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Homodimerization propensity of the intrinsically disordered N-terminal domain of Ultraspiracle from *Aedes aegypti*

Joanna Pieprzyk, Agnieszka Zbela, Michał Jakób, Andrzej Ożyhar, Marek Orłowski *

Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland

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ABSTRACT

The mosquito *Aedes aegypti* is the principal vector of dengue, one of the most devastating arthropod-borne viral infections in humans. The isoform specific A/B region, called the N-terminal domain (NTD), is hypervariable in sequence and length and is poorly conserved within the Ultraspiracle (Usp) family. The Usp protein together with ecdysteroid receptor (EcR) forms a heterodimeric complex. Up until now, there has been little data on the molecular properties of the isolated Usp-NTD. Here, we describe the biochemical and biophysical properties of the recombinant NTD of the Usp isoform B (aaUsp-NTD) from *A. aegypti*. These results, in combination with in silico bioinformatics approaches, indicate that aaUsp-NTD exhibits properties of an intrinsically disordered protein (IDP). We also present the first experimental evidence describing the dimerization propensity of the isolated NTD of Usp. These characteristics also appear for other members of the Usp family in different species, for example, in the Usp-NTD from *Drosophila melanogaster* and *Bombyx mori*. However, aaUsp-NTD exhibits the strongest homodimerization potential. We postulate that the unique dimerization of the NTD might be important for Usp function by providing an additional platform for interactions, in addition to the nuclear receptor superfamily dimerization via DNA binding domains and ligand binding domains that has already been extensively documented. Furthermore, the unique NTD–NTD interaction that was observed might contribute new insight into the dimerization propensities of nuclear receptors.

1. Introduction

Nuclear receptors play essential roles in regulating the differential expression of target genes that are involved in development, reproduction and metabolic homeostasis in animals, by binding to specific DNA response elements [1]. Many well-known nuclear receptors are ligand-dependent transcription factors whose activity is controlled by binding lipophilic ligands, including hormones and other metabolic molecules [2]. Members of the nuclear receptor superfamily share common structural and functional features. They are composed of a hypervariable in a sequence and length of the A/B region, called the N-terminal domain (NTD), and of highly evolutionarily conserved functional domains: the DNA binding domain (DBD) and the ligand binding domain (LBD). Nuclear receptors may act as homodimers or heterodimers using dimer interfaces that are located within the DBDs and especially within the LBDs [3].

Ecdysteroids, the principal steroid hormones in arthropods, are molecules involved in regulating essential biological processes such as molting, development, metamorphosis and reproduction [4–6]. The naturally occurring ecdysteroid hormone, 20-hydroxyecdysone (20E), manifests its action through a functional receptor that is a heterodimer of two members of the nuclear receptor superfamily: the ecdysone receptor (EcR) [7]and the homologue of the vertebrate retinoid X receptor (RXR)-Ultraspiracle (Usp) [8–10], described for the first time for *Drosophila melanogaster* [9,10]. Ecdysteroids promote binding of the Usp/EcR heterodimer to ecdysteroid-responsive elements to stimulate the transcription of target genes [9–12].

Nuclear receptors and other factors involved in ecdysteroid signaling pathways from *D. melanogaster* are valuable models for studying the mechanism of transcription modulation in vertebrates [13]. Mosquitoes, on the other hand, are the primary vectors of many devastating human diseases such as malaria and dengue, because they require blood as a nutrient source to promote egg development [14,15]. The yellow fever mosquito, *Aedes aegypti* is the main vector of dengue, which is the most serious arthropod-borne viral infection in humans [15]. In

Abbreviations: NTD, N-terminal domain; DBD, DNA binding domain; LBD, ligand binding domain; 20E, 20-hydroxyecdysone; EcR, ecdysone receptor; RXR, retinoid X receptor; Usp, Ultraspiracle; HR38, hormone receptor 38; IDP, intrinsically disordered protein; EcR-NTD, N-terminal domain of ecdysone receptor; HR38-NTD, N-terminal domain of hormone receptor 38; aaUsp-NTD, N-terminal domain of Usp-B from *Aedes aegypti*; dmUsp-NTD, N-terminal domain of Usp from *Drosophila melanogaster*; bmUsp-NTD, N-terminal domain of Usp from *Bombyx mori*; SEC, size-exclusion chromatography; SV-AUC, sedimentation velocity analytical ultracentrifugation; CD, circular dichroism; IMAC, immobilized-metal affinity chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoroacetic acid; GdmCl, guanidine hydrochloride; TMAO, trimethylamine N-oxide; TFE, 2,2,2-trifluoroethanol; BS^3, bis(sulfosuccinimidyl) suberate; R_S, Stokes radius; V_H, hydrodynamic volume; V_e, elution volume; MG, molten globule; PMG, pre-molten globule; N, native; U, unfolded

* Corresponding author. Tel.: +48 71 320 62 86; fax: +48 71 320 63 37.
E-mail address: marek.orlowski@pwr.edu.pl (M. Orłowski).

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mosquitoes, 20E plays a crucial role in regulating blood meal-triggered vitellogenesis [4,16]. In *A. aegypti*, two Usp isoforms (AaUspA and AaUspB) and two EcR isoforms (AaEcRA and AaEcRB) have been cloned and identified [11,17,18]. Although both *A. aegypti* Usp isoforms are capable of forming functional heterocomplexes with *A. aegypti* EcR isoforms, and although they recognize the same ecdysteroid-responsive elements, aaUspB is thought to be a major heterodimerization partner for EcR during the vitellogenic response to 20E [11,17].

Usp has received increasingly more attention due to its homology with RXR, which has an ability to be a heterodimeric partner for other nuclear receptors. In particular, Usp is an obligatory heterodimeric partner, not only for EcR, but also for other proteins, e.g., hormone receptor 38 (HR38) and the Seven-up protein with which it forms heterocomplexes [12,19]. Like other members of the nuclear receptor family, Usp proteins exhibit a typical modular structure. The major differences in the known isoforms of Usp are in their NTDs. In stark contrast to the highly evolutionarily conserved DBDs and LBDs, isolated NTDs of the studied nuclear receptors, e.g., glucocorticoid, estrogen and androgen receptors or their fragments are disordered in solution [20–22]. Moreover, direct interaction between a particular NTD and the right protein partner induces folding of the NTD, as has been shown for GR, ERα and AR [21,23,24]. Thus, the above-mentioned examples have shown NTDs as having features characteristic of intrinsically disordered proteins (IDPs) [25]. IDPs are characterized by the lack of a stable and unique three-dimensional structure under physiological conditions either entirely or in regions. They are able to adopt distinct structures by interacting with particular protein partners or due to other factors, such as ligand binding [26]. Structural intrinsic disorder is thought to be essential for these proteins, as their various biological functions stem either directly from this state or from some local folding/ordering in molecular recognition [25]. The experiments recently performed in our laboratory showed that recombinant *Drosophila* EcRA-NTD, EcRB-NTD [27] and *Drosophila* HR38-NTD [28] exhibit characteristic attributes of IDPs.

Currently, nothing is known about the molecular mechanisms involved in the function of the NTDs of *A. aegypti* Usp, let alone in the context of full-length Usp. In particular, questions about the three-dimensional structure and biophysical characteristics of this domain are still waiting to be answered. For the first time in this article we characterize some of the biophysical and biochemical properties of the *A. aegypti* N-terminal domain of Usp-B (aaUsp-NTD). We established an efficient two-step procedure for the expression and purification of the recombinant aaUsp-NTD. The results of in vitro experiments along with in silico bioinformatic analyses demonstrated that aaUsp-NTD exhibits characteristics of an IDP. Detailed analysis showed aaUsp-NTD to be an asymmetric, elongated elliptically-shaped protein domain. What was unexpected was that three independent experiments: size-exclusion chromatography (SEC), specific chemical crosslinking and sedimentation velocity analytical ultracentrifugation (SV-AUC) revealed that aaUsp-NTD readily forms dimers. This tendency is conserved with different strength in *Drosophila* Usp-NTD and *Bombyx mori* Usp-NTD. However, aaUsp-NTD exhibits the strongest homodimerization potential. The data obtained in our research suggest that the unique NTD–NTD interaction might play an important role in the dimerization of Usp by providing an additional interface for inter- or intra-domain interaction. Moreover, information on the dimerization of the NTD provides new insight on the previously documented dimerization events within the nuclear receptor superfamily. Our findings can contribute to further studies on the structure and molecular function of aaUsp-NTD in mosquito Usp and Usp/EcR heterocomplex activity during vitellogenesis and in mosquito reproductive pathways.

2. Materials and methods

2.1. Buffer composition

Buffer L (lysis buffer; 20 mM Na_2HPO_4, 150 mM NaCl, 1 mM dithiothreitol (DTT; Sigma), pH 7.4); buffer A (wash buffer; 50 mM Na_2HPO_4, 300 mM NaCl, 1 mM β-mercaptoethanol (Pierce), pH 7.5); buffer AA (elution buffer; 50 mM Na_2HPO_4, 300 mM NaCl, 200 mM imidazole,1 mM β-mercaptoethanol, pH 7.5); buffer B (gel filtration buffer; 50 mM Na_2HPO_4, 150 mM NaCl, 1 mM β-mercaptoethanol, pH 7.5); Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol); PBST buffer (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM, pH 7.4, 0.1% Tween 20); blocking buffer (5% non-fat dry milk in PBST buffer).

2.2. DNA construct

The cDNA for the aaUsp-NTD, isoform B was used as a template for PCR (1–112 amino acids), obtained from Prof. Alexander S. Raikhel, (Department of Entomology and the Institute for Integrative Genome Biology, University of California, USA) [29]. The corresponding NTD was cloned using the following forward and reverse primers: 5′-gcccggggatccATGG ACCCCAGCGATCGAGG-3′, 5′-gcccgg*aagctt*TCACAGATGCTTGGA CCCGCT G-3′, respectively. The small letters in the sequences represent nucleotides added for cloning purposes, the letters typed in italics represent restriction sites (*Bam*HI and *Hin*dIII) and the uppercase letters indicate a sequence derived from aaUsp-NTD cDNA. The amplified fragment was double-digested with *Bam*HI and *Hin*dIII and ligated into the corresponding sites of the pQE-80L *Escherichia coli* expression vector in a frame with the sequence that encodes the N-terminal His-tag (Qiagen). The sequence was verified by DNA sequencing.

2.3. Protein expression and purification

The protein was expressed in *E. coli* BL21(DE3)pLysS expression cells (Novagen). A 2 l bacterial culture was grown in Luria Broth medium (LB; Invitrogen), supplemented with 100 μg/ml carbenicilin and 35 μg/ml chloramphenicol, at 29 °C, 182 rpm to an A_{600} of 0.5 and was subsequently induced with 0.25 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Roth). After 2.5 h of incubation, the culture was centrifuged (15 min, 4 °C,4000 ×*g*), washed with 10 ml of buffer L, resuspended in the same buffer (1 g of cell pellet in 10 ml of buffer) and frozen at −80 °C. Cells were lysed by quick thawing in a 25 °C water bath. Immediately after, 0.2 mg/ml PMSF, 20 μg/ml DNase and 20 μg/ml RNase were added to the lysate. The resulting suspension was incubated for 1 h and centrifuged (40 min, 4 °C, 17,500 ×*g*). The supernatant was supplemented with 0.2 mg/ml PMSF (phenylmethylsulfonyl fluoride) and incubated for 20 min with 0.8 ml TALON™ Metal Affinity Resin (GE Healthcare), which had been previously washed with 20 ml of buffer L. After incubation, the resin was packed onto a disposable gravity flow column (20 ml) and washed with 20 ml of buffer A. In the next step, the resin was loaded onto a 5/50 Tricorn™ column (Amersham Biosciences). All subsequent purification steps were carried out by the ÄKTA explorer system (Amersham Bioscience). The resin was washed with buffer A containing 15 mM of imidazole. The fusion protein was eluted with buffer AA at a flow rate of 0.25 ml/min. The 0.25 ml fractions were collected, pooled and concentrated to 0.3 ml by ultrafiltration on the Amicon Ultra-4 Centrifugal Filter Unit (Millipore) with a cut-off limit of 3 kDa. The final step was gel filtration (Superdex75 10/300 GL; GE Healthcare) in buffer B at a flow rate of 0.5 ml/min. The elution profile was monitored at 280 nm. The 0.25 ml fractions were collected, pooled and concentrated to approximately 1.5 mg/ml. Protein samples were collected at every stage of purification and analyzed by SDS-PAGE (see below). Protein purity was estimated using densitometric analysis (Image Lab™ Software; Bio-Rad).

Details of the purification of the N-terminal domain of Usp from *D. melanogaster* (dmUsp-NTD) and the N-terminal domain of Usp from *Bombyx mori* (bmUsp-NTD) will be published in separate articles.

2.4. SDS-PAGE and Western blot

The protein samples were analyzed by SDS-PAGE according to Laemmli [30]. Proteins were separated on 15% SDS-PAGE gels. Samples