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Chaperone function of two small heat shock proteins from maize

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ABSTRACT

Small heat shock proteins (sHsps) are molecular chaperones that protect cells from the effect of heat and other stresses. Some sHsps are also expressed at specific stages of development. In plants different classes of sHsps are expressed in the various cellular compartments. While the Class I (cytosolic) sHsps in wheat and pea have been studied extensively, there are fewer experimental data on Class II (cytosolic) sHsps, especially in maize. Here we report the expression and purification of two Class II sHsps from *Zea mays* ssp. *mays* L. (cv. Oh43). The two proteins have almost identical sequences, with the significant exception of an additional nine-amino-acid intervening sequence near the beginning of the N-terminus in one of them. Both ZmHsp17.0-CII and ZmHsp17.8-CII oligomerize to form dodecamers at temperatures below heat shock, and we were able to visualize these dodecamers with TEM. There are significant differences between the two sHsps during heat shock at 43 °C: ZmHsp17.8-CII dissociates into smaller oligomers than ZmHsp17.0-CII, and ZmHsp17.8-CII is a more efficient chaperone with target protein citrate synthase. Together with the previous observation that ZmHsp17.0-CII but not ZmHsp17.8-CII is expressed during development, we propose different roles in the cell for these two sHsps.

Introduction

Small heat shock proteins play many roles in the cell

Small heat shock proteins (sHsps) play important roles in the cells of almost every organism in all three kingdoms of life [1–4]. Especially diverse in plants, sHsps are expressed in large quantities in response to heat shock. For instance, the sHsps constitute more than 1% of total protein in heat-stressed pea leaves [5]. First characterized as molecular chaperones that bind to cellular proteins and prevent heat-induced aggregation [6,7], sHsps also protect against many other stresses such as cold, drought, and metal ions [8–10]. sHsps are now recognized as crucial "protein stability sensors" [11], ATP-independent "holdases" [12,13] that bind to hydrophobic residues of partly unfolded proteins, then pass the target proteins along to either ATP-dependent folding systems such as HSP70 or degradation by ubiquination or macroautophagy [14]. In addition to stress-induced expression some sHsps are expressed during specific developmental stages in many organisms [15]. Furthermore, sHsps are expressed constitutively in mammalian muscle, nervous tissue, and lens of the eye. Because their function is of such fundamental importance, it is not surprising that mutations in sHsps result in a variety of disease states such as cataract, neuropathy, and myopathies [16–19], and that regulation of sHsp expression is important in protein mis-folding diseases such as Alzheimer's and Parkinson's [16,20,21].

sHsps share a conserved α-crystallin domain, variable N-terminus and C-terminal extension

The α-crystallin domain (ACD), named for the crystallin proteins that are responsible for the refractive power of the vertebrate lens [22], is the shared motif among the sHsps. This highly conserved C-terminal domain consists of 90–100 amino acids that form a stable β-sheet sandwich structure. In the crystal structures of the sHsps from hyperthermophilic archaeon *Methanococcus jannaschii* (reclassified as *Methanocaldococcus jannaschii*) and wheat (*Triticum aestivum*), dimers are formed by β6-strand exchange between the ACDs of the paired monomers [23,24]. In *Xanthomonas citri* pv. *citri* [25] and vertebrate crystal structures dimers are stabilized by antiparallel interactions between β-strands from each monomer, which form an extended β-sheet [22,26,27]. With some exceptions [28] the dimers further associate into large oligomeric

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complexes that vary from 12 to more than 48 monomers in different organisms [29]. Below heat-shock temperature the oligomers are storage structures; as the temperature rises, there is extensive subunit exchange and formation of both smaller and larger assemblies [30–34]. Various experimental approaches, notably nanoelectrospray mass spectrometry, have shown that smaller oligomers are the effective chaperones that stabilize target proteins [35].

In contrast to the genetic and structural stability of the central ACD, the N-terminus and the C-terminal extensions of sHsps have highly variable sequences. In the full-length sHsps that have been successfully crystallized the N-terminus is either partly or fully disordered [23–25]; thus far only truncated ACDs have been crystallized from vertebrate sHsps [22,26,27,36]. Even when the sHsps form stable complexes in solution with target proteins, the N-terminal arm continues to be locally dynamic [12]. Although the N-terminus appears to be intrinsically disordered, there is convincing evidence that this part of the sequence provides the variability needed for association into oligomers as well as recognition and binding to multiple target proteins [14,15,28,37,38].

Plant sHsps exhibit remarkable variety

Perhaps because of their inability to move away from stressors, land plants express a remarkable range of sHsps. There are 12 gene subfamilies of plant sHsps; each family is expressed in a particular cellular compartment and may include several members [39]. Class I (CI) and Class II (CII) sHsps, which are found in the cytoplasmic/nuclear compartment, are the most studied sHsps. These two subfamilies share sHsp consensus I and plant sHsp consensus II in the ACD; they are differentiated by class-specific consensus regions in the N-terminus. (See Fig. 1 for sequence comparisons of CI and CII sHsps.) Experimental studies of sHsps from pea, tomato, and rice have shown that CI and CII sHsps stabilize target proteins during heating both *in vitro* and *in vivo* [40–42]. Likewise, both classes are expressed during seed development/germination in pea [43], during tomato ripening in fruit and seeds [40] and in varied tissues of the rice plant, especially in seeds [44]. However, CI and CII demonstrate differences in oligomerization behavior with heating, efficiency of target protein protection, and characteristics of heat-shock complexes [42,45–48], leading to the conclusion that the differences in N-terminal sequence affect expression and function of CI and CII sHsps.

Two Class II sHsps from maize have intriguing differences in function

The two CII sHsps from maize examined in this study, ZmHsp17.0-CII and ZmHsp17.8-CII differ only slightly in sequence: ZmHsp17.8-CII has a glycine- and proline-rich intervening sequence of nine amino acids in the N-terminus that is missing in ZmHsp17.0-CII. With the exception of this intervening sequence, 93% of the amino acids in the two proteins are identical; 97% are closely similar when conserved or semi-conserved substitutions are included.

In Fig. 1 sequences of some plant CI and CII sHsps are compared, showing the N-terminal CI and CII-specific sequences, plant-specific consensus II, eukaryotic sHsp consensus I, and the proline-rich C-terminal extension with I/VxI/V motif in the tail. Another distinction between CI and CII sequences is the abundance of methionine residues in the N-terminus of CII sHsps. Translation is initiated by these internal AUGs as well as the initial AUG in maize during heat shock and microsporogenesis [53], giving rise to isoforms that may respond to specific protein targets, stresses, or developmental signals. These methionines may also interact with exposed aromatic residues in unfolding proteins [54], thus contributing to the region's role in target recognition and binding. We note that intervening sequences similar to that which differentiates Zm17.8-CII from Zm17.0-CII is present in some other sHsps in both classes I and II.

Both of these CII sHsps are expressed during heat shock, but only ZmHsp17.0-CII is expressed early in anthers during microsporogenesis and in radicles under metal-ion stress [8,51,55]. The difference in developmental expression suggested a possible difference in function for the two sHsps. Intrigued by these differences that correlate with a relatively small difference in N-terminal primary structure, we expressed and purified the two proteins in order to investigate the oligomerization behavior and efficiency of these two sHsps in protecting a target protein, citrate synthase (CS), from thermal stress.

Material and methods

Engineering the vector construct

A uniform strategy was employed in cloning the three ZmHsp17-CII coding regions for expression of their polypeptides in *Escherichia coli* using the IMPACT-CN™ system (NEB). The N-terminal primer for amplifying each coding region comprised six spacer bases followed by CAT (so that the resulting amplicon contained an NdeI restriction site) followed by a segment matching the coding sequence from its natural ATG start codon and extending through enough bases to give a T_m in the upper 50s °C. The C-terminal primer had a 3′ segment matching the protein-coding segment of the sequence over its final anti-codons extending through enough bases to give a T_m in the mid-60s °C. The region 5′ to this comprised a cysteine codon replacing the stop codon of the coding region and an inverted SapI restriction site plus seven spacer bases. The restriction site was positioned so that cleavage of the amplicon would expose the three bases of the cysteine codon as the 3′ extension of the coding region, allowing in-frame fusion to the intein sequence of the vector as discussed below.

Amplification was performed using the cDNA clone templates for ZmHsp17.8-CII and ZmHsp17.6-CII (formerly designated 18-3 and 18-9 [50]) and ZmHsp17.0-CII (formerly designated 18-1 [51]) and with their respective primer pairs using the FailSafe™ system (Epicenter) as follows. Reaction Mix: 10 μM Forward and Reverse primers, 1.2 μL each; 500 pg/mL cDNA plasmid, 0.8 μL; 2× D-mix, 6.0 μL; FailSafe polymerase mix, 0.1 μL; dH_2O, 2.7 μL. PCR conditions: (1) 94 °C, 5 min; (2) 94 °C, 30 s; (3) 64 °C, 30 s; (4) 68 °C, 2 min; (5) go to #2 4×; (6) 90 °C, 30 s; (7) 68 °C, 30 s; (8) 68 °C, 2 min; (9) go to #6 29×; (10) 10 °C, hold.

The amplicons were initially cloned into the TOPO™ vector pCRII (Invitrogen) and transformed into *E. coli* host strain DH5α. Colonies with inserts of the expected size were identified by colony-PCR performed using the universal M13 primers for the pCRII vector using the Green Taq™ system (Promega) as follows. Reaction Mix: 10 μM Forward primer and Reverse primer, 0.3 μL each; pick of colony in 25 μL dH_2O, 0.4 μL; Green Taq mix, 3.0 μL; dH_2O, 2.0 μL. PCR conditions: (1) 95 °C, 5 min; (2) 95 °C, 30 s; (3) 54 °C, 30 s; (4) 72 °C, 2 min; (5) go to #2 29×; (6) 72 °C, 4 min; (7) 10 °C, hold. Plasmid DNA of isolates was prepared from 4-mL liquid cultures of positive colonies and DNA sequencing performed using the same universal M13 primers for the pCRII vector in order to identify a completely accurate copy for each ZmHsp17-CII. Once identified, these were excised by a double-digest with NdeI and SapI, and then gel-purified. They were then ligated into IMPACT™ expression vector pTYB1 (NEB) that had been double-digested with NdeI and SapI. The pTYB1 vector contains an IPTG-inducible T7 promoter. Once made, the ligations were re-transformed into *E. coli* host strain DH5α. Insert-positive clone colonies were identified by PCR using the Impact T7 forward and Intein reverse primers and the Green