The crystal structure of S100A4 – NMIIA complex

Due to the ambiguous results in the literature my first goal was to determine the affinity and stoichiometry of the S100A4 − NMIIA interaction. The results of the ITC measurements with a 45-residue long monomeric NMIIA fragment indicated an unexampled high affinity and a unique stochiometry in the S100 family. We successfully crystallized an S100A4 variant (C3S, F45W, C81S, C86S) with the 45-residue long NMIIA fragment, which indeed revealed a novel interaction mode in the S100 family. A single, predominantly α-helical NMIIA chain is wrapped around the S100A4 dimer interacting simultaneously with the two canonical binding clefts. Note that the sequentially most diverse region in the S100 family, which comprises the target binding cleft has to be conformationally promiscuous enough to interact with the two NMIIA termini having different chemical characteristics. Beside the flexibility of the canonical binding clefts, a new interaction surface on S100 proteins was revealed. This wide, shallow groove (the "waist" of the dimer) is located between the two binding clefts connecting them. Observing the known, open state S100 structures, this surface also exists on other S100 proteins as well, consequently it may function as a binding groove in the case of other S100 proteins as well.

A crystal structure that supports this finding was published recently. In the case of S100A10, the two Annexin A2 helical peptides interact in a symmetrical way with the two canonical S100 clefts, while the unstructured AHNAK peptide binds to the waist of the dimer. It is important to note that my PhD work promoted the extensive investigation of the protein-protein interaction of S100(A4) protein(s) in our laboratory This research aims to assess the relevance and prevalence of the asymmetric binding mode observed by the S100A4 − NMIIA interaction. As a result, we have structural information on different S100 − target interactions, which support the idea that the asymmetric binding mode could be more general in S100 family than previously thought.

S100A4 negatively discriminates NMIIB

The sequence comparison of the NMII isoforms reveals that the non-helical tailpiece is the most diverse part of the heavy chain. Therefore one can hypothesize that this region has a crucial role in the isoform selectivity. However, I demonstrated that NMIIC binds to S100A4 as strongly as NMIIA, while its non-helical binding region is more similar to that of NMIIB

Additionally, deletion of this region causes a much smaller decrease in binding affinity than the deletion of the N-terminus of the binding site. Consequently, we can conclude that the key position in the selectivity is probably localized in the helical region of the S100A4 binding site. Using phage display I carried out combinatorial paralog scanning. The results of this experiment indicated that the substitution of a single amino acid position between the low- and high-affinity isoforms causes a 60-fold difference in the affinity of NMII fragments. Furthermore, I investigated the role of the non-helical tailpiece in S100A4-binding. The tailpiece of NMIIA was the most favorable in terms of S100A4-binding, and this region affected significantly the association rate constant of dimeric NMII variants. Interestingly, I found that the NMIIB coiled-coil is much more stable than that of the NMIIA, which could explain why the association rate constant of NMIIA coiled-coil with NMIIB tailpiece (NMIIBA) exceeded the k_a value measured in the case of NMIIAB.

The model of S100A4-induced NMIIA filament disassembly

On the one hand, I showed that S100A4 binds to soluble and filament-forming dimer NMIIA fragments with the same stoichiometry than to the monomeric NMIIA peptides. On the other hand, the NMIIA coiled-coil unwinds besides the S100A4 binding-site as well upon S100A4-binding leading to the partial denaturation of ACD. Transient kinetic measurements revealed that the NMII non-helical tailpiece affects the association rate of the S100A4 binding to coiled-coil NMII fragments. Based on these results we propose the following model:

1. an S100A4 dimer “docks” to the non-helical tailpiece,
2. unwinds the C-terminal coiled-coil while a 30-residue long part of the latter is wrapped around the S100A4 dimer,
3. approximately two heptades outside of the S100A4 binding site uncoil leading to the partial destabilization of the assembly competence domain,
4. which, along with the steric constraints caused by the two S100A4 dimers bound to the myosin rod, results in NMIIA filament disassembly and inhibition of filament assembly.

RELATED PUBLICATIONS

Kiss B, Duelli A, Radnai L, Kékesi KA, Katona G, Nyitray L. *Crystal structure of the S100A4-nonmuscle myosin IIA tail fragment complex reveals an asymmetric target binding mechanism.* Proc Natl Acad Sci U S A, 2012. **109**(16): p. 6048-53.

Duelli A, **Kiss B**, Lundholm I, Bodor A, Petoukhov MV, Svergun DI, Nyitray L, Katona G. *The C-Terminal Random Coil Region Tunes the Ca^{2+}-Binding Affinity of S100A4 through Conformational Activation.* PLoS One, 2014. **9**(5): p. e97654.