Role of Posttranslational Cleavage in Glycinin Assembly

Craig D. Dickinson, Ebtissan H. A. Hussein,* and Niels C. Nielsen

United States Department of Agriculture/Agricultural Research Service, Department of Agronomy, Purdue University, West Lafayette, Indiana 47907

Glycinin, like other 11S seed storage proteins, undergoes a complex series of posttranslational events between the time proglycinin precursors are synthesized in endoplasmic reticulum and the mature glycinin subunits are deposited in vacuolar protein bodies. According to the current understanding of this process, proglycinin subunits aggregate into trimers in endoplasmic reticulum, and then the trimers move to the vacuolar protein bodies where a protease cleaves them into acidic and basic polypeptide chains. Stable glycinin hexamers, rather than trimers, are isolated from mature seeds. We used a re-assembly assay in this study to demonstrate that proteolytic cleavage of the proglycinin subunits is required for in vitro assembly of glycinin oligomers beyond the trimer stage. The possibility that the cleavage is a regulatory step and that it triggers the deposition of 11S seed storage proteins as insoluble aggregates in vivo is considered.

INTRODUCTION

Large quantities of storage protein are accumulated in developing seeds of leguminous plants that function as a reserve of carbon and nitrogen to be used during germination and early growth (Boulter and Barber, 1963). These proteins are deposited in an aggregated form within specialized organelles of the seed called protein bodies (Graham and Gunning, 1970). In soybeans (Glycine max.) the predominant seed storage protein is a globulin called glycinin. It can be extracted from the seeds as a 12S hexamer using dilute salt solutions. Proteins homologous to glycinin exist in a variety of monocots and dicots, and are known collectively as 11S globulins or legumin-type proteins (Derbyshire et al., 1976).

Glycinin, like the other 11S seed storage proteins, is translated as a precursor subunit that requires two proteolytic cleavages for maturation (Sengupta et al., 1981; Turner et al., 1981; Barton et al., 1982). The first cleavage involves the removal of a signal peptide that is involved in transport of the protein into the lumen of the endoplasmic reticulum (ER) (Tumer et al., 1981). The second cleavage takes place posttranslationally. By analogy with pea legumin, this second cleavage probably occurs within vacuolar protein bodies (Chrispeels, 1984), and results in fragmentation of the subunit into its constituent 40-kD acidic and 20-kD basic polypeptide chains (Turner et al., 1981, 1982). The site of the second cleavage is between an asparagine and glycinin residue, a site that has been highly conserved during the evolution of the 11S globulins (Croy et al., 1982; Marco et al., 1984; Simon et al., 1985; Baumlein et al., 1986; Chian et al., 1986; Takaia and Larkins, 1986).

Assembly of the 11S complex begins in the ER while the subunits are in precursor form. In soybeans these precursors are referred to as proglycinin. By pulse-labeling of cotyledons it has been shown that legumin of pea (Pisum sativum) assembles into an 8S species soon after synthesis (Chrispeels et al., 1982). This 8S species is thought to be a trimer of subunits. Hexamers, like those isolated from mature seeds, are not observed until after the proteins appear in vacuoles. Barton et al. (1982) have suggested that the assembly of glycinin proceeds in a similar stepwise fashion. They observed glycinin oligomers of about 180 kD in the ER shortly after pulse-labeling. It therefore appears that proglycinin subunits are transported from the ER to the vacuoles as trimers. The pathway for transport to the protein bodies is presumed to be through the Golgi apparatus, as has been shown for the phytohemagglutinin of bean, Phaseolus vulgaris (Chrispeels, 1983). Although it has been suggested that assembly of glycinin into the 12S form may be associated with posttranslational cleavage (Barton et al., 1982), a direct relationship between these two events has not been demonstrated.

Recently we described a system by which the assembly of glycinin precursor-subunits could be studied in vitro (Dickinson et al., 1987). The assay was based on in vitro transcription-translation technology (Melton et al., 1984). Transcription from a modified cDNA was accomplished with SP6 RNA polymerase, and then the transcripts were

1 Current address: Department of Biology C016, University of California at San Diego, La Jolla, CA 92093.
2 Current address: Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt.
3 To whom correspondence should be addressed.
translated in rabbit reticulocyte lysates. Proglycinin produced by this method self-assembled into trimers in reticulocyte lysates. However, even after prolonged incubations, no evidence for hexamer formation was observed (Dickinson et al., 1987). We were unable to determine whether the absence of hexamer formation was the result of a deficiency of the in vitro assay, or the result of an inherent difference between the precursor and mature forms of glycinin subunits.

The objective of research described in this report was to determine whether posttranslational cleavage was required for assembly of proglycinin trimers into hexamers. The assembly properties of radiolabeled glycinin precursors were evaluated using an assay in which mature glycinin subunits were dissociated and then re-assembled in the presence of proglycinin. The results provide direct evidence that there is a relationship between post translational cleavage of proglycinin precursor subunits into acidic and basic polypeptide chains and the assembly of trimers into hexamers.

RESULTS

Dissociation and Re-assembly of Native Glycinin

Wolf and Briggs (1958) described conditions in which glycinin could be partially dissociated in solutions of low (\( \mu = 0.01 \)) ionic strength. While repeating this work, we analyzed the sedimentation properties of glycinin in sucrose gradients and discovered that it could be dissociated completely when dialyzed extensively at an even lower ionic strength (e.g., 2 mM Tris, 10 mM 2-mercaptoethanol, pH 8). Figure 1, A and B, shows that glycinin 12S hexamers dissociate into 9S and 3S components under these conditions. By analogy to previous cross-linking studies, we considered the 9S component to consist of trimers and the 3S component to consist of monomers. A similar proportion of 9S to 3S species occurred when the dissociation was carried out either in the presence or absence of 2-mercaptoethanol (data not shown). Dissociation of the hexamers of glycinin subunits was found to be reversible. Figure 1C shows that restoration of high ionic strength (\( \mu = 0.5 \)) by the addition of buffer (0.4 mM NaCl/35 mM phosphate, pH 7.5) resulted in the conversion of about 90% of the dissociated glycinin subunits back into 12S hexamers.

The proteins in the major sucrose gradient peaks shown in Figure 1 were resolved by electrophoresis to characterize the dissociation and re-assembly of glycinin. Figure 2 shows that the native 12S hexamers in Figure 1A consisted of 60-kD subunits (lane 5) and that these subunits could be resolved into the 40-kD acidic and 20-kD basic polypeptides upon exposure of the samples to 2-mercaptoethanol (e.g., compare lanes 1 and 5). It should be noted that the staining intensity of the 20-kD basic polypeptides was similar to that of the 40-kD acidic polypeptides when subunits in the native hexamers were resolved by electrophoresis. A more complex picture emerged when these experiments were repeated using the 9S and 3S fractions.
Re-assembly with Glycinin Precursors

The re-assembly system described above was used to evaluate the ability of proglycinin precursors to become incorporated into glycinin oligomers. Figure 3 outlines these experiments schematically. G4 proglycinin subunits were produced from plasmid pSP65/248 by in vitro transcription with SP6 RNA polymerase and translation with rabbit reticulocyte lysate (Dickinson et al., 1987). Protein produced in this manner (Figure 3, solid circles) had the same primary structure as authentic G4 proglycinin (Nielsen et al., 1989), except that there was a methionine for isoleucine substitution at the NH₂ terminus. Nanogram quantities of radioactively pure proglycinin were synthesized by this technique. Proglycinin subunits produced in vitro were permitted to self-assemble into 9S trimers in the rabbit reticulocyte lysate, and the trimers that resulted were purified using a sucrose density gradient (Dickinson et al., 1987). The radiolabeled proglycinin trimers prepared in this way were incubated with NaCl and saturating amounts of unlabeled, mature glycinin monomers and trimers (Figure 3, open circles). Re-assembly under these conditions was driven by the dissociated glycinin subunits because the concentration of the monomers and trimers was at least 10,000-fold greater than that of the radiolabeled precursors. To avoid reassembly of mature glycinin monomers and trimers into 12S hexamers prior to addition of proglycinin precursors, the increase in ionic strength needed to drive re-assembly was provided as NaCl and buffer in the solution that contained the radiolabeled trimers. Figure 4A shows the distribution of radiolabeled products from the re-assembly reactions after analysis in sucrose density gradients. Under the conditions used for these experiments, the majority of the mature, unlabeled glycinin from the seed would have re-assembled into 12S hexamers, but the radiolabeled proglycinin trimers persisted as 9S trimers after re-assembly. There was no evidence that proglycinin trimers had partitioned into the 12S hexamer fraction. We concluded from this experiment that there must be a barrier, perhaps a conformational incompatibility, to the incorporation of proglycinin trimers into hexamers during the re-assembly.

The behavior of proglycinin monomers in the re-assembly system was also tested. G4 proglycinin was synthesized in vitro but not allowed to self-assemble into trimers. Instead, the radiolabeled monomers were mixed with NaCl and saturating concentrations of unlabeled, mature glycinin. Figure 4B shows the result of one such re-assembly experiment. Although mature glycinin subunits efficiently re-assembled into 12S hexamers under the conditions used for these experiments, only 20% of the proglycinin monomers became associated with glycinin hexamers. This result indicated that, although a small proportion of G4 proglycinins were incorporated into hexamers, the remainder were blocked to assembly beyond the 9S trimer.
Figure 3. Conceptual Diagram of the Re-assembly Experiment Performed with Radiolabeled Proglycinin Trimers.

Solid circles represent 3H-labeled proglycinin subunits synthesized at the direction of clone pSP65/248. The open circles represent mature glycinin subunits purified from seeds and which had undergone posttranslational cleavage to yield disulfide linked acidic and basic polypeptides.

stage. Together, the results indicated that there were differences between the mature subunits from seeds and the immature unprocessed subunits that comprised the proglycinin monomers and trimers that were produced in vitro. The barrier to assembly could be structural and could reflect changes in conformation that occur when the proglycinin subunits are cleaved posttranslationally in protein bodies.

Effect of Papain Treatment on Precursor Assembly

Because the lack of posttranslational cleavage into acidic and basic polypeptide chains might have been responsible for the trimer stage block exhibited by the trimers of proglycinin subunits, we considered the possibility that processing could be simulated by partial proteolysis of proglycinin using a nonspecific protease. To examine this possibility, G4 proglycinin was synthesized in vitro and permitted to partially self-assemble into trimers, and then the differential effects of proteolysis on trimers versus monomers were determined. Half of this radiolabeled proglycinin was treated with papain and loaded onto a sucrose gradient, and the remainder was treated identically except that papain was excluded. Mild conditions of papain treatment were selected that did not destroy the sedimentation properties of the proglycinin trimers in the sucrose gradients. Figure 5A shows that monomers were more sensitive to papain treatment than the trimers because the 3S peak was rapidly destroyed by papain treatment. The papain treatment appeared to decrease sedimentation of trimers slightly, but the proglycinin trimers clearly persisted. The extent of proteolysis of the trimers was determined by isolating trimer peaks from sucrose gradients and separating the proteins in this fraction by electrophoresis. Figure 5B shows that cleavage took place at multiple sites in proglycinin subunits in the trimers, presumably at exposed sites located at the surface of the trimers. However, judging from the sedimentation characteristics of the trimer complex in the sucrose gradients after proteolysis, the basic structure of the trimers was preserved.

To examine the effect of partial proteolysis on assembly, the trimers were isolated from sucrose gradients (Figure 5A) and tested in the re-assembly system. Trimmers treated with papain were mixed with NaCl and then with mature glycinin that had been dissociated. The results from a typical experiment are shown in Figure 5, C and D. A prominent peak due to molecules sedimenting in the sucrose gradient with a rate of about 12S was observed in the case of papain-treated precursors (Figure 5D), but not in the control (Figure 5C). Similar, although less striking, results were obtained when the experiment was repeated using V8 protease (data not shown). The results demonstrated that proglycinin trimers were capable of forming

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Figure 4. Sucrose Density Gradients Showing the Behavior of Proglycinin Trimers and Monomers in the Re-assembly Assay.

(A) Self-assembled G4 proglycinin trimers were isolated from a sucrose gradient, and (after dialysis to remove the sucrose) the trimers were tested in the re-assembly system. A Bradford assay (1976) was performed on the fractions to show that mature glycinin had re-assembled into 12S hexamers.

(B) Same as (A) except that proglycinin monomers were used. Sedimentation standards are shown at the top.
that a complex between a radiolabeled acidic polypeptide and a free basic polypeptide from dissociated glycinin monomers might form, and these would mimic "mature" subunits that had been cleaved posttranslationally to remove the trimer block. A plasmid was constructed that could be used to produce only the acidic portion of a glycinin subunit. For this purpose, the region between two HincII sites in pSP65/248 was deleted (Figure 6), and the resulting plasmid was called pSP65/248ΔHH. This deletion resulted in a change in the reading frame and produced a chain termination codon near the end of the region encoding the acidic polypeptide chain. The amino acid sequence of the mutant G4-ΔHH acidic polypeptide produced from plasmid pSP65/248ΔHH was identical to G4 from pSP65/248 until amino acid 301 in the hypervariable region of the subunit. Two amino acids, a glutamine and proline, were located at the COOH terminus of the G4-ΔHH that were not found in G4. Although the mutant G4-ΔHH polypeptide produced by this construction lacked approximately one-half of the hypervariable region, this modification in structure was not expected to alter the ability of the mutant subunit to assemble into hexamers. The entire hypervariable region, which is about 100 amino acids in length in G4 (Nielsen et al., 1989), can be eliminated with no measurable effect on self-assembly of the G4 glycinin subunits into trimers (Dickinson, 1988).

Figure 7 characterizes the behavior of the G4-ΔHH polypeptide during assembly. The G4-ΔHH polypeptides had a mobility in SDS gels that was expected for a 35-kD protein (Figure 7A), and they did not self-assemble (Figure 7B) under conditions where G4 proglycinin completely self-assembled into hexamers once they had been modified by treatment with protease. Partial proteolysis with papain and V8 protease apparently mimicked the effect of posttranslational processing and removed a barrier to their incorporation into hexamers.

Re-assembly with a Glycinin Acidic Chain

To test the possibility that posttranslational cleavage of proglycinin subunits in trimers eliminated a block to hexamer assembly, we tested the ability of radiolabeled acidic polypeptides to re-assemble into hexamers. The idea was...
assembled into trimers (Dickinson et al., 1987). Figure 7C shows the behavior of G4-ΔHH during re-assembly. The majority of the 3H-labeled G4-ΔHH polypeptides were found at the 3S position in sucrose gradients after re-assembly, but there was also a radiolabeled peak in the 12S hexamer position in the gradient. The composition of the minor peak was determined by electrophoresis. Figure 7D shows that, when the radiolabeled proteins in the 12S peak were treated with 2-mercaptoethanol, their mobilities in the electrophoresis gels were reduced from one expected for a 55-kD protein to one expected for a 35-kD protein. This result indicated that all of the 3H-labeled G4-ΔHH polypeptides that were contained in the 12S fraction had formed a cystine linkage with unlabeled basic chains. Figure 7C also shows that, whereas 12S hexamers were produced during re-assembly, 9S trimers were not detected in the sucrose gradients. This observation was important because it showed that a trimer stage block did not interfere with re-assembly of the G4-ΔHH acidic polypeptides into 12S glycinin hexamers to the same extent as when G4 proglycinins were used. Because the combination of 3H-G4-ΔHH acidic and unlabeled basic chains corresponded to "mature" subunits, their assembly into hexamers suggested that the lack of posttranslational cleavage interfered with the assembly of proglycinins into hexamers.

Re-assembly with a Glycinin Basic Chain

Experiments analogous to those carried out with the acidic polypeptide chains were done using basic polypeptides. A plasmid that directed synthesis of only the basic portion of G4-proglycinin was produced by inserting an oligonucleotide containing an in-frame ATG at an appropriate position in plasmid pSP65/248 (see "Methods"). The resulting plasmid, pG4B17 (Figure 6), encoded a G4 basic polypeptide (G4-B17) whose amino acid sequence was identical to the basic chain from G4 except that it contained five additional amino acids at its NH2 terminus (MetGluThrArgAsn). Figure 8A shows that a polypeptide with the expected electrophoretic mobility was produced from plasmid pG4B17. As with basic polypeptides isolated from soybean seeds, the G4-B17 basic polypeptide tended to precipitate from solution, and this property prevented a self-assembly assay with the protein. Figure 8B shows the distribution of 3H-labeled G4-B17 in a sucrose gradient after re-assembly.
with monomers and trimers from mature glycinin. Although radioactivity was recovered in the 9S trimer fractions, the 12S hexamer fractions were much more heavily labeled. About half of the radiolabeled G4-B17 basic polypeptides were linked to acidic polypeptides in both the 9S trimer and 12S hexamer fractions (data not shown). The distribution of radioactivity in the sucrose gradients was markedly different when the G4-B17 basic polypeptides were used for reassembly compared with when G4 proglycinin subunits were used. The 12S hexamer fraction was by far the most heavily labeled fraction when G4-B17 was used (Figure 8B) for reassembly, whereas the 3S and 9S fractions were the most heavily labeled when G4 proglycinin was used (Figure 4B). These results would be expected if the blockage to incorporation of proglycinin was relieved by the posttranslational modification that produced the disulfide-linked acidic and basic polypeptides.

**DISCUSSION**

We previously demonstrated that G4 precursors synthesized in vitro could self-assemble into trimers of subunits (Dickinson et al., 1987). However, no evidence for assembly into hexamers was obtained, even after prolonged incubations and complete assembly to trimers. In this report we described a glycinin re-assembly assay that was used to study the assembly of a G4 glycinin precursor subunit. We defined conditions in which mature glycinin hexamers were completely dissociated into 9S trimers and 3S monomers. Although the monomer fraction contained mainly acidic polypeptide chains that were linked to basic ones via a cystine bond, the fraction also contained some free acidic and basic chains. Re-assembly of glycinin was accomplished by returning the dissociated glycinin solution to conditions of high ionic strength.

Proglycinin monomers and trimers synthesized in vitro behaved differently from mature glycinin in the re-assembly assay. Proglycinin trimers remained as trimers when mixed with saturating amounts of mature glycinin trimers that were undergoing re-assembly (Figure 4A). Some proglycinin monomers were incorporated into 12S hexamers when glycinin was re-assembled, but the majority were recovered in the 3S fraction and as 9S trimers. The conceptual basis for these results is shown in Figure 9, A and B. Three points can be inferred from these observations: (1) trimers composed entirely of precursor subunits were incapable of interacting with trimers composed of mature subunits during re-assembly of hexamers, (2) assembly of proglycinin subunits is blocked at the trimer stage, and (3) the assembly defect exhibited by proglycinin is likely to be conformational in nature since there was apparently enough flexibility in the glycinin hexamer to accommodate individual proglycinin subunits.

Figure 5 shows that digestion of proglycinin trimers with papain partially removed the trimer stage block exhibited by the precursors in the re-assembly assay. This result indicated that, to the extent that the trimer block was removed, the papain treatment had an effect on the assembly properties of a glycinin subunit which mimicked the effect of posttranslational cleavage. The results support the idea that the posttranslational cleavage that occurs in vivo is responsible for the removal of a barrier to assembly of glycinin trimers into hexamers. An acidic chain synthesized in vitro was unable to assemble in the self-assembly assay. In the re-assembly assay, however, some of the in vitro synthesized acidic polypeptides formed hexamers. Importantly, no glycinin trimers were evident. A model that explains the results of this experiment is shown in Figure 9C. A minor proportion of the radiolabeled acidic polypeptides associated with free basic polypeptides to form, in effect, “mature” processed subunits. Mature subunits formed in this way were capable of uninhibited assembly to hexamers because no evidence for a trimer fraction was evident in the re-assembly profile. This model was verified by showing that the acidic chains which had assembled were cystine-linked to a basic chain. The results indirectly demonstrated that the effect of processing on glycinin assembly is to facilitate hexamer formation because the assembly of a mature subunit produced in vitro was not blocked at the trimer
stage. Were this not the case, a re-assembly result similar to that obtained with proglycinin (e.g., exhibiting more trimers than hexamers, Figure 4B) would have been observed. Similar results were obtained with a basic chain synthesized and re-assembled into glycinin hexamers in vitro.

The COOH terminus of the acidic and NH₂ terminus of the basic polypeptide chains produced in vitro did not exactly match those that would be expected from post-translational cleavage of G4 proglycinin. However, these modifications were not expected to perturb the ability of the chimeric subunits consisting of radiolabeled acidic and native basic chains (and vice versa) to assemble. The modifications were located at the junction between the COOH terminus of the acidic polypeptide chain and the NH₂ terminus of the basic polypeptide chain. This region varies in length among authentic glycinin subunits (Nielsen et al., 1989), and it can tolerate large insertions and deletions without measurable effect on the self-assembly properties of the G4 subunits (Dickinson, 1988). The fact that trimers containing chimeric glycinin subunits could reassemble into hexamers, but trimers consisting of proglycinin subunits could not, may indicate that posttranslational modification mediates a conformational change that is required before further assembly of the glycinin complex can take place.

It can be concluded that posttranslational cleavage of the glycinin subunits is required for glycinin assembly beyond the trimer stage. Although this conclusion is based on in vitro studies, it fits well with the available in vivo data. Pulse-chase studies with radiolabeled cotyledons suggested that proglycinin (Barton et al., 1982) and prolegumin (Chrispeels et al., 1982) did not assemble beyond the trimer stage in vivo. Assembly to the hexamer stage occurred in the vacuoles after posttranslational cleavage. The in vivo results, together with the in vitro evidence presented in this report, indicate that posttranslational cleavage of glycinin subunits is required for assembly beyond the trimer stage.

The assembly observed with the acidic chain also has important implications about the structure of the oligomer. The results demonstrate that the basic chain is critical for assembly, because assembly of an acidic chain was not observed unless it had associated with and became cysteine-linked to a basic chain. The importance of the basic domain for trimer formation is also supported by an earlier observation that a 21 amino acid deletion in the basic chain of G4 proglycinin inhibited self-assembly into trimers (Dickinson et al., 1987). The acidic domain was found to be important for the solubility of a glycinin subunit. Free basic chains precipitated from solution, whereas free acidic chains remained soluble. One of the functions of the acidic chain may be to protect the basic chain from solvent. The results also support the idea put forward by Argos et al. (1985) that the acidic and basic chains constitute semiautonomous domains because the relatively mild conditions of low ionic strength were shown to be capable of reversibly dissociating the acidic and basic chains.

The observation that disulfide bonds between the acidic and basic chains in re-assembled glycinin had re-formed may indicate that they are important for the stability of a subunit. Interchain disulfide bonds were also demonstrated with radiolabeled basic chains re-assembled with mature acidic polypeptides of glycinin (Figure 8B). The conservation of the disulfide bond among all 11S seed storage proteins studied to date (Staswick et al., 1984; Derbyshire et al., 1976; Nielsen et al., 1989) underscores its importance for the function of these proteins.

Low angle x-ray diffraction studies on crystals of 11S globulins from a variety of plants indicate 32 symmetry (Coleman et al., 1980; Pleitz et al., 1987). These data, together with the dissociation data for glycinin, are consistent with the models shown in Figure 9. In the simplest case (e.g., assuming symmetry), two types of subunit interactions can be expected, one type for trimer formation and another for hexamers. Thus, it is reasonable that hexamer formation was dependent on processing, whereas trimer formation was independent of the proteolysis. There is precedent for posttranslational cleavage causing a conformational change with resultant consequences upon subunit assembly. Posttranslational cleavage of the capsid proteins is required for stability of the black beetle virus capsid (Gallagher and Rueckert, 1998; J.E. Johnson, personal communication). Similarly, procollagen must be converted to collagen before assembly occurs.

Proteolytic processing may be an important mechanism by which assembly and packaging of 11S storage proteins are regulated. It has been shown for legumin in pea that the posttranslational cleavage takes place soon after the arrival of precursors in the vacuolar protein bodies (Spencer and Higgins, 1980; Chrispeels et al., 1982). We speculate that posttranslational processing functions to bring about the precipitation or higher order assembly of 11S subunits in the organelle in which they are to be deposited. With such a model, the conservation of the cleavage site across a wide evolutionary range of plants can be understood in terms of its importance to the function of these storage proteins. Processing would be vital for packaging and the realization of a storage function.

Our findings may also have relevance for the development of seed protein body organelles. Craig et al. (1980) have shown that large vacuoles of developing pea (P. sativum) cotyledon parenchyma cells fragment to give rise to the small protein bodies found in mature seeds. Prior to this fragmentation, seed storage proteins accumulate in the vacuoles in an aggregated form at the periphery of the vacuole and are associated with tonoplast membranes (Craig et al., 1979). Deposition of protein at the tonoplast membranes may be a prerequisite to protein body formation. Thus, the posttranslational cleavage of 11S storage proteins may not only trigger their precipitation and depo-
sition, but (by virtue of their high concentration in the storage parenchyma cells) may also trigger the genesis of seed protein bodies.

METHODS

Purification of Glycinin

Glycinin was purified according to previously published procedures (Moreira et al., 1979).

Dissociation and Re-assembly of Glycinin

Glycinin was dissociated by dialysis of an 18 mg/mL sample against dissociating buffer (2 mM Tris, pH 8/10 mM 2-mercaptoethanol) for 2 days at 4°C. Re-assembly was accomplished by the addition of a 5x solution to give a final concentration of 5.5 mg/mL protein in 35 mM phosphate, pH 7.5, 0.4 M NaCl, 10 mM 2-mercaptoethanol.

In Vitro Synthesis and Self-assembly

In vitro transcription with SP6 RNA polymerase and translation with rabbit reticulocyte lysates were accomplished as described earlier (Dickinson et al., 1987). Self-assembly assays with the acidic polypeptide derived from glycinin (8 mg/mL) was added and vortexed. Another 8 mL of 5x extraction buffer was quickly added to the mixture and vortexed. This mixture was dialyzed against 35 mM phosphate, pH 7.5, 0.4 M NaCl, 10 mM 2-mercaptoethanol overnight. Prior to loading the products of re-assembly onto a sucrose density gradient, the solution was diluted with 100 μL of 1x extraction buffer without 2-mercaptoethanol. For re-assembly with proglycinin trimers, the 9S peak isolated from a sucrose gradient was dialyzed against 1x extraction buffer and then mixed directly with dissociated glycinin. (The subsequent steps were performed as above.)

Constitution of pSP65/248HH and pGB17

Plasmid pSP65/248 (Dickinson et al., 1987) was digested with Hincll (Figure 6) and subsequently religated in dilute buffer. A resulting plasmid, pSP65/248HH, contained a deletion of the sequences between the Hincll sites and was sequenced by the dideoxy method using the G4 primer described previously (Dickinson et al., 1987). This plasmid was used in the same way as pSP65/248 to synthesize a glycinin polypeptide chain which terminated in the hypervariable region (Argos et al., 1985).

Plasmid pG4B17 was produced by first placing a synthetic ATG contained in the partially complementary oligonucleotides pGATCTGGAGGAAAAATTATATG and pGATCCATAATTTTTT-CCTCCA into the BamHl (see Figure 6) site of pSP65/248. The orientation was such that the BamHl site at the 5' side of the oligonucleotide was destroyed, whereas the other site was not. This plasmid clone was linearized with SalI, digested with Bal31, digested with BamHl (which cut immediately after the ATG), digested with mung bean exonuclease, and recircularized with T4 DNA ligase. A resulting clone, pG4B17, contained the synthetic ATG in-frame with the position 352 glutamate codon.

SDS-PAGE

For analysis of SDS-PAGE, samples were dialyzed against (or diluted with 10 volumes of) sample buffer (0.03 M Tris-Cl, pH 6.8/2% SDS/2% 2-mercaptoethanol/2.5 M urea/10% glycerol) and were boiled for 2 min. For non-reduced samples the 2-mercaptoethanol was omitted, and the samples were heated to 68°C for 5 min rather than boiling. Samples were separated electrophoretically in a 12.5% polyacrylamide gel (Laemmli, 1970). After electrophoresis, the gels were stained with Coomassie Blue, and when appropriate, treated with ENHANCE (Du Pont-New England Nuclear) and dried, and the proteins visualized by fluorography.

Sucrose Density Gradient Centrifugation

Fractionation of 7% to 25% linear sucrose density gradients has been described previously (Dickinson et al., 1987). When reticulocyte lysates were loaded, the solution was first diluted with 1.5 volumes of 1x extraction buffer. The buffer of sucrose gradients containing dissociated glycinin was adjusted with dissociating buffer. Fractions were assayed by the protein dye binding method (Bradford, 1974) or by TCA precipitations followed by scintillation counting. Sedimentation standards included soy proteins (Hill and Breidenbach, 1974), hemoglobin (4S), or 9S G4 proglycinin (Dickinson et al., 1987).

TCA Precipitations

The sample (100 μL) was mixed with 0.25 mL of 1.5 M NaOH, 2.25% hydrogen peroxide and incubated at 37°C for 10 min. Then 1.5 mL of 25% trichloroacetic acid, 2% casamino acids were added and mixed, and the mixture was placed on ice for at least 30 min. When re-assembly reactions were analyzed, the volumes were scaled up by a factor of 3. Samples were collected on glass fiber filters, washed twice with 10 mL of 10% TCA, and subsequently washed with 5 mL of ethanol. The filters were then dried and counted in 10 mL of ACS scintillation fluid (Amersham).

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