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Identification in Pea Seed Mitochondria of a Late-Embryogenesis Abundant Protein Able to Protect Enzymes from Drying[1]

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Late-embryogenesis abundant (LEA) proteins are hydrophilic proteins that accumulate to a high level in desiccation-tolerant tissues and are thus prominent in seeds. They are expected to play a protective role during dehydration; however, functional evidence is scarce. We identified a LEA protein of group 3 (PsLEAm) that was localized within the matrix space of pea (*Pisum sativum*) seed mitochondria. PsLEAm revealed typical LEA features such as high hydrophilicity and repeated motifs, except for the N-terminal transit peptide. Most of the highly charged protein was predicted to fold into amphiphilic α-helixes. PsLEAm was expressed during late seed development and remained in the dry seed and throughout germination. Application of the stress hormone abscisic acid was found to reinduce the expression of PsLEAm transcripts during germination. PsLEAm could not be detected in vegetative tissues; however, its expression could be reinduced in leaves by severe water stress. The recombinant PsLEAm was shown to protect two mitochondrial matrix enzymes, fumarase and rhodanese, during drying in an in vitro assay. The overall results constitute, to our knowledge, the first characterization of a LEA protein in mitochondria and experimental evidence for a beneficial role of a LEA protein with respect to proteins during desiccation.

Late-embryogenesis abundant (LEA) proteins are overwhelmingly hydrophilic proteins that accumulate to high levels in the latter stages of seed maturation and disappear following germination (Galau et al., 1986). While almost ubiquitous in the plant kingdom, data mining has revealed the widespread occurrence of LEA proteins in prokaryotes and eucaryotes (Garay-Arroyo et al., 2000). Historically clustered in five main groups based on primary structure analysis (Dure et al., 1989; Cuming, 1999), the LEA protein classification was recently reexamined using statistically based bioinformatic tools (Wise, 2003).

LEA protein expression, which often appears abscisic acid (ABA) dependent, can also occur in vegetative tissues subjected to water deficit associated with drought, salt, or cold stress (for review, see Ingram and Bartels, 1996; Thomashow, 1998; Cuming, 1999). Both the pattern of expression and the structural features of LEA proteins suggest a general protective role in desiccation tolerance (Ingram and Bartels, 1996; Cuming, 1999). This hypothesis was recently supported by the discovery of a LEA protein in an anhydrobiotic nematode (Browne et al., 2002) as well as by the sensitization to desiccation induced by mutational inactivation of LEA genes in the prokaryote *Deinococcus radiodurans* (Battista et al., 2001). In view of the apparent lack of well-ordered tertiary structure of LEA proteins preventing their use as catalysts, several mechanisms have been proposed to relate their structural features to the protection of cellular structures required by a dehydrated state: water replacement, ion sequestering, macromolecules, and membrane stabilization (Close, 1996, 1997; Cuming, 1999). Experimentally, several LEA proteins have been shown to behave in vitro as cryoprotectants (Kazuoka and Oeda, 1994; Houde et al., 1995; Bravo et al., 2003) and as stabilizers of glassy states (Wolkers et al., 2001). Another LEA protein from maize, DHN1, was demonstrated to undergo conformational changes when binding specifically lipid vesicles, suggesting a role in membrane stabilization during stress (Koag et al., 2003). By expressing the wheat LEA protein Em6 in yeast, Swire-Clark and Marcotte (1999) brought to light the osmoprotective properties of the protein. However, although transgenic plants overexpressing LEA proteins exhibited improved stress tolerance (Xu et al., 1996; Sivamani et al., 2000), experimental evidence for the function of LEA protein remains faint (Cuming, 1999). More recently, a dehydrin was shown to bind calcium in a phosphorylation-regulated mode (Alsheikh et al.,

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2003), and another LEA protein was assigned a role in iron transport in the phloem of *Ricinus communis* (Krüger et al., 2002).

At the subcellular level, several LEA proteins have been localized in the cytosol and nucleoplasm (Roberts et al., 1993; Houde et al., 1995), in chloroplast (Artus et al., 1996; NDong et al., 2002), in protein bodies and amyloplasts (Rinne et al., 1999), and in vacuoles (Heyen et al., 2002). Heat-soluble proteins cross-reacting with dehydrin antibodies have been detected in mitochondrial fractions from cereals (Borovskii et al., 2000, 2002), but neither their primary sequence nor their subcellular localization has been demonstrated. In a survey of pea (*Pisum sativum*) mitochondrial proteome during development, a putative seed mitochondrial protein exhibited peptide tag sequence similarities with a soybean protein annotated in databases as a LEA-like protein (Bardel et al., 2002). The molecular cloning, subcellular localization, and expression profiling of the corresponding polypeptide that are reported here provide, to our knowledge, the first characterization of a LEA protein in mitochondria. This protein is expected to participate in the protection of seed mitochondria against desiccation, allowing the rapid resumption of mitochondrial respiration during seed imbibition.

RESULTS

cDNA Cloning and Sequence Analysis of PsLEAm

Two peptide sequence tags that were derived from spot S3 in a previous proteomic analysis of pea mitochondria (Bardel et al., 2002) were used to clone the corresponding cDNA from pea seeds by a reverse transcription (RT)-PCR strategy. The resulting full-length cDNA encoded a polypeptide of 358 amino acids that was named PsLEAm (accession no. AJ628940). Since the N-terminal sequence of the protein had been determined, PsLEAm appeared to be synthesized as a precursor with a 37-N-terminal amino acid extension. The deduced molecular mass (34,771 D) and pI (5.10) of the mature PsLEAm polypeptide

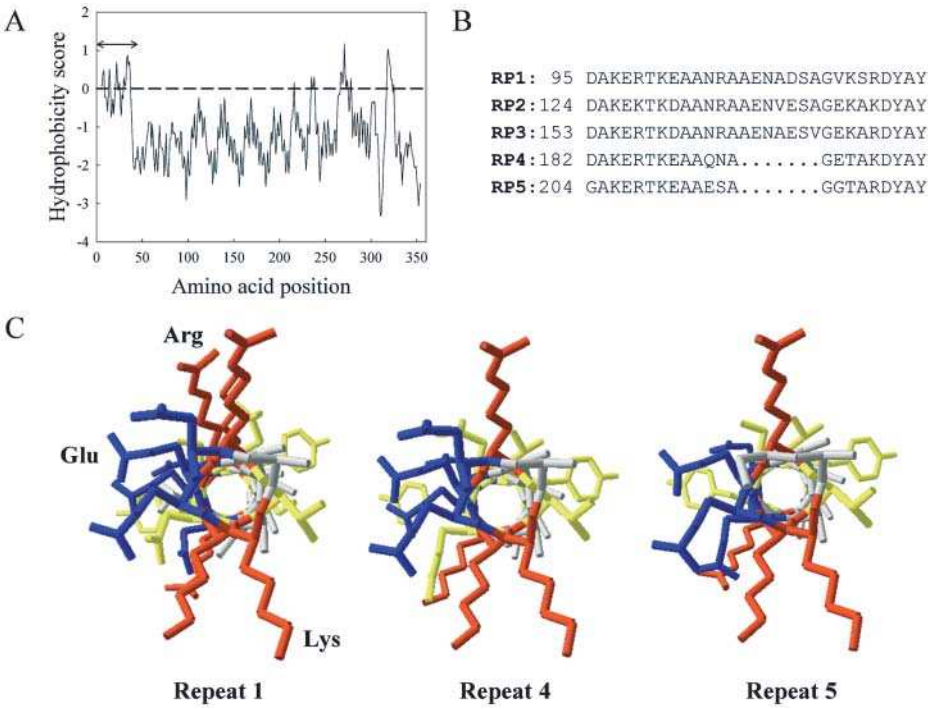

Figure 1. Analysis of PsLEAm primary structure. A, Kyte and Doolittle hydropathy analysis of PsLEAm. Values below the zero line (dashed) are negative and hydrophilic and were averaged over a moving window of nine amino acids. The numbers on the *x* axis refer to the amino acid positions. The arrow signals the position of the presequence. B, Alignment of the repeating 29- and 22-mer motifs found in the PsLEAm protein sequence. Alignment is optimized with gaps introduced as dots in the truncated versions. Motifs are labeled Rp1 to Rp5 and numbers refer to the amino acid position of the first residue. C, Three-dimensional models of the repeated motifs Rp1, Rp4, and Rp5 designed with the Swiss-PdbViewer program. The motifs are shown with the first residue in front, and the amino acids have been colored according to their properties. Charged amino acids are indicated in red (basic, K and R) or blue (acid, D and E) and other residues in yellow (polar or hydrophobic: N, S, T, and Y) or gray (nonpolar or hydrophobic: A, G, and V). The lateral chains of three residues are indicated on the repeat 1 model for the sake of clarity.

were in agreement with the experimental values of spot S3.

The primary sequence of the mature PsLEAm protein was found to be highly enriched in hydrophilic (58% of D, E, K, N, R, S, and T) and charged residues (40% of D, E, K, and R). Five residues (A, D, E, G, and K) comprised 61% of the sequence, which was devoid of Cys, Phe, and Pro. Five tandemly repeated motifs were found, the first three 29-mer repeats being almost identical while the last two repeats appeared shorter (Fig. 1B). Although remnants of the repeats could be found further on the sequence, they were highly degenerated (data not shown). The hydropathy profile of PsLEAm appeared emblematic of LEA proteins with an overwhelming hydrophilicity and allowed a clear visualization of the repeats (Fig. 1A). The N-terminal extension displayed a completely different profile with a succession of hydrophilic and hydrophobic stretches that could feature a transit peptide (Fig. 1A). The secondary structure analysis of the mature protein carried out with several programs predicted a high proportion of alpha helixes (GOR 4, 68.85%; PHDSec, 78.8%; PROF, 79.13%). The repeated motifs were modeled with the Swiss-Pdb viewer program using a filamin helical structure as a template. The models clearly highlighted the amphiphatic properties of the repeats with half of the helix side exposing negatively charged residues framed by positively charged residues, the other half being constituted of hydrophobic or neutral residues (Fig. 1C). Interestingly, although each of the last two repeats was interrupted by a seven-residue gap corresponding to two turns of helix (pitch 3.6), the amphiphatic properties were conserved (Fig. 1C). Scanning of the mature PsLEAm protein sequence with the COILS program revealed two regions between residues 50 to 150 and 200 to 270 that could be involved in coiled-coil structures. Several classes of LEA proteins have been defined on the basis of their primary and secondary sequences (for review, see Wise, 2003). The hydrophilicity, high α-helix content, and amino acid composition of PsLEAm strongly suggest that it belongs to group 3 of LEA proteins. Although PsLEAm exhibited repeated motifs, another characteristic of group 3, these did not correspond to the historical 11-mer repeat TAQAAKEKAXE (Dure, 1993). When PsLEAm primary sequence was blasted against the Pfam database, the protein was clearly assigned to the Pfam LEA4 family (accession PF02987) that corresponds to group 3 of LEA proteins.

Mitochondrial and Submitochondrial Localization of PsLEAm

Although highly purified, pea seed mitochondria were found to be contaminated by protein bodies (Bardel et al., 2002; Benamar et al., 2003), and the ultimate annotation of PsLEAm as a mitochondrial protein thus required some considerable effort to validate its localization. The fact that PsLEAm appeared to be synthesized with a presequence suggested a possible mitochondrial localization, and the sequence of the precursor protein was thus subjected to bioinformatic analysis with subcellular targeting programs. Two programs (MitoProt, PSORT) predicted a high probability of mitochondrial localization, while two others (PREDOTAR, TargetP) ranked first a plastidial followed by a mitochondrial targeting probability. Such an outcome is not surprising since discriminating the respective targeting sequences remains a difficult task. To clarify the subcellular localization of PsLEAm, an expression plasmid was constructed to express in plants a translational fusion of the first 49 amino acids of PsLEAm upstream of a synthetic green fluorescent protein (GFP) in a reporter plasmid. The selected N-terminal sequence comprises the putative transit peptide and cleavage site. The recombinant construct was then transformed into pea leaf protoplasts for 35S promoter-driven expression. After incubation, the protoplasts were examined by confocal fluorescence microscopy. The expressed fusion protein appeared to be localized as numerous small green spots (0.5–1 μm) as shown in Figure 2a. Protoplasts were simultaneously labeled with the mitochondria-specific fluorochrome Mito-

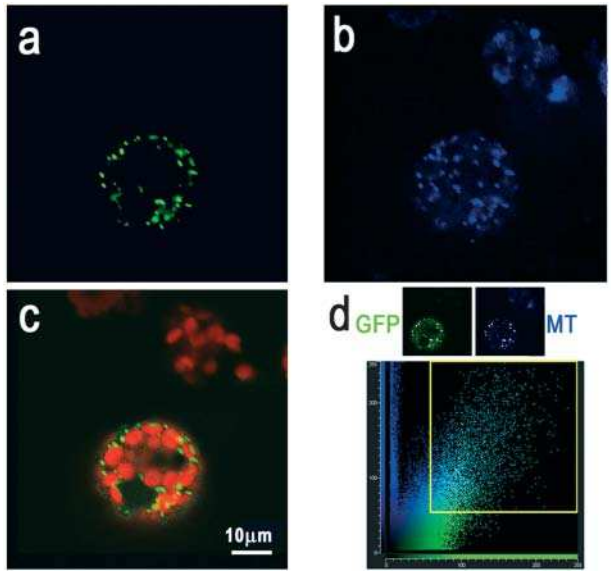

Figure 2. Mitochondrial targeting of a translational fusion of PsLEAm presequence with GFP. The presequence of PsLEAm (amino acids 1–49) including the first 12 residues of the mature protein was translationally fused to S65TsGFP in an expression vector designed for constitutive expression in plants. The construction was transformed into pea leaf protoplasts that were observed after transient expression by confocal microscopy. a, GFP fluorescence of a transfected protoplast; b, MT fluorescence in a transfected and in a nontransfected protoplast; c, overlay of GFP and chlorophyll fluorescence. Images a, b, and c correspond to an optical section (0.7 μm depth) obtained with an oil immersion 40 $\times$ objective (NA:1,25). d, The graph indicates the intensity ratio between green (GFP) and blue (MT) pixels. Dots within the yellow square, corresponding to colocalization, are indicated in white in the reduced images of GFP and MT fluorescence shown above.

Tracker (MT) CM-H2TMRos, which colocalized with the GFP label in the transformed protoplast and revealed a neighboring nontransformed protoplast (Fig. 2b). The quantification of pixel colocalization confirmed the visual examination, most of the GFP label being associated with MT (Fig. 2d). The presence of some single-labeled areas (GFP or MT) can be explained by cytoplasmic movements between scans and by the occurrence of mitochondria with a lower level of imported GFP or lower mitochondrial membrane potential, since the MT dye is actively sequestered in functioning mitochondria. The superposition of green GFP fluorescence with the red fluorescence of chlorophyll allowed us to clearly visualize plastids, which had obviously not imported the fusion protein (Fig. 2c). Such in vivo import experiments indicate that PsLEAm presequence is able to target a reporter protein selectively to mitochondria. From all these converging data, it can be inferred that PsLEAm is a genuine mitochondrial protein.

Since the proteomic analyses were performed using total soluble proteins, and thus comprise both matrix and intermembrane space, the question of submitochondrial localization of PsLEAm had to be addressed. Intact seed mitochondria, which are normally kept in a medium with 0.6 M mannitol, were suspended in serially diluted media exhibiting increasing osmotic potential. Organelles were then pelleted by centrifugation, and the release of PsLEAm was followed immunochemically in the supernatant together with the activities of fumarase (matrix marker) and adenylate kinase (intermembrane space marker). Clearly, PsLEAm was released together with fumarase. For instance, the protein was not detected in the 0.4 M mannitol condition in which almost one-half of the adenylate kinase activity was released (Fig. 3). It can therefore be concluded that PsLEAm is a matrix mitochondrial protein.

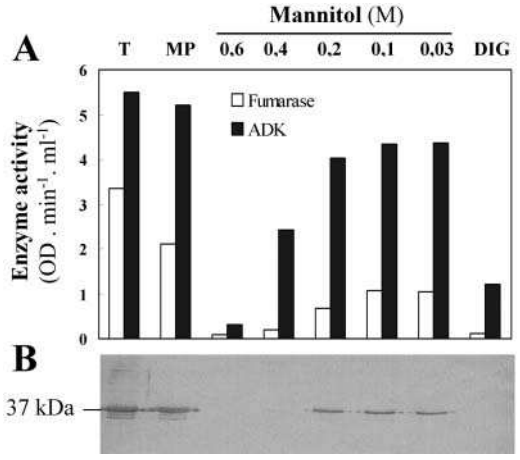

Figure 3. Submitochondrial localization of PsLEAm. Purified intact mitochondria (MP) were osmotically shocked by incubation in different concentrations of mannitol (0.6–0.03 M). Alternatively, the outer membrane was ruptured by a low concentration (200 μg/mL) of digitonin (DIG), or mitochondria were disrupted by the addition of Triton X-100 0.1% (v/v; T). Following the treatments, organelles were pelleted by centrifugation and the supernatants were assayed for marker enzymes activities (section A) or PsLEAm immunodetection (section B). The activities of the matrix enzyme fumarase (white bar) and the intermembrane space adenylate kinase (ADK, black bar) were measured spectrophotometrically, and values (V_{max}) are expressed as absorbance per minute and per milliliter of supernatant.

Expression Profile of PsLEAm

The pattern of expression of PsLEAm was examined during seed development and germination. Pea pods from field-grown plants were harvested from the same nodal stage at different times during ripening, and four seed developmental stages (P1, P2, P3, and P4) were defined according to seed dry weight and water content (Fig. 4A). P1 corresponds to the beginning of seed filling (29 mg dry weight), P2 and P3 to intermediate stages (160 and 239 mg dry weight, respectively), with P4 being a late stage prior to the final desiccation period (242 mg dry weight). The four stages correspond to chlorophyllous green seeds, while the dry mature seeds were yellow, with an average dry weight of 259 mg. The water content of the different stages (Fig. 4A) exhibits the expected drop during maturation and increase during germination. PsLEAm expression was monitored by northern and western blotting at the different developmental stages and during seed germination (Fig. 4A). PsLEAm transcripts, which were faintly detected at stage P2, became very abundant at stages P3 and P4 and remained in the dry seeds. Upon imbibition, the level of transcripts dropped, no signal being detected in late germination. Western-blot analysis revealed that PsLEAm accumulated at the P3 stage and remained almost constant during late development and germination (Fig. 4A). These results indicate that PsLEAm expression is triggered during late seed filling, the protein being stable all along germination and even later since it was still detected in young seedlings after 72 h of imbibition.

To evaluate a possible induction of PsLEAm expression in response to water deficit, 3-week-old pea plants were subjected to water stress for several days, and leaf RNAs were probed with a PsLEAm cDNA by northern blotting (Fig. 4B). The mRNA could not be detected in leaves of watered control plants, in which water content remained in the 90% (fresh weight basis) range over the experimental period (Fig. 4B). However, in stressed leaves where water content gradually decreased, a signal corresponding to PsLEAm transcript was detected after 5 d of stress (Fig. 4B). The level increased for the next 4 d, after which the water content had dropped to 50% fresh weight (Fig. 4B). A western-blot analysis was performed using heat soluble protein extracts from the leaf samples. A sharp signal corresponding to PsLEAm protein was detected after 7 d of water shortage and remained constant afterward (Fig. 4B). In agreement with the transcript

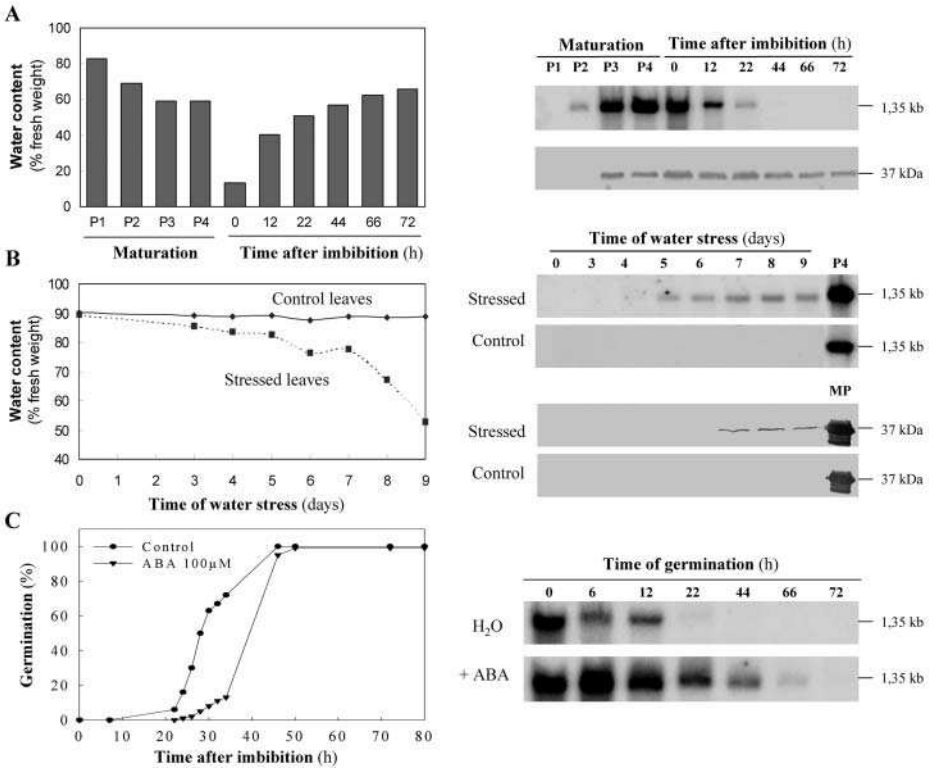

Figure 4. Expression profile of PsLEAm. A, Seed developmental expression. PsLEAm expression was analyzed by northern blot (top) and western blot (bottom) at different developmental stages of pea seed maturation (P1–P4) or during germination with reference to the time of imbibition. Time zero corresponds to the dry mature seed, and radicle protrusion occurred around 24 h of imbibition. The water content (fresh weight basis) of seeds or seedlings is indicated in the bar graph on the left. Northern analysis was carried out with 10 μg of total seed RNA and western-blot analysis with 20 μg of total seed protein extract subjected to heat treatment. B, Water stress-induced expression of PsLEAm in pea leaves. Water stress was triggered by interrupting water supply of 3-week-old pea plants. Water content of control (solid curve) and stressed (dash curve) leaves is indicated on the basis of fresh weight as a function of time on the left graph. Expression of PsLEAm in leaves of stressed and control plants was monitored by northern blot (top) and western blot (bottom). Total RNA (10 μg) was subjected to northern analysis and detected using a full-length PsLEAm probe. A pea seed maturation stage, P4, was included in the northern blot as a positive control. Leaf protein samples (200 μg) and mitochondria (MP) sample (10 μg) were heated at 95°C during 10 min and the soluble fractions were analyzed by western blot. C, ABA-induced expression of PsLEAm during germination. The effect of exogenous ABA upon germination rate is shown in the left graph (black triangle) in comparison with the control (black circle). Expression of PsLEAm transcripts was monitored by northern blotting using 10 μg total RNA. Equal loading of the membranes was verified by methylene blue staining.

analysis, PsLEAm protein was not detected in the watered control plants (Fig. 4B). A two-dimensional PAGE western blot carried with the 8-d stressed leaf extract confirmed the identity of the signal and indicated that the expression level was much lower in stressed leaves than in seeds (data not shown).

Considering the developmental and stress-induced expression of PsLEAm, and since ABA-induced expression is a general (but not exclusive) feature of LEA proteins (Ingram and Bartels, 1996; Cuming, 1999), we investigated whether PsLEAm expression could be elicited by the hormone ABA. When seeds were germinated in the presence of 100 μM ABA, germination was delayed with a T_{50} (time for 50% of germination) of 40 h compared to 28 h for the control (Fig. 4C). A comparative northern-blot analysis revealed that in the presence of ABA, there was a much higher steady-state level of PsLEAm transcript during early imbibition (Fig. 4C). Even when the ABA-treated seeds had finally germinated, the mRNA levels were still high, in marked contrast with the control (Fig. 4C). These observations indicate that the addition of ABA stimulates the expression of PsLEAm transcript.

Protective Effect of PsLEAm upon Mitochondrial Enzyme Dehydration

A recombinant PsLEAm was produced and purified in order to evaluate potential protective effect upon isolated enzymes subjected to drying. Fumarase and rhodanese were selected because they are mitochondrial matrix enzymes. In addition, rhodanese is a classical model for studying chaperone-mediated refolding (Mendoza et al., 1991).

In the designed drying test, the enzyme was mixed with PsLEAm or bovine serum albumin (BSA; as

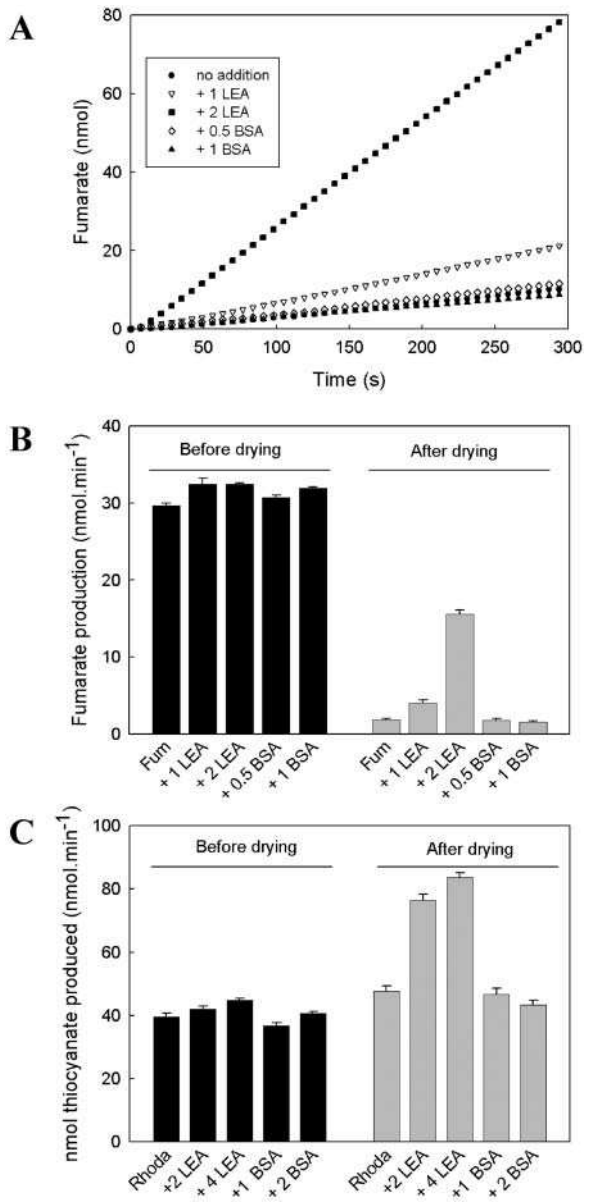

Figure 5. Protective effect of recombinant PsLEAm upon mitochondrial enzymes exposed to dehydration in vitro. A, An example of the kinetics of fumarase activity following overnight drying of the enzyme (250 ng = 2 μL) alone or in the presence of different amounts (125–250 ng) of PsLEAm or BSA as indicated in the insert. B, Summary of the fumarase activities monitored before (black bar) or after (gray bar) drying the enzyme alone or in the presence of different amounts (125–250 ng) of PsLEAm or BSA, in a total volume of 5 μL. The values correspond to the means of seven replicates shown with SES. C, The rhodanese activities monitored before (black bar) or after drying (gray bar) the enzyme (200 ng) alone or in the presence of different amounts (250–500 ng) of PsLEAm or BSA, in a total volume of 2 μL. The values correspond to the means of eight replicates shown with SES.

a control) in a few microliters to either proceed to drying or to a direct enzyme assay. The dried sample was assayed 16 h later. Without drying, neither PsLEAm nor BSA had major effects upon either enzyme (Fig. 5, B and C). After drying, fumarase activity was dramatically decreased by more than 90% (Fig. 5B). However, when the enzyme was dried together with PsLEAm, a protective effect was obvious since up to one-half of the original activity could be retained with the highest concentration of PsLEAm (Fig. 5, A and B). In the same conditions, BSA was unable to provide any protection. Surprisingly, rhodanese activity was found to be slightly increased after drying (Fig. 5C). Nevertheless, PsLEAm provoked a marked stimulation of rhodanese activity after drying (up to 175%), while BSA was ineffective (Fig. 5C). These results indicate that PsLEAm is able to provide beneficial effects upon the two enzymes with respect to desiccation.

DISCUSSION

We report here the first characterization, to our knowledge, of a LEA protein in mitochondria. The occurrence of a LEA protein in pea seed mitochondria was alleged when a previous proteomic analysis pointed out a protein spot exhibiting peptide sequence tags related to LEA-like proteins through data mining (Bardel et al., 2002). However, evidence of contamination of the mitochondrial preparation by other organelles precluded the reliable localization of the protein, a major concern when dealing with organelle proteomics (Brunet et al., 2003; Taylor et al., 2003). Besides, the peptide tag sequence information was not even sufficient to decide upon the LEA protein annotation. To clarify whether such a protein was a genuine mitochondrial LEA protein, we proceeded to its molecular characterization using cDNA cloning. The primary sequence of the protein that was called PsLEAm clearly revealed its LEA protein features and the presence of an N-terminal presequence that markedly diverged from the overwhelming hydrophilic profile of the mature protein. In silico predictions with several programs indicated a high probability of mitochondrial or plastidial localization for PsLEAm. Since mitochondrial and plastidial transit peptides share common features (length and composition) and similar import machineries, their discrimination remains hazardous, and in addition some proteins are found to be dual targeted (Peeters and Small, 2001; Zhang and Glaser, 2002). The difficulty of predicting the mitochondrial localization of proteins was recently highlighted by a thorough examination of the Arabidopsis (*Arabidopsis thaliana*) mitochondrial proteome in which only one-half of the proteins were correctly predicted (Heazlewood et al., 2004). To confirm the subcellular targeting of PsLEAm, a translational fusion of the transit peptide of PsLEAm with S65T-sGFP was transiently expressed in pea leaf protoplasts. The reporter protein appeared clearly localized within mitochondria, indicating that the presequence of PsLEAm was a specific mitochondrial transit peptide. Finally, the submitochondrial localization of PsLEAm revealed the protein was located within the matrix space. Based on the several pieces of evidence resulting from biochemical and molecular

analysis of PsLEAm, we conclude that pea seed mitochondria contain an authentic LEA protein. Although previous reports quoted the cold-increased accumulation of dehydrin-like protein in mitochondria from cereals, neither the nature nor the actual localization of the proteins was demonstrated (Borovskii et al., 2000, 2002). Nevertheless, our work strengthens such observations, and it would be of significant interest to identify stress-related proteins liable to accumulate in mitochondria in response to cold. Another report suggested that HIC6, a 14-kD Chlorella LEA protein, might be localized in mitochondria since the transgenic protein in tobacco (*Nicotiana tabacum*) seemed to cosediment with cytochrome oxidase in linear Suc gradient fractionation (Honjoh et al., 2001).

Structurally, the most notable features of PsLEAm are its enrichment in charged amino acids (40%) and its high predicted content of α-helical structures. Pioneer bioinformatic analysis of the 11-mer motif, which was a signature for group 3 protein, highlighted the amphiphatic properties of the helical structure, the latter of which was expected to serve as a basis for higher order structure (Dure et al., 1989; Dure, 1993). Structure modeling of the tandem repeats of PsLEAm revealed the remarkable conservation of the amphiphatic properties in spite of deletions in the shorter repeats. It is therefore anticipated that such a structure, which spans at least 120 residues, should be a key determinant for the function of the protein.

Expression analysis of PsLEAm revealed a typical seed LEA profile with strong expression peaking during late maturation, and hence PsLEAm can be considered as a canonical LEA protein (Wise, 2003). The expression of PsLEAm was found to occur, albeit at very low levels, in response to severe dehydration in pea leaves. Such a correlation between PsLEAm expression and developmental or stress-induced desiccation raised the question of ABA signaling with regard to PsLEAm expression. Like many drought-induced genes, a number of LEA protein are known or expected to respond to the plant stress hormone ABA (Ingram and Bartels, 1996; Cuming, 1999), and LEA protein mRNAs accordingly showed up in the transcriptome of ABA-treated plants (Seki et al., 2002). However, it must be stressed that the ABA response involves complex and interacting signaling pathways (Finkelstein and Rock, 2002), and, accordingly, LEA protein expression in response to ABA appeared not uniform and varied for individual members of the LEA family (Hughes and Galau, 1989; Parcy et al., 1994; Delseny et al., 2001).

Exogenous ABA was found to markedly stimulate PsLEAm transcript accumulation during early imbibition, suggesting a possible control by ABA.

The expression profile of PsLEAm and its subcellular localization is circumstantial but compelling evidence that the protein is involved in desiccation protection of seed mitochondria. Such a trait is expected since germination is an energy-demanding process that requires mitochondria to perform their bioenergetic and metabolic roles as early as possible to sustain cell metabolism. Desiccation tolerance is a complex trait that involves at least a down-regulation of metabolism, the immobilization of cytoplasm by vitrification, and the stabilization of macromolecules and membranes (Hoekstra et al., 2001). While seed mitochondria can be expected to benefit from cytosolic protective systems, the accumulation of PsLEAm would participate in protection within the matrix space. It should be stressed that protective sugars that play a major role in cytosol stabilization are normally not present within mitochondria. The question of the molecular function of PsLEAm can be addressed only in the context of various hypothetical functions of LEA proteins that include maintenance of water shells, ion sequestration, and membrane and macromolecule stabilization (Cuming, 1999). We have shown here that recombinant PsLEAm had a beneficial activity upon two mitochondrial enzymes that were subjected to complete drying. Fumarase appeared very sensitive to drying, which may be related to the tetrameric state of the enzyme that could be disrupted during dehydration. PsLEAm, which provided a significant protection of enzyme activity, might help in preserving the quaternary structure of fumarase. In marked contrast with fumarase, the activity of rhodanese, a monomer, was not affected by drying. However, drying rhodanese together with PsLEAm resulted in an increase in enzyme activity. To explain such an effect, one should consider that not all rhodanese molecules are potentially active in the original enzyme solution. Such a hypothesis is conceivable since rhodanese exhibits a two-independent-domain structure that is especially prone to misfolding (Mendoza et al., 1991). In this context, PsLEAm would act during drying by favoring the correct folding of inactive rhodanese molecules. The observed stimulation of both enzyme activities by PsLEAm implies a direct interaction at the protein-protein level that does not occur in the hydrated state but is favored during dehydration when protein concentration increases. Interestingly, it has been shown that the folding of a nematode LEA protein was induced by desiccation (Goyal et al., 2003), which implies that such a protein is functional at low water content. The nature of the molecular mechanism that results in the observed effects of PsLEAm remains to be established and will require thorough biochemical and biophysical studies of protein-protein interactions.

To our knowledge, this is the first report of enzyme protection by a LEA protein during total dehydration. Protective effects of LEA proteins upon enzymes has often been demonstrated using lactate dehydrogenase freezing assays (Bravo et al., 2003). However, Crowe et al. (1990) have pointed out that freezing and dehydration were very different stress vectors since only the latter is able to completely remove the nonfreezable water near macromolecules. For these reasons, the enzyme-drying assay seems adequate to mimic the water conditions expected during seed desiccation.

The high proportion of charged amino acid residues in PsLEAm would favor a role in ion sequestration or buffering during dehydration. Experimental evidence

supporting a role of LEA protein members in ion binding was recently reported. An iron-binding protein in the phloem of castor bean was identified as a LEA protein (Krüger et al., 2002), and the ionic-binding properties of ERD14, an Arabidopsis dehydrin (group 2 LEA protein), were recently demonstrated and shown to depend upon phosphorylation, suggesting a role in calcium buffering (Alsheikh et al., 2003). However, in the case of PsLEAm, such an ion-buffering role is unlikely to explain the protective effect upon dehydration of enzymes, since the experiments were carried out in salt-free dilute solutions.

Another interesting hypothesis based on computational analysis of LEA proteins suggested a structural role reminiscent of microfilaments for helical LEA proteins liable to form coiled-coils (Goyal et al., 2003; Wise, 2003; Wise and Tunnacliffe, 2004). Seed desiccation and rehydration is accompanied by important changes in volume, also likely to involve mitochondria, and the reinforcement of the organelle structure by a filamentous network might be an asset. Although two regions of PsLEAm could be predicted as potential coiled-coil motifs, it will be essential to provide experimental evidence in support of the hypothesis.

The discovery of a LEA protein in pea seed mitochondria raises the question of its species distribution and evolutionary origin. Although we have no proof of the presence of orthologs of PsLEAm in other seeds, a number of plant proteins annotated as LEA proteins in databases, and which exhibited sequence and hydrophilicity profile similarities with PsLEAm, could be predicted as putative mitochondrial proteins (data not shown). While this suggests a distribution in the plant kingdom, it is noteworthy that PsLEAm was classified in the only Pfam family that also includes LEA proteins from nonplant kingdoms, namely animals, fungi, protists, and bacteria. Owing to the early endosymbiotic origin of mitochondria (Emelyanov, 2003) and to the probable coincidence of the evolution of desiccation tolerance with land colonization by primitive plants, it will be of interest to decipher the evolutionary history of PsLEAm.

In conclusion, PsLEAm appears as the first LEA protein identified in mitochondria from any organism. Its pattern of expression and its reported activity suggest that PsLEAm could participate in the stabilization of mitochondrial matrix proteins in the dry state and hence contribute to desiccation tolerance of the seed.

MATERIALS AND METHODS

Plant Material

Pea (*Pisum sativum*) L. cv Baccara seeds were grown locally by the agronomical research institute Fédération Nationale des Agriculteurs Multiplicateurs de Semences, Brain-sur-l'Authion (France) and stored in sealed plastic bags at 5°C (70% relative humidity). Seeds at different stages of maturation were collected in the field, flash frozen in liquid nitrogen, and stored at −80°C until use. Seed germination assays were carried out in the dark at 20°C as described by Benamar et al. (2003). Mature plants were grown in trays (46 × 29 × 8 cm) of compost in a growth cabinet (16-h/8-h photoperiod at 250 μmol photons m^{-2} s^{-1}, 22°C/18°C) and were watered every day. Water stress was initiated by interrupting the water supply of 3-week-old plants, and leaves were collected from 0 to 9 d of stress. Samples were flash frozen in liquid nitrogen and kept at −80°C until use. For ABA treatment, the germination assays were carried out with 100 μM ABA (Sigma-Aldrich, St. Louis). Water content of seeds or plant tissues was measured by the difference between fresh and dry mass (24 h at 100°C) and expressed on the basis of fresh weight.

cDNA Cloning of PsLEAm

The presence of a LEA-like protein in pea seed mitochondria was suggested by a previous proteomic analysis (Bardel et al., 2002). An RT-PCR strategy was developed to clone a cDNA corresponding to the target polypeptide for which N-terminal and internal peptide sequence tags had been obtained. The oligonucleotides (pPsLEAm-a, 5′-GGCATGCGGCCGCTTCGGCNGCNGAYGGNAARMG, and pPsLEAm-b, 5′-GGCAGCGTCCGTGACGGACYTCNCCNGTNGTYTC), derived, respectively, from the peptide sequences AADGKR and ETTGEV, were tailored with GC rich sequences (underlined) to increase the specificity of amplification. Total RNA (2 μg) from dry seeds was reverse transcribed with M-MuLV reverse transcriptase (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. The PCR reactions were carried out with 2 μL of the cDNA mixtures as templates in PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9, 0.1% [v/v] Triton X-100, 1.5 mM $MgCl_2$) containing dNTPs (100 μM each), specific primers (10 pmol each), 0.5 units Taq DNA polymerase (Promega, Madison, WI), and water to 20 μL. After a preliminary denaturation step (4 min, 94°C), amplifications were performed on a Mastercycler gradient thermocycler (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) according to the following scheme: 1 min, 94°C; 2 min, 40.5°C; 2 min, 72°C for 5 cycles and 20 s, 94°C; 1 min, 62°C; 2 min, 72°C for 30 cycles, followed by a final elongation step (10 min, 72°C). The analysis by agarose gel electrophoresis revealed the presence of a main fragment (700 bp) that was cloned using the TOPO TA Cloning kit for sequencing (Invitrogen, Groningen, The Netherlands). The 5′ and 3′ ends of the cDNA were obtained by RNA-ligated mediated RACE using specific internal primers and kit primers (GeneRacer kit; Invitrogen) according to the manufacturer's instructions using a previously PCR-generated cDNA library (Duval et al., 2002). To confirm the sequence of the full-length cDNA encoding PsLEAm protein, a final RT-PCR experiment was carried out to amplify a 1,100-bp DNA fragment that was cloned into the plasmid pCR4-TOPO to yield the recombinant plasmid pTOPLEAm, which was transformed into TOP10 *Escherichia coli* (Invitrogen). Publication grade sequencing of the insert (MWG-Biotech AG, Ebersberg, Germany) yielded the sequence of the full-length cDNA encoding PsLEAm. The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number AJ628940.

Bioinformatic Analyses

Sequence similarities were examined with the GenBank/EMBL database using the BLAST program. A hydropathy plot was established with the ProtScale program (http://au.expasy.org/tools/protscale.html) using the Kyte and Doolittle (1982) algorithm. Secondary structure analysis was performed with the GOR IV (Garnier et al., 1996) and the PHDsec (Rost and Sander, 1993) programs available through the Expasy server (http://au.expasy.org). Helical wheel models of the PsLEAm repeats were generated with the Swiss-PdbViewer (Guex and Peitsch, 1997; http://www.expasy.org/spdbv/) by threading the primary sequences onto the three-dimensional template of filamin (PDB accession no. 1GK7). Subcellular localization of PsLEAm was predicted using the following four programs: TargetP (Emanuelsson et al., 2000; www.cbs.dtu.dk/services/TargetP), PREDOTAR version 0.5 (www.inra.fr/predotar/), PSORT (psort.nibb.ac.jp/form.html.), MitoProt (Claros and Vincens, 1996; http://ihg.gsf.de/ihg/mitoprot.html). Scanning for coiled-coil regions was performed with the COILS program, version 2.2 (Lupas, 1996; www.ch.embnet.org/software/coils/COILS_doc.html).

RNA Isolation and Northern-Blot Analysis

Pea samples were pulverized under liquid N_2 and total RNA was extracted from powder as described (Verwoerd et al., 1989). Northern blotting was

performed with 10 μg glyoxal-denaturated total RNA separated on 1.2% agarose gel. The gel was blotted onto a Hybond-N membrane (Amersham Biosciences, Piscataway, NJ), and membrane prehybridization and hybridization were performed according to manufacturer's instructions. The PsLEAm-specific probe was the full-length PCR-generated cDNA that was ^{32}P-radiolabeled using Rediprime kit (Amersham Biosciences). A pea 18S rRNA probe (427 pb) was used to check for equal loading. Blots were exposed to a phosphor screen (Eastman Kodak Company, New York) and analyzed using a phosphoimager (Molecular Imager FX Pro; Bio-Rad, Hercules, CA).

Transient Expression of GFP Fusion Protein

The nucleotide sequence coding the N-terminal putative transit peptide and cleavage site (residues 1–49) of PsLEAm (TP49LEAm) was fused using recombinant techniques in frame with a modified GFP harbored in the reporter plasmid described by Chiu et al. (1996). The resulting Pro_{35S}:TP49LEAm:sGFP(S65T) plasmid was then used for protoplast transformation.

Leaves (2 g) of 3-week-old plants, sliced into 1-mm-wide strips, were infiltrated under vacuum in the protoplast isolation medium (0.5 M sorbitol, 5 mM 2-[*N*-morpholino] ethane-sulfonic acid, pH 6.2, 1 mM $CaCl_2$, 1% [w/v] Suc, 1% [w/v] polyvinylpyrolidone-40) containing 1% (w/v) Cellulase RS (Kyowa Chemical Products, Osaka) and 0.3% (w/v) Pectolyase Y23 (Seishin Corporation, Tokyo). After incubation for 2 h at 22°C in the light, protoplasts were filtered through Miracloth (CalBiochem, San Diego) and washed twice by gentle centrifugation (10 min, 80 g) in isolation medium. Protoplast polyethylene glycol-mediated transformation was carried out with 20 μg of reporter plasmid following the method of Abel and Theologis (1994). Prior to microscopy observation, protoplasts were incubated for 15 min with 50 nM fluorescent dye MitoTracker Orange (CM-H2TMRos, Molecular Probes, Eugene, OR). Confocal laser-scanning microscopy was performed using a Leica TCS-SP2 operating system (Leica, Deerfield, IL). GFP (S65T-sGFP), MT, and chlorophyll were excited and fluorescences were collected sequentially (400 Hz, line by line), by using the 488-nm line of an Ar laser for GFP, the 543-nm line of an He-Ne laser for MT, and the 633-nm line of another He-Ne laser for chlorophyll. Fluorescences were collected between 498 nm and 533 nm, 553 nm and 607 nm, 645 nm and 704 nm, for GFP, MT, and chlorophyll, respectively.

Submitochondrial Localization

Pea seed mitochondria with high respiratory control and more than 95% integrity of outer membrane were isolated after 22 h of imbibition according to Benamar et al. (2003). To test the submitochondrial localization of PsLEAm, mitochondria were subjected to an osmotic shock or detergent treatment followed by western-blot analysis. Mitochondria (500 μg proteins) were diluted into osmotic shock medium (20 mM MOPS, pH 7.5, 2 mM EDTA, and either 0.6 M, 0.4 M, 0.2 M, 0.1 M, or 0.03 M mannitol) to adjust the osmotic potential. Digitonin (200 μg/mL) was used to specifically rupture the outer membrane and release intermembrane space proteins. Triton X-100 (0.1% v/v) was added to trigger the lyses of mitochondria and release intermembrane space and matrix proteins. After 15 min of incubation on ice, organelles were pelleted by centrifugation (10,000*g*, 10 min) and supernatants subjected directly to enzyme assays using a SpectraMAXPlus microplate reader (Molecular Devices, Sunnyvale, CA). Fumarase, a matrix enzyme, was assayed at 250 nm by monitoring the production of fumarate in a medium containing 50 mM Tricine, pH 7.5, 0.1% (v/v) Triton X-100, and 50 mM malate as a substrate. Adenylate kinase, an intermembrane space enzyme, was assayed at 340 nm (NADPH production) in a medium containing 50 mM Tris, pH 8.0, 15 mM Glc, 5 mM $MgCl_2$, 5 mM KCN, 3 mM ADP, 0.75 mM $NADP^+$, 0.5 units/mL Glc-6-P dehydrogenase, 4 units/mL hexokinase. The presence of PsLEAm in the heat-soluble fraction of the 14,000*g* supernatant was examined by western blot as described below.

Protein Extraction and Western-Blot Analysis

Samples pulverized under liquid N_2 were homogenized in cold extraction buffer (50 mM Tris-HCl, pH 7.8, 2 mM EDTA, 0.1% [v/v] Triton X-100), and the slurry was clarified by centrifugation at 15,000*g*, 4°C for 20 min. To take advantage of the heat stability of PsLEAm, supernatant was heated at 95°C for 10 min, cooled on ice, and centrifuged at 14,000*g* for 15 min at 4°C. For seed samples, supernatants were directly subjected to SDS-PAGE according to standard protocols using 13.5% (w/v) polyacrylamide gels and a Tris/Tricine split buffer system. For leaf samples, heat-soluble proteins were precipitated by the addition of 5 V of cold acetone and incubation for 30 min at −20°C. After centrifugation (14,000*g*, 10 min), the pellets were suspended in 20 mM Tris-HCl, pH 7.5 and subjected to SDS-PAGE. The immunodetection was performed according to Duval et al. (2002) with a rabbit anti-PsLEAm serum raised against a C-terminal peptide (KGYGENKGYDQNRGY; dilution 1:20,000; Neosystem, Strasbourg, France). Immunodetection was performed by adding to the blot 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium Blue Liquid Substrate System for Membranes (Sigma-Aldrich). The anti-PsLEAm antibodies appeared specific for PsLEAm since they did not react with other LEA proteins that were abundant in the seed protein extracts.

Production of Recombinant PsLEAm

A synthetic cDNA encoding the mature PsLEAm was cloned into the expression vector pETY-3a (Sauge-Merle et al., 1999). The engineered vector was used to drive recombinant protein expression into *E. coli* BL21-AI (Invitrogen). The actual expression of the recombinant PsLEAm was confirmed by peptide mass fingerprinting of the overexpressed spot appearing in two-dimensional PAGE analysis of induced-bacteria extract (data not shown). Cells from 1.5-L cultures were concentrated by centrifugation (4,000*g*, 20 min) and suspended in Tris-EDTA (TE) buffer (Tris-HCL 20 mM, pH 7.4, EDTA 1 mM). The suspension was subjected to two freeze-thaw cycles and then centrifuged (100,000*g*, 1 h) at 4°C. The soluble fraction was brought to 50% saturation with ammonium sulfate and stirred on ice for 1 h. After 15 min centrifugation (14,000*g*), the pellet was suspended in TE buffer and dialyzed overnight at 4°C in the same buffer. The protein extract was subjected to anion-exchange chromatography on a MonoQ 5/50 GL column connected to an AKTA Purifier system (Amersham Biosciences). After loading and washing with five-column volumes of TE buffer, a two-step NaCl gradient (0–0.2 M in 36 mL/0.2 −0.5 M in 16 mL) was developed with a flow rate of 1 mL min^{-1}. The recombinant PsLEAm was detected by western blotting and found to elute at low salt concentrations (150 mM). The fractions containing PsLEAm were pooled, dialyzed, and concentrated by ultrafiltration using Amicon Ultra-15 concentrator (Millipore, Bedford, MA). The procedure yielded 175 μg of protein, which appeared pure when 5 μg were visualized after SDS-PAGE and Coomassie Blue staining.

Dehydration Protection Assays

Fumarase from porcine heart and rhodanese from bovine liver were purchased from Sigma-Aldrich. Fumarase was dialyzed against 20 mM Tris, pH 8.0, to remove ammonium sulfate. The principle of the dehydration assay was to mix the enzymes with known amounts of PsLEAm or BSA before spotting equal volumes (2 μL for fumarase assay and 5 μL for rhodanese assay) into the wells of two different microplates. One microplate was immediately used to monitor enzyme activity, while the other was placed in a box containing desiccated Silicagel and kept under vacuum for 16 h at room temperature in the dark. Fumarase activity was measured at 30°C as described above, except that both control and dried protein mixtures were preincubated with 270 μL of fumarase reaction buffer at 30°C for 10 min before starting the reaction by addition of malate. Rhodanese activity (thiosulfate sulfurtransferase) was measured with both control and dried protein mixtures (without preincubation) at 30°C according to Papenbrock and Schmidt (2000) in a final volume of 250 μL/well. After 10 min of incubation, the reaction was stopped by addition of the acidic iron reagent and thiocyanate determined using a calibration curve.

Distribution of Material

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AJ628940.

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