

Statements of the PhD Thesis

**Analysis of Structure and Function
of the Intrinsically Disordered Dehydrin
ERD14**

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Introduction

Dehydrins are a class of plant stress proteins that belong to the family of late embryogenesis abundant (LEA) proteins. They are known to be involved in the late stages of plant embryogenesis, i.e. seed development, as well as in the plant's response to particular abiotic stress effects, such as cold, dehydration, and high salinity. However, their exact role within stress response is unknown, although several investigations have aimed at the determination of the molecular function of dehydrins. Possible functions that have been suggested range from hydration buffers to chaperones, from membrane stabilizers to radical scavengers, and may be different (i.e., not uniform) among different dehydrins. In specific, for the dehydrin investigated in the present work, ERD14 (Early Response to Dehydration 14), its potent chaperone function and membrane binding capacity were shown *in vitro*¹.

Dehydrins are generally highly variable in sequence, but contain specific conserved regions, so called segments. The most important segment seems to be the K-segment, which is present in each and every dehydrin at least once, though other segments such as the Y-, S- and ChP-segments are – if present within a given dehydrin – highly conserved as well. Molecular functions that have been proposed to be related to specific segments include membrane binding and an antibacterial effect (K-segment), phosphorylation and calcium binding (S-segment), as well as chaperone function, phosphorylation and nuclear targeting (ChP-segment).

From a structural point of view, dehydrins are intrinsically disordered proteins (IDPs), i.e., proteins that do not adopt a well-defined folded structure but stay flexible even in their native environment. This pops up the question, how e.g. the potent chaperone function of ERD14 can be explained without the involvement of structure. In a classical view, chaperones are large protein machines of elaborate structure that assist in the folding of proteins or RNA molecules. However, similarly to ERD14, also other partially or fully disordered chaperones have been identified and investigated. Nevertheless, the molecular mode of function of such ID chaperones is still elusive.

Furthermore, no in depth structural study – to identify eventual residual structure within their intrinsically disordered state – has been performed on dehydrins until the beginning of the present work.

¹ Dénes Kovács et al., “Chaperone Activity of ERD10 and ERD14, Two Disordered Stress-related Plant Proteins,” *Plant Physiol* 147, no. 1 (2008): 381–90.

Aims

The goal of my PhD thesis was to investigate the dehydrin ERD14 from *Arabidopsis thaliana* from a structural as well as functional point of view, under *in vitro* as well as *in vivo* conditions. In specific, experiments aimed at the detailed characterization of the disordered state of the protein, at the verification of its chaperone function under *in vivo* conditions, and the identification of functionally important sequence regions.

For the structural characterization of ERD14 a thorough structural analysis by NMR under *in vitro* conditions was planned, including the expression of specifically isotope labeled, purified ERD14 for the measurement, assignment and structural as well as dynamic characterization of the protein. The sparse literature about the applicability of general interpretation methods of chemical shifts to IDPs (as available at the time of beginning of the work) indicated also a need for a detailed comparison of the relevant possibilities and the validation of their use on ERD14. In order to also assess the structural state of ERD14 under *in vivo* conditions, in-cell NMR experiments were planned, consisting of the preparation and measurement of samples of single labeled ERD14 within living *E. coli* cells.

The functional analysis aimed to verify the known *in vitro* chaperone function of ERD14 also under *in vivo* conditions. For this, a novel *in vivo* chaperone assay was designed, using *E. coli* cells as an easy-to-handle, living model environment. The implementation of several different stress treatments – comparable to those abiotic stress effects that invoke dehydrin response in plants – was planned.

Furthermore, the preparation and investigation of deletion mutants (lacking the conserved segments hypothesized to be important in function) was planned for their comparison of structural and functional traits with respect to the wild type protein.

Methods

For the recombinant expression and purification of ERD14 I used standard biochemical procedures. I prepared mutant ERD14 constructs by a PCR reaction using mutagenic primers and transformed, expressed, and purified the proteins in analogy to the wild type protein. I compared mutant and wild type ERD14 by gel electrophoresis, CD spectroscopy and size exclusion chromatography.

I used Nuclear Magnetic Resonance Spectroscopy (NMR) for an in depth structural investigation of wild type ERD14 under *in vitro* conditions. For the respective NMR measurements I expressed and purified single (^{15}N) and double (^{15}N - ^{13}C) labeled samples of ERD14. I used the resulting spectra to achieve the full backbone assignment and to interpret the resulting chemical shift information and relaxation behavior in terms of structure and dynamics of ERD14. Within this process, I compared the available sets of reference chemical shifts for secondary chemical shift calculation (SCS) and the interpretation methods of SCS values with respect to their use on ERD14 (and on IDPs in general).

In order to assess the structural state of wild type ERD14 under *in vivo* conditions, I also performed in-cell NMR experiments. I acquired spectra of single (^{15}N) labeled ERD14 within living *E. coli* cells, and compared them to the according reference and control spectra.

For the functional analysis of ERD14, I designed and implemented a novel method for the assessment of its *in vivo* chaperone effect. I applied four different stress conditions on protein-containing and control *E. coli* cells. Deviating from other examples in the literature, I detected the relative viability of cells with the application of the BacTiter-GloTM Microbial Cell Viability Assay (from Promega).

Results

- Through the in depth analysis of ERD14 by NMR I confirmed its overall intrinsically disordered state under *in vitro* conditions. I achieved the full backbone assignment of ERD14 and interpreted in detail the obtained chemical shift and relaxation data. I identified five regions of restricted motion, which overlap with the conserved regions of ERD14 (K-, S- and ChP-segments) and show helical propensities of about 5-15%. These regions might represent preformed structural elements (PSEs) for the function of the protein.
- I compared the available sets of reference chemical shifts for secondary chemical shift (SCS) calculation and the interpretation methods of SCS values with respect to their use on ERD14 (and on IDPs in general). I pointed out typical errors that can arise due to the use of an inappropriate set of reference chemical shifts, and identified the three sets that are most appropriate for the use with IDPs under near native conditions.
- I showed by several low-resolution techniques, that mutants of ERD14 (lacking the conserved segments one-by-one) have the same overall intrinsically disordered state as the wild type protein. These results prove that no stable tertiary contacts are formed within wild type ERD14.
- Through the performed in-cell NMR measurements I demonstrated that ERD14 is intrinsically disordered within living *E. coli* cells similarly as under *in vitro* conditions. I identified three regions – which fall within the three K-segments of the protein – that seem to undergo binding within the cell, indicating the possible functional importance of these regions.
- I developed and implemented a novel *in vivo* chaperone assay using *E. coli* cells and three different stress treatments, as well as an extra alternative approach to test the *in vivo* protective effect of ERD14 and other dehydrins with chaperone function.
- Using the novel *in vivo* chaperone assay I demonstrated that ERD14 has potent *in vivo* chaperone function under all four tested conditions. Through the measurement of a mutant of ERD14 in which all three K-segments have been deleted I showed that this function is connected to the presence of the K-segments.

Conclusions

In conclusion, both the structural as well as the functional analysis underline the importance of the K-segments of ERD14 under *in vivo* conditions and confirm the intrinsic disorder of ERD14 in its functional state within the living cell.

The importance of these results is twofold: the structural investigations confirmed that ERD14 is an IDP, which retains its intrinsic disorder even in the cell, and the *in vivo* chaperone assay demonstrated that intrinsic disorder is the active, functional state of ERD14. This contradicts the general conception in the IDP field that (most) IDPs have to fold upon binding to become active, as was generally supposed so far to be the case for dehydrins as well. Furthermore, the presented results implicate that ERD14 is an IDP that does survive in the cell even while being disordered, which is – speaking of IDP proteins in general – still subject of heated discussions.

My experiments showed both that the conserved K-segments of ERD14 contain preformed helical elements for binding within the cell, and that these segments are essential for the function of the protein. The K-segment is the most conserved region of dehydrins, and the results presented herein confirm its importance in function.

As for the molecular mechanism of chaperone function, I propose that ERD14 might bind to substrates by the use of its K-segments and help in the refolding of misfolded proteins either passively (by preventing aggregation through entropic exclusion) or actively (by providing some entropic energy for the rescue from a folding trap). Though, to set up a more detailed mechanistic model of the chaperone function of ERD14, various mutants will have to be tested using either the presented assay or similar investigations². However, the protective effect of dehydrins within the cell might be even more complex. Water retention, ion buffering and radical scavenging potentials of dehydrins may all operate in addition to the chaperone effect investigated herein. This shows that although important results could be presented in my work, still a lot of further investigations are needed to be able to fully understand the mode of action of dehydrins, or of ID chaperones in general.

² in progress, manuscript in preparation; **Bianka Szalainé Ágoston** et al., “Functional Disorder of Plant Dehydrin ERD14 *in vivo*,” 2013.

Publications

Publications related to the thesis:

- **Szalai** **Ágoston B.**, Kovács D., Tompa P., Perczel A.: „*Full Backbone Assignment and Dynamics of the Intrinsically Disordered Dehydrin ERD14*“ *Biomolecular NMR Assignments* (2011) 2: 189-193.
- Kovács D., **Ágoston B.**, Tompa P.: "*Disordered plant LEA proteins as molecular chaperones*" *Plant Signaling and Behavior* (2008) 3: 710-713.

Other publications:

- Kovács D., Rakács M., **Ágoston B.**, Lenkey K., Semrad K., Schroeder R., Tompa P.: „*Janus chaperones: assistance of both RNA- and protein-folding by ribosomal proteins*” *FEBS Letters* (2009) 583, 88-92.
- Mohammed-Ziegler I., Tánzos I., Hórvölgyi Z., **Ágoston B.**: "*Water-repellent acylated and silylated wood samples and their surface analytical characterization*" *Colloids and Surfaces A: Physicochemical and Engineering Aspects* (2008) 319: 204-212.
- Tollinger M., Kloiber K., **Ágoston B.**, Dorigoni C., Lichtenecker R., Schmid W., Konrat R.: "*An Isolated Helix Persists in a Sparsely Populated Form of KIX under Native Conditions*" *Biochemistry* (2006) 45: 8885-8893.