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12 November 1987; accepted 28 February 1988

Aggregation of Lysine-Containing Zeins into Protein Bodies in *Xenopus* Oocytes

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Zeins, the storage proteins of maize, are totally lacking in the essential amino acids lysine and tryptophan. Lysine codons and lysine- and tryptophan-encoding oligonucleotides were introduced at several positions into a 19-kilodalton zein complementary DNA by oligonucleotide-mediated mutagenesis. A 450–base pair open reading frame from a simian virus 40 (SV40) coat protein was also engineered into the zein coding region. Messenger RNAs for the modified zeins were synthesized in vitro with an SP6 RNA polymerase system and injected into *Xenopus laevis* oocytes. The modifications did not affect the translation, signal peptide cleavage, or stability of the zeins. The ability of the modified zeins to assemble into structures similar to maize protein bodies was assayed by two criteria: assembly into membrane-bound vesicles resistant to exogenously added protease, and ability to self-aggregate into dense structures. All of the modified zeins were membrane-bound; only the one containing a 17-kilodalton SV40 protein fragment was unable to aggregate. These findings suggest that it may be possible to create high-lysine corn by genetic engineering.

THE SEED STORAGE PROTEINS OF CEREALS, prolamines, are noted for their high content of proline and glutamine and the virtual absence of lysine. Since prolamines account for approximately half of the total seed protein, cereals are generally deficient in lysine. Previously, the only means by which this essential amino acid could be increased was by selecting strains with low prolamine content (*1*); however, the softer kernels and smaller yields of such strains have limited their usefulness (*2*). Since genes encoding storage proteins with higher lysine contents do not normally exist in cereals, an alternative to conventional plant breeding is to modify existing genes in vitro by genetic engineering and to express them in transgenic plants (*3*).

The prolamines of corn, *Zea mays* L., are known as zeins (*4*). These proteins perform no known enzymatic function and thus appear to be ideal candidates for amino acid modification by genetic engineering. The three types of zeins (α, β, and γ) (*5*) are synthesized on rough endoplasmic reticulum and aggregate within this membrane in dense deposits called protein bodies. The mechanisms responsible for protein body formation are thought to involve hydrophobic and weak polar interactions between zeins (*6*). The absence of lysine in these proteins may reflect the fact that charged amino acids would adversely affect protein aggregation and thus the formation of normal protein bodies.

Earlier studies (*7–9*) demonstrated that injection of zein messenger RNA (mRNA) into *Xenopus* oocytes results in the synthesis and processing of zein proteins into membrane-enclosed structures with the physical characteristics of protein bodies from maize. With this system it is possible to study the consequences of lysine substitutions and other modifications to zein proteins on their ability to form protein bodies. We have assayed two properties relating to the assembly of modified zeins into protein bodies in oocytes: (i) transport into membrane-bound structures and (ii) aggregation into vesicles sedimenting with a high density. Surprisingly, we find that only the most severe modification of a zein protein hinders its ability to form protein bodies.

Figure 1 shows the amino acid sequence of a 19-kD α zein arranged in a manner that corresponds to the structural domains proposed by Argos *et al.* (*6*); the indicated protein changes were made from three types of modifications to the corresponding complementary DNA (cDNA) clone. Single and double amino acid substitutions incorporate lysines into several positions in place of neutral amino acids; oligopeptide insertions place lysine- and tryptophan-rich peptides within the zein molecule; and the large peptide insertion, pMZ44-SV40 (simian virus 40), places 17 kD of an unrelated, hydrophilic protein (from the SV40 VP2 protein) into the NH_2-terminal region.

In order to synthesize the modified zeins and assay their ability to form protein bodies, we produced artificial mRNAs for them in vitro by using SP6 RNA polymerase. These mRNAs were injected into *Xenopus* oocytes, and the proteins were labeled by subsequent injection of [^{3}H]leucine. After fractionation, the zeins were extracted and subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Amino acid–substituted zeins were detected, and their molecular size was the same (migration in SDS-PAGE) as that of the unmodified protein. The signal peptides of zeins are correctly removed in *Xenopus* oocytes (*7*), and this is also true of the single and double lysine-substituted zeins. The actual incorporation of lysines into the modified zeins was independently confirmed by isoelectric focusing (IEF) (*10*). We conclude that the introduction of lysine into various positions in the molecule does not significantly affect its synthesis, stability, or the cleavage of its signal peptide. Injection of RNAs for peptide insertion mutants and the pMZ44-SV40 zein similarly resulted in production of stable proteins of the expected molecular size.

In that the modified zeins were faithfully synthesized and processed in oocytes, we next examined their assembly into protein bodies. Hurkman *et al.* (*8*) showed that total zein mRNA injected into oocytes can direct the synthesis and assembly of zeins into dense, membrane-bound structures similar to maize protein bodies.

The appearance of zein in membrane-bound organelles was assayed by the protein's resistance to proteolytic digestion. A wild-type 19-kD zein was synthesized in oocytes by injection of synthetic mRNA, and the homogenized eggs were treated with protease K. The zein was resistant to digestion with protease (up to 1 mg/ml) unless 1% Triton X-100 was present, in which case it was completely digested (Fig. 2A). To demonstrate that this resistance is the result of internalization within membranous structures and not merely a consequence of hydrophobic interactions with membranes, we performed the following experiment (Fig. 2B). Purified, labeled zein

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was added to an oocyte homogenate and treated with protease K. In this case the zein was sensitive to protease even in the absence of detergent.

To show the effect of a major alteration to a zein on its ability to be transported into membranes, we coinjected oocytes with RNAs for the wild type, the highly modified zein (pMZ44-SV40), and human β-globin. Whereas the cytosolic globin protein was readily degraded by protease, both the wild-type and modified zeins were resistant unless detergent was present (Fig. 2C). Thus, even the gross modification apparently does not hinder the transport of zein into membrane vesicles. Similar results were obtained for the other, less severely modified zein constructs. Probably the signal peptide can alone target zeins to the endoplasmic reticulum, where sequences in the protein itself direct assembly into a protein body.

We also assessed protein body formation by isopycnic banding of oocyte-generated protein bodies in density gradients. Oocyte homogenates were separated into various components by centrifugation on metrizamide gradients (top of Fig. 3) (*8*). These were fractionated as shown, and the zeins were extracted and analyzed by SDS-PAGE. A large portion of the zeins produced by injection of native zein mRNA bands at a density corresponding to that of maize protein bodies (Fig. 3A) (*8*). Some of the radioactive zein that sediments near the top of the gradient is associated with membranes and probably arises from the rupture of vesicles during homogenization. When SP6 mRNA encoding the 19-kD zein was injected alone, a significant proportion of the protein was also found in vesicles that sedimented far into the gradient (Fig. 3B). Purified zein added to homogenized oocytes will not band at a high density (Fig. 3C), showing that it is not passive association of zein with dense oocyte particles that produces the above results. Thus the presence of all types of zeins does not appear to be necessary for the formation of a protein body; a single α-type zein will aggregate into a dense structure. Although the protein bodies formed from the α zein alone are not as dense as those containing the full complement of zeins, they are of sufficient density to indicate that proper aggregation has taken place. The ability of pure α zein to assemble into a protein body is consistent with the structural model of Argos *et al.* (*6*). The other types of zeins may increase the density of a protein body, perhaps by regulating its size and thus changing the ratio of protein to lipid (*9*).

Single and double lysine substitution mutants (Fig. 3, D and E) and peptide insertion mutants (Fig. 3, F and G) all gave sedimen-

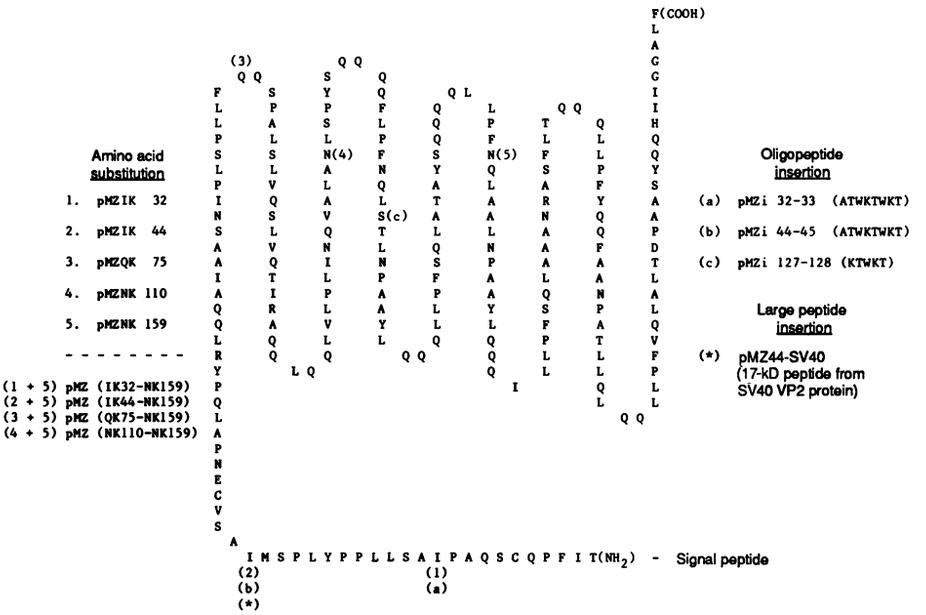


Fig. 1. Modified zein constructs showing the positions of amino acid substitutions, oligopeptide insertions, and a large peptide insertion made in a mature zein amino acid sequence. The sequence corresponds to the 19-kD zein cDNA clone cZ19c1 (*12*). It is laid out according to the proposed secondary structure of Argos *et al.* (*6*), where the parallel regions represent α helix and the unpaired section the NH_2-terminal turn region. The signal peptide of 21 amino acids is not shown. The names of the constructs (at the sides of the figure) indicate the sites of the substitution or insertion (amino acid number is that of the preprotein) and the amino acids substituted or inserted. Amino acid substitution mutants were synthesized by oligonucleotide-mediated mutagenesis (*13*). They have been positioned in the long NH_2-terminal "turn" region, in the glutamine-rich turns between the α helices, and within the α helices themselves. The mutagenesis was designed such that the one nucleotide change resulted in the formation of a new restriction site in addition to a lysine codon. Thus, double-stranded DNA preparations could be screened by restriction enzyme digestion to obtain the mutant clones. The base changes were further confirmed by DNA sequence analysis. Peptide and protein insertion mutants were created by the cloning of double-stranded DNA fragments into restriction sites in the zein coding region (either natural or the ones created). For the peptide insertions, synthetic oligonucleotides were inserted to give the amino acid sequences indicated. For the protein insertion, a 447-bp Hind III fragment of the SV40 VP2 coat protein (positions 1046–1493) was cloned into the Hind III site created in amino acid substitution mutant pMZIK44. The modified zein constructs were subcloned into the vector pSP64 containing a zein 3′ polyadenylated sequence derived from a zein cDNA clone (*10*). Capped mRNA was synthesized as described by Galili *et al.* (*14*).

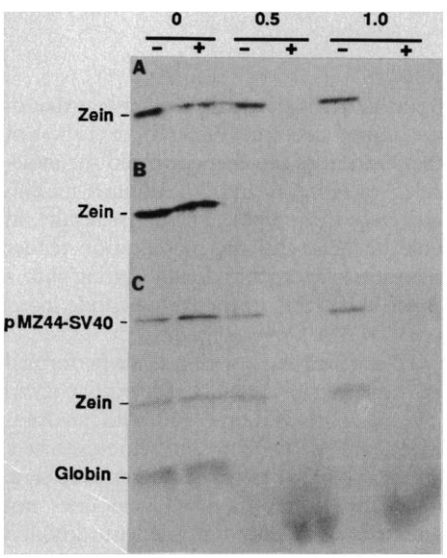


Fig. 2. Protease treatment of oocyte protein bodies. (**A**) SP6 mRNA (5 to 10 ng) was injected into each of four stage 6 oocytes. After 24 hours 1.1 μCi of [^{3}H]leucine (New England Nuclear, 2.2 Ci/mmol) was injected per oocyte. After a 4-hour labeling period, they were resuspended in 300 μl of buffer B [20 m*M* tris-HCl (*p*H 7.6), 50 m*M* KCl, 10 m*M* $MgCl_2$, 300 m*M* NaCl, 2 m*M* EDTA, and 10% sucrose] and then homogenized by passage ten times through the tip of a 200-μl micropipetter. The homogenate was divided into six equal parts, three of which were made 1% in Triton X-100. Samples with and without detergent were digested with 0, 0.5, or 1.0 mg of protease K per milliliter for 30 minutes at 4°C. Reactions were stopped by the addition of phenylmethylsulfonyl fluoride to a concentration of 1.0 mg/ml, and zeins were extracted by addition of 120 μl of ethanol containing 1% β-mercaptoethanol (β-ME) and incubated for 15 minutes at 65°C. After centrifugation for 5 minutes, the supernatants were evaporated to dryness, resuspended in sample buffer, and subjected to SDS-PAGE and fluorography. (**B**) Unmodified ^{3}H-labeled zein was extracted from four oocytes as described above, then resuspended in 20 μl of 70% ethanol containing 1% β-ME. This was added, while vortexing, to four uninjected oocytes homogenized in 300 μl of buffer B. Treatments with protease and SDS-PAGE followed. (**C**) Same as for (A) except that oocytes were coinjected with SP6 mRNAs for human β-globin, wild-type zein, and pMZ44-SV40 zein. (Human β-globin is soluble under these conditions.)

Fig. 3. Densities of protein bodies from oocytes. Eight to 12 oocytes were injected and treated, as described for Fig. 2, with only 250 μl of buffer B. The extracts were layered on 4 ml of 10 to 50% gradients of metrizamide in buffer B containing 15% sucrose. The samples were centrifuged for 18 hours at 45,000 rpm at 4°C in a SW60Ti rotor. The banding positions of oocyte components were clearly visible and are shown in the drawing (L, lipids; E, endomembranes; M, mitochondria; YP, yolk platelets) (*7*). Fractions (0.4 ml) were collected, and zeins were extracted by adding 0.7 ml of ethanol containing 1% β-ME as for Fig. 2. Unlabeled zein (100 μg) was added to each sample, which was then evaporated to near dryness. The residue was dissolved in 1.5 ml of 0.03*M* sodium acetate, *p*H 5.3, and left overnight at 4°C. The water-insoluble zeins were sedimented by microcentrifugation for 15 minutes at 4°C. The pellets were taken up in sample buffer and subjected to SDS-PAGE and fluorography. Injection of: (**A**) native zein mRNA; (**B**) SP6 mRNA for wild-type 19-kD zein; (**C**) exogenous ^{3}H-labeled zein added to oocyte homogenate as in (B); (**D**) pMZIK 32 mRNA; (**E**) pMZ (NK110–NK159) mRNA; (**F**) pMZi 32–33 mRNA; (**G**) pMZi 127–128 mRNA; (**H**) pMZ44-SV40 mRNA. The sizes of the zeins in (A) are shown; the migration of all others was equivalent at 19 kD except for that of the pMZ44-SV40 zein (H), which was 35 kD.

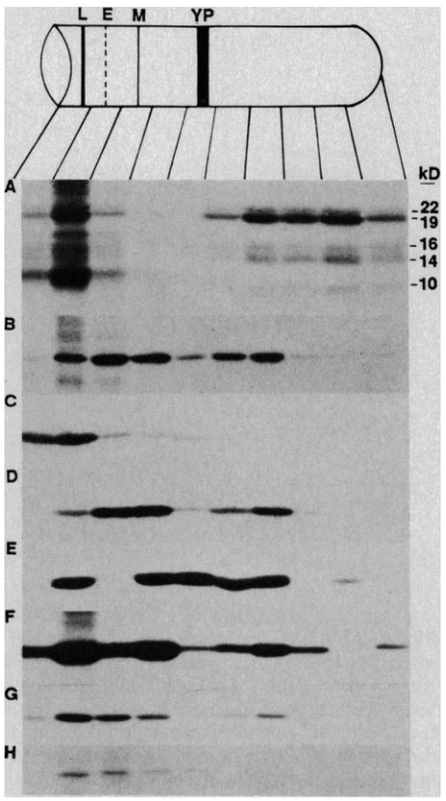
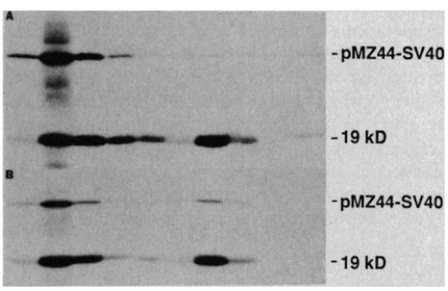


Fig. 4. Density of hybrid protein bodies. Sample processing was as described for Fig. 3. (**A**) Separate batches of oocytes injected with mRNAs for either the unmodified 19-kD zein or the pMZ44-SV40 construct were pooled and then homogenized and fractionated together. (**B**) Oocytes were coinjected with both wild-type and pMZ44-SV40 mRNAs.



tation patterns essentially the same as that of the unmodified zein (Fig. 3B), regardless of the position of the change within the molecule (see Fig. 3, F and G). All other modifications of these types gave similar results. In only one case did the modification render the protein incapable of aggregating into a dense body: that of the large peptide insertion pMZ44-SV40 (Fig. 3H).

To confirm the last result, we performed an experiment in which separate oocytes injected with wild-type and with pMZ44-SV40 RNAs were mixed, homogenized, and fractionated in the same gradient (Fig. 4A). The larger, modified zein does not sediment deep into the gradient although the normal one does. Surprisingly, when wild-type and pMZ44-SV40 RNAs were coinjected (Fig. 4B), some of the altered zein bands at a high density. It appears that the modified zein can interact with the wild-type protein and assemble into protein bodies. The SV40 protein is inserted into a portion of the molecule believed not to interact directly with other zeins to form a tight complex (*6*) (Fig. 1). Perhaps the smaller, wild-type molecules are able to interact with the α-helical repeats of the larger molecules and allow aggregation.

Although a frog oocyte certainly does not have the same intracellular environment as maize endosperm, we have shown that it can translate and package wild-type and modified α zeins into protein bodies. The observation that a single, cloned α zein can assemble into protein bodies similar to those that contain several types of zeins supports a simple model of protein body formation (*5*). The β and γ zeins apparently are not necessary for the aggregation in vivo of the α types. This is not too surprising since the α zeins are generally found at the surfaces of protein bodies (*9, 11*).

In that neither lysine nor tryptophan has been found in any of the several zeins whose sequences are known, we were surprised to discover that introduction of as many as two lysines and two tryptophans has no major effect on α zein aggregation, even when inserted into a region thought to be important to zein tertiary structure. Only a zein with an insertion of 17 kD of foreign protein was unable to assemble into protein bodies. This augurs well for the possibility of eventually expressing high-lysine zeins, or perhaps other useful proteins, in the seeds of maize plants.

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15. We thank J. S. Lindell and C. Logan for help in the preparation of this manuscript and L. D. Smith for *Xenopus* oocytes and facilities for microinjection. Supported by a grant from Lubrizol Genetics, Inc. (B.A.L.), NSF postdoctoral fellowships in plant biology (J.C.W. and M.A.S.), and a fellowship from the Weizmann Institute of Science, Israel (G.G.). This is journal paper 11,362 of the Purdue Agriculture Experiment Station.

29 October 1987; accepted 16 February 1988