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Regulatory plant 14-3-3 proteins markedly differ in stability and provide insights into the plant stress resistance and molecular evolution

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Motivation and aim: Proteins unusually named as 14-3-3 are known as regulators of key physiological processes in plants. Numerous studies indicate their importance in gaining agriculturally important traits, for instance, starch accumulation, resistance to drought and salt stresses, and plant immunity. Despite over 30 years of research, the plant 14-3-3 protein family remains poorly characterized at the molecular level. The protein sequence analysis classifies plant 14-3-3s onto two large phylogenetic groups called epsilon and non-epsilon, but the biochemical and functional criteria underpinning the phylogeny were unknown. We systematically analyzed 14-3-3 proteins from phylogenetically distant plants and revealed how the phylogenetic group affiliation of a particular 14-3-3 contributes to the protein stability, which provides new routes towards molecular studies of plant stress resistance and incites new hypotheses in the fields of molecular adaptations and protein evolution.
Materials and methods: We obtained genetic constructions encoding 14-3-3 proteins: 12 of *Arabidopsis thaliana* and 3 proteins of *Selaginella moellendorffii*. We heterologously expressed the proteins in *E. coli* and purified them to homogeneity by means of two-step immobilized metal-affinity chromatography (IMAC) and size-exclusion chromatography (SEC). The proteins underwent systemic analysis with multiple biochemical approaches. The oligomeric state was determined by SEC coupled with multiangle light scattering (MALS) detection. Thermal stability was measured with differential scanning fluorimetry (DSF). Surface hydrophobicity was probed with an environmentally sensitive fluorescent dye bis-ANS. Thermodynamic stability of 14-3-3s was estimated by analyzing protein migration patterns at the increasing denaturant concentrations on native gel electrophoresis. We studied 14-3-3 dimer dissociation by SEC of gradually diluted protein samples and by running native gels. To assess the resistance to proteases, we probed the proteins with a model protease chymotrypsin and also analyzed protein degradation in plant lysates. As a functional test, we studied the interaction of 14-3-3s and a model client protein phosphopeptide by fluorescence polarization. Structure-guided site-directed mutagenesis was applied to analyze the effect of mutating the key amino acid positions responsible for protein stability. We conducted bioinformatic analysis to determine the conservation of the key residues in phylogeny.
Results: We showed that all analyzed 14-3-3 proteins of *A. thaliana* form homodimers, a typical attribute of 14-3-3s. However, surprisingly, the four epsilon group proteins monomerized upon dilutions to low protein concentrations (100 nM) and in the native gel, which was in striking contrast with the non-epsilon group proteins behaving as stable dimers under the same conditions. This effect had nothing to do with phosphorylation of the proteins studied, as their treatment with unspecific phosphatase did not change the observed pattern. The monomerizing epsilon group proteins had enhanced surface hydrophobicity, indicating the dissociation occurs already in the intact protein solution. Remarkably, the epsilon-type proteins were much less thermostable than non-epsilon-type (the decrease in the melting temperature exceeded 20 °C). Epsilon group proteins were readily degraded by proteolysis and elevated temperatures in the model experiments. The outstanding instability of epsilon-type 14-3-3 inspired us to search for a mechanistic explanation of the observed phenomena in the spatial structure and polypeptide sequences. By point mutagenesis of 14-3-3 iota (epsilon-type), we found two key amino acid positions, P1 and P2, contributing to the 14-3-3 stability. The identified positions correlated well with the epsilon/non-epsilon demarcation on a wide taxonomic scale: Lycophytes, Ferns, Gymnosperms, Monocots and Dicots. We experimentally verified the predicted demarcation in *S. moellendorffii* and showed that epsilon 14-3-3 is less stable than the two non-epsilon paralogs. Interestingly, multiple biochemical approaches demonstrated that *S. moellendorffii* 14-3-3 are more biochemically stable than *A. thaliana* 14-3-3. Using protein melting temperature data, we demonstrated that in the course of evolution, thermostability of the epsilon and non-epsilon groups decreased and the melting temperature gap between the two groups instead increased.
Conclusions: We showed that in two plant models from evolutionary distant taxa 14-3-3 proteins from the epsilon group are substantially less stable than the non-epsilon paralogs. This provided the first evidence on the biochemical and functional difference of the epsilon and non-epsilon groups of 14-3-3 proteins. The identified strong relationship between the biochemical properties of 14-3-3 and their phylogenetic affiliation resulted in the formulation of an accurate criterion predicting protein biochemistry from sequence of a given 14-3-3 from organisms across all vascular plants. Decrease of thermostability in the course of evolution sheds light on the origin of plant 14-3-3, suggesting that an ancestral protein was stable and non-epsilon-like.
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