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Molecular analysis of the A/B region of ecdysteroid receptor from *Aedes aegypti*.

Abstract:

Aedes aegypti is the primary vector of flaviviruses, the cause of dengue fever, Chikungunya, yellow fever and Zika. The essential biological processes in arthropods such as molting, development and metamorphosis are regulated by two hormones: juvenile hormone (JH) and 20-hydroxyecdysone (20E) and their nuclear receptors (NRs) (Raikhel 2005). The insect steroid hormone 20E is a ligand of the functional receptor (Laudet *and* Gronemeyer 2001) which act as heterodimer of two members of the nuclear receptor superfamily: the ecdysone receptor (EcR) and the homologue of the vertebrate retinoid X receptor (RXR)-Ultraspiracle (Usp) (Cho, Kapitskaya *et al.* 1995, Kapitskaya, Wang *et al.* 1996). Two Usp isoforms, Usp-A and Usp-B, which reside in the N-terminal domain (NTD), occur through alternative splicing and alternative promoter usage (Wang, Li *et al.* 2000). In this region of typical NR N-terminal activation domain (activation function 1 [AF1]) is located. AF1 generates functional interactions with regulators of transcription thereby activating transcription in a ligand-independent manner (Bocquel, Kumar et al. 1989, Wärnmark, Treuter et al. 2003). Nothing is known about the tertiary structure and molecular mechanisms involved in the function of the NTD of *A. aegypti* Usp (*aa*Usp-NTD). Due to the fact that *A. aegypti* is a primary vector of many devastating human diseases, it is important to collect knowledge about endocrinology of this mosquito to regulate population growth. There is no specific treatment available for virus infection or diseases transmitted by *Aedes* species mosquitoes. The biggest challenge is to elucidate the aaUsp-NTD function in the context of the functional ecdysone receptor which is a key element in reproduction and development of this mosquito.

Therefore it was decided to investigate molecular properties of aaUsp-NTD. In this study, recombinant plasmid containing NTD of *A. aegypti* Usp was obtained. This construct was overexpressed in the *E. coli* cells as a His-tagged fusion protein. An efficient procedure for the expression and purification of the recombinant aaUsp-NTD was established. Two step purification processes included immobilized metal affinity (IMAC) and size-exclusion (SEC) chromatography. In addition, to investigate the influence of the NTD on the molecular properties of a full-length Usp (aaUsp), the research was expanded by adding aaUsp and Usp shortened by NTD deletion (aaUsp-ΔNTD). For this purpose, the aaUsp and aaUsp-ΔNTD

constructs were obtained also with an N-terminal His-tag. The purification procedure of the recombinant aaUsp-ΔNTD includes IMAC and SEC, same as for aaUsp-NTD. However, the same procedure used for aaUsp did not allow to obtain a homogeneous preparation, therefore a heparin affinity chromatography step was added before SEC. Identity of the purified proteins was confirmed by immunoblotting and their molecular mass was confirmed by electrospray ionisation mass spectrometry (ESI-MS).

The results of *in vitro* experiments along with *in silico* bioinformatic analyses demonstrated that *aa*Usp-NTD exhibits characteristics of an intrinsically disordered protein (IDP). IDPs are characterized by the lack of a stable and unique three-dimensional structure under physiological conditions, which allows them to adopt distinct structures and interact with many partners (Tompa 2005). For the above mentioned reasons, NTD seems to be an extremely interesting object of scientific research. Circular dichroism (CD) showed presence of residual, ordered secondary structure. The decreased electrophoretic mobility, increased hydrodynamic radius observed in SEC experiments and analytical ultracentrifugation sedimentation velocity (SV-AUC), suggested that *aa*Usp-NTD exhibits characteristics of IDPs. Larger hydrodynamic radius means less compact conformation in comparison with globular proteins. Moreover SV-AUC analysis showed *aa*Usp-NTD to be an asymmetric, elongated elliptically-shaped protein domain. Based on collected data in this study, *aa*Usp-NTD cannot be unambiguously classified as existing in a specific conformational IDP state. It is possible that aaUsp-NTD exhibits both pre-molten globule (PMG) and molten globule (MG) characteristics under specific conditions. SEC and CD performed in presence of denaturant showed that aaUsp-NTD may have intermediate conformational states, which is reflected in denaturation curve shape. Moreover, aaUsp-NTD has tendency to adopt more ordered structure in response to external influences, what was manifested in CD measurements in presence of 2,2,2-trifluoroethanol (TFE). All the obtained results demonstrate that aaUsp-NTD is a dynamic molecule and has the potential to form a wide range of conformational states under denaturing and structure-forming conditions. Due to these features aaUsp-NTD may have the ability to bind to several different partner proteins.

Surprisingly, three independent experiments: size-exclusion chromatography (SEC), specific chemical crosslinking and sedimentation velocity analytical ultracentrifugation (SV-AUC) revealed that aaUsp-NTD readily forms dimers. The aaUsp-NTD mainly exists in solution as a monomer and in minority as a dimer. This tendency is conserved with different strengths in *Drosophila* Usp-NTD and *Bombyx mori* Usp-NTD. However, aaUsp-NTD exhibits the strongest homodimerization potential. Homodimerization in the IDP family is rare however recently more frequently reported in the literature.

Additionally the influence of *aa*-Usp-NTD on full-length *aa*UspB structure was investigated. Therefore hydrogen-deuterium exchange monitored by mass spectrometry (HDX-MS) experiments were performed. The results confirmed that aaUsp-NTD is intrinsically disordered and for the full length aaUsp the well-known organization of globular domains and their linker in the structure of NRs was reflected. The aaUsp-NTD remained disordered in full-length *aa*UspB.

The SEC and SV-AUC experiments carried out on *aa*Usp i *aa*Usp-ΔNTD showed that NTD does not significantly affect the hydrodynamic properties of full-length *aa*UspB, which suggests that NTD is located between DNA binding domain (DBD) and ligand binding domain (LBD). Moreover, in HDX-MS measurements observed conformational changes in DBD in full-length protein suggest a possible cross-domain interaction between NTD and DBD.

In this study the influence of NTD on DNA binding properties of full-length protein was investigated too. Microscale thermophoresis (MST) revealed that NTD increases protein-DNA interaction. Furthermore, EMSA results showed that the presence of the NTD decreases the dimerization tendency of aaUspB bound to hormone response element (HRE).

The structural and functional characterisation of *aa*Usp-NTD observed in this study makes it reasonable to suggest that the *aa*Usp-NTD might play an important role in many protein-protein interactions by providing an additional interface for inter- or intra-domain interaction, especially due to the fact that Usp coordinates many signaling pathways related with activity of different NRs, regulated by different signaling molecules.

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