The Plant Cell, Vol. 9, 2037–2050, November 1997 © 1997 American Society of Plant Physiologists

Role of the Sulfhydryl Redox State and Disulfide Bonds in Processing and Assembly of 11S Seed Globulins

Rudolf Jung,[a,b,1,2] Young-Woo Nam,[a,1,3] Isolde Saalbach,[b] Klaus Müntz,[b] and Niels C. Nielsen[a,4]

[a] United States Department of Agriculture, Agricultural Research Service, and the Departments of Agronomy and Biochemistry, Purdue University, West Lafayette, Indiana 47907-1150

[b] Institute of Plant Genetics and Crop Plant Research, Corrensstrasse 3, D-06466 Gatersleben, Germany

Seed legumins contain two conserved disulfide bonds: an interchain bond (IE) connecting the acidic and basic chains and an intrachain bond (IA) internal to the acidic chain. Mutant subunits were constructed in which these disulfide bonds were disrupted. Oxidized glutathione stimulated the rate of assembly of trimers with unmodified prolegumin subunits. Stimulation was not detected during assembly of IE mutant subunits and was diminished for the IA mutant. Hexamer assembly with trimers of mature unmodified subunits required oxidizing conditions. Trimers composed of mature IE mutants did not form hexamers. Both mutant and non-mutant subunits accumulated in hexamers when the cDNAs were expressed in tobacco. Hexamer assembly in seeds probably involved trimers with a mixture of mutant and non-mutant subunits. Similarly, mixed trimers that were a mixture of mutant and non-mutant subunits assembled into hexamers in vitro. The results demonstrate the importance of disulfide bonds during the assembly of 11S globulins.

INTRODUCTION

The 11S globulins are abundant storage proteins found in the seeds of many plants (Nielsen et al., 1997). They are synthesized and assembled during a complex process that involves a series of post-translational modifications as the proteins are transported to and deposited within protein storage vacuoles (Müntz, 1989; Shotwell and Larkins, 1989). Mature 11S globulins, which are encoded by a small family of genes (Heim et al., 1989; Nielsen et al., 1989), are extracted from seeds as hexameric complexes (Badley et al., 1975). Each 11S subunit consists of an acidic and a basic polypeptide; these peptides are joined by a single disulfide bond (Nielsen, 1984). Cleavage of a subunit propeptide into these acidic and basic chains takes place at an evolutionarily conserved asparagine–glycine bond by a specific asparaginyl endopeptidase (Scott et al., 1992; Hara-Nishimura et al., 1995) and is prerequisite for the formation of mature hexamers (R. Jung, M.P. Scott, Y.W. Nam, T.W. Beaman, R. Bassüner, I. Saalbach, K. Müntz, and N.C. Nielsen, submitted manuscript). Before the formation of 11S hexamer complexes in protein storage vacuoles, the globulin precursors are part of 9S trimers that form in the endoplasmic reticulum (ER) (Chrispeels et al., 1982). The early events in this process involve the synthesis of prepropeptides by polysomes associated with membranes (Püchel et al., 1979). After translocation of the precursors into the ER, a signal sequence is removed (Ereken-Tumer et al., 1982; Bassüner et al., 1984). The folding and assembly of translocated propeptides into trimers are considered to be mediated by components in the ER (Vitale et al., 1993), although these events are not well characterized.

One factor that influences folding and assembly of newly translated secretory proteins is the sulfhydryl redox state in the ER. The redox state of the lumen of the ER has recently been demonstrated to be oxidizing compared with the reducing state found in the cytosol (Hwang et al., 1992). This condition is the consequence of a higher ratio (1:2) of oxidized glutathione (GSSG)/reduced glutathione (GSH) in the ER compared with the cytosol (Hwang et al., 1992). An oxidative redox state should accelerate the folding of proteins that contain disulfide bonds between cysteine residues (Creighton, 1983; Gilbert, 1990). Such processes are often facilitated by protein disulfide isomerase (PDI), an abundant ER lumenal enzyme (Noiva and Lennarz, 1992; Freedman et al., 1994). In this regard, the cotranslational formation of disulfide bonds in γ-gliadin imported into dog pancreatic microsomes is dependent on PDI (Bulleid and Freedman, 1988).

The 11S globulins contain two pairs of highly conserved cysteine residues (Casey et al., 1986). One forms an intrachain disulfide bond that occurs in the acidic chain, whereas the other forms an interchain disulfide bridge that connects the acidic and basic chains of the mature subunit (Horstmann, 1983; Staswick et al., 1984). The conservation

[1] These two authors contributed equally to this work.

[2] Current address: Trait and Technology Development, Pioneer Hi-Bred International Inc., 7300 N.W. 62nd Ave., Johnston, IA 50131-1004.

[3] Current address: Department of Plant Pathology and Microbiology, Texas A & M University, College Station, TX 77843.

[4] To whom correspondence should be addressed. E-mail nnielsen@dept.agry.purdue.edu; fax 765-494-6508.

of these residues implies a functional role of these bonds in the structure of 11S globulins. Utsumi et al. (1993) constructed plasmids encoding mutant G3 glycinin propeptides in which the disulfide bonds were disrupted. Examination of the assembly of the protein products expressed in *Escherichia coli* revealed that the mutant proglycinins were still able to attain a trimer conformation. This observation is not consistent with a functional role of these residues. However, during experiments designed to determine processing and maturation of 11S globulins, we observed that the sulfhydryl redox state had a pronounced impact on the kinetics of subunit cleavage and assembly. Moreover, an oxidative redox state appeared to be an absolute requirement for assembly of processed proglobulin subunits into hexamers in vitro (Beaman, 1996).

To determine whether the cysteine residues of the 11S globulins, or other unknown redox-dependent factors in our experimental system, were responsible for the kinetic effects observed, we studied the influence of mutations involving these residues both by using the in vitro assembly system described by Dickinson et al. (1987) and by using transgenic tobacco. Results obtained from these experiments demonstrate a critical role of the disulfide bonds in the maturation and assembly of 11S globulins.

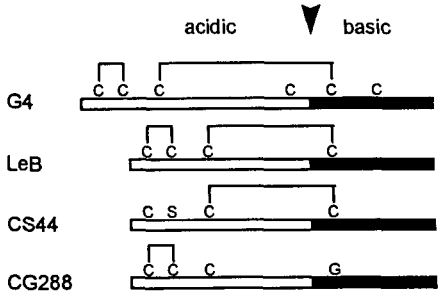

Figure 1. 11S Proglobulins and Cysteine Mutant Derivatives.

G4 of soybean contains six cysteine residues, of which four are involved in the formation of two disulfide bonds. In contrast, LeB from fava bean contains four cysteine residues, all of which participate in the formation of the two conserved disulfide bonds. Two mutant derivatives of LeB, denoted CS44 and CG288, were constructed in which each of these disulfide bonds was disrupted. Mutant CS44 has a cysteine-to-serine replacement at position 44, whereas the cysteine at position 288 was changed to glycine in mutant CG288. An intrachain disulfide bond was disrupted in the acidic chain of CS44, whereas the mutation in CG288 eliminated the interchain disulfide bond between the acidic and basic chain of the LeB proprotein. The arrowhead indicates the position where post-translational processing takes place. This event gives rise to the acidic (open bars) and basic (black bars) polypeptides of mature proglobulin subunits.

RESULTS

Involvement of Sulfhydryl Redox State in 11S Proglobulin Assembly

An Oxidative Redox State Stimulates in Vitro Assembly of Proglobulin Trimers

Proglycinin G4 (G4) from soybean and prolegumin B4 (LeB) from fava bean (*Vicia faba*) were used as models in this study. The two proteins were encoded by expression plasmids pSP65/248 (Dickinson et al., 1987) and pLeMS (Scott et al., 1992), respectively. The essential features of these proteins are shown schematically in Figure 1. Whereas G4 contains six cysteine residues, of which four are involved in the formation of two disulfide bonds, LeB contains only the four conserved cysteine residues that are required to form the two disulfide bonds. mRNAs from pSP65/248 or pLeMS were translated in vitro in a rabbit reticulocyte lysate, and the products were permitted to self-assemble into trimers (Dickinson et al., 1987). Previous studies established that the sedimentation properties of these trimers are similar to those of the 11S precursors found in the ER from developing cotyledons (Dickinson et al., 1987; Jung et al., 1993).

Figure 2 shows the time course of in vitro assembly of G4 that was monitored by sucrose density gradient centrifugation. Typically, assembly reached a maximal level after incubation for 24 hr in the reticulocyte lysate; ~70% of the labeled polypeptides were recovered in the 9S fractions (trimer) and 30% in the 3S fractions (monomer) (Figure 2). After 6 hr, only a small proportion of G4 monomers had assembled into trimers (Figure 2).

To investigate the role of redox state in the assembly of proglobulin subunits, we used the in vitro synthesis and assembly system described above. The cell-free rabbit reticulocyte lysate has a reducing sulfhydryl redox potential (Scheele and Jacoby, 1982; Kaderbhai and Austen, 1985; Jagus, 1987). In a determination of whether changes in the redox state of the translation mixture affected 11S subunit assembly, the redox potential was made oxidizing by the addition of GSSG. Preliminary results indicated that concentrations of GSSG up to 10 mM did not adversely affect translation efficiency in the lysate as determined by incorporation of ^{3}H-leucine into protein per unit time (data not shown). When G4 proproteins were synthesized in the presence of 5 mM GSSG and allowed to self-assemble for 6 hr, assembly reached a maximal level that normally could be attained only after 24 hr in the absence of GSSG (Figure 2). Strikingly, a shift in the redox state of the lysate to a more oxidizing condition stimulated the rate of assembly of G4 trimers approximately fourfold.

Different concentrations of GSSG (0.5 to 50 mM), GSH (0.5 to 5 mM), and diamide (diazenedicarboxylic acid *bis*-[*N*,*N*-dimethylamide]) (1 to 5 mM) as well as different concentration ratios between GSSG and GSH were tested. As shown in Table 1, GSSG concentrations between 1 and 10 mM were optimal at stimulating trimer formation. Therefore, 5 mM GSSG was used in the reaction mixtures as the stan-

dard condition throughout this study. The stimulatory effect of GSSG was mimicked by diamide (Kosower et al., 1969), an oxidizing agent that elicits disulfide formation. On the other hand, addition of either GSH or GSSG/GSH in 1:2 ratios had an adverse or little effect on the assembly of G4 trimers.

Table 1 also shows the effect of GSSG on the assembly of LeB subunits. As with G4, the rate at which LeB polypeptides assembled into trimers was nearly doubled by the addition of GSSG. However, the effect of GSSG on LeB assembly appeared less pronounced than with G4 because LeB assembles more rapidly than does G4 in the absence of GSSG. When GSSG was added after termination of translation in 1 hr, assembly of G4 subunits was accelerated only marginally, indicating a substantial decrease in the stimulatory effect. This result suggests that the disulfide bonds are formed early during the folding of G4 polypeptides, and their formation is probably rate determining to trimer assembly.

Because disulfide-bridge formation in the seed protein γ-gliadin is catalyzed by PDI (Bulleid and Freedman, 1988), we performed a similar experiment by adding GSSG together with an active mammalian PDI preparation (Noiva and Lennarz, 1992) to the in vitro translation mixture. No additional stimulation of trimer formation more than that caused by the addition of GSSG alone was detected (results not shown).

Effect of an Oxidative Redox State on Trimer Assembly of Cysteine Mutants of Legumin B Propeptides

Because LeB contains only the four evolutionarily conserved cysteine residues that form the intrachain and interchain disulfide bonds, the function of the disulfide bonds in 11S globulin assembly was examined further by using two mutant constructs derived from the plasmid pLeMS that contained mutations disrupting the formation of disulfide bonds. In unmodified LeB (Figure 1), a conserved interchain disulfide bridge is formed between Cys-87 and Cys-288, whereas an intrachain bond connects Cys-11 and Cys-44 (Horstmann, 1983). The two mutants, CS44 and CG288 (Figure 1), contain mutations that disrupt the intrachain and interchain disulfide bonds, respectively. Mutant CS44 contains a Cys-44–to–Ser-44 mutation, whereas CS288 has a Cys-288–to–Gly-288 mutation.

Effects of these mutations on the assembly of LeB subunits were then examined (Figure 3). When analyzed after 6 hr of assembly, GSSG stimulated trimer formation of CS44 polypeptides by a factor of two (Figure 3B). Thus, the extent of stimulation observed with subunits prepared from this mutant was very similar to that observed when unmodified LeB was used (Figure 3A). Contrary to this result, trimer formation of CG288 polypeptides was insensitive to the addition of GSSG (Figure 3C). Analysis of the aliquots taken from the same batches of the three samples at an earlier

Table 1. Effects of Different Redox Additives and Their Concentration on in Vitro Assembly of G4 and LeB Subunits

Proglobulin Subunit[a]	Additive[b]	Concentration (mM)	Assembly[c] Monomers (%)	Assembly[c] Trimers (%)
G4	None	—[d]	83.5 (28.4)[e]	16.5 (71.6)[e]
	GSSG	0.5	81.8	18.2
	GSSG	1.0	38.5	61.5
	GSSG	5.0	34.8 (64.3)[f]	65.2 (35.7)[f]
	GSSG	10	55.0	45.0
	GSSG	25	80.3	19.7
	GSH	2.5	94.4	5.6
	GSSG/GSH	1.25/2.5	82.7	17.3
	Diamide	1.0	51.9	48.1
LeB	None	—[d]	59.2 (34.4)[e]	40.8 (65.6)[e]
	GSSG	5.0	33.7 (36.3)[f]	66.3 (63.7)[f]

[a] G4 and LeB proglobulin subunits were synthesized in a rabbit reticulocyte lysate from mRNAs derived from pSP65/248 and pLeMS, respectively.

[b] Additives were supplemented in the initial mixture of translation reactions.

[c] Assembly of ^{3}H-leucine–labeled G4 or LeB subunits was permitted to occur for 6 hr. After sucrose gradient centrifugation of the assembly mixtures, individual fractions were analyzed by trichloroacetic acid precipitation and scintillation counting. Radioactive counts associated with fractions corresponding to the 3S or 9S peaks were combined to calculate the percentage of monomers or trimers of the total proglobulins synthesized.

[d] —, not applicable.

[e] Assembly was permitted to occur for 24 hr.

[f] Additives were supplemented after termination of translation in 1 hr.

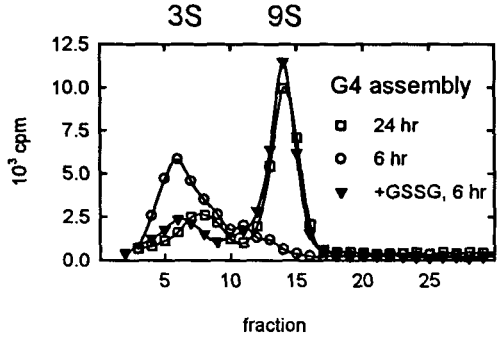

Figure 2. Effect of GSSG on in Vitro Assembly of G4 Subunits.

mRNAs encoding G4 proprotein were translated in a rabbit reticulocyte lysate in the absence (open squares and open circles) or presence (black triangles) of 5 mM GSSG. Translation was terminated in 1 hr, and the synthesized ^{3}H-leucine–labeled G4 subunits were permitted to self-assemble in the lysate at 30°C either for 6 hr (open circles and black triangles) or for 24 hr (open squares). The mixtures were then centrifuged in 6 to 22% linear sucrose density gradients. Fractionated samples were analyzed by trichloroacetic acid precipitation and scintillation counting. 3S and 9S designate sedimentation coefficients characteristic of monomers and trimers, respectively.

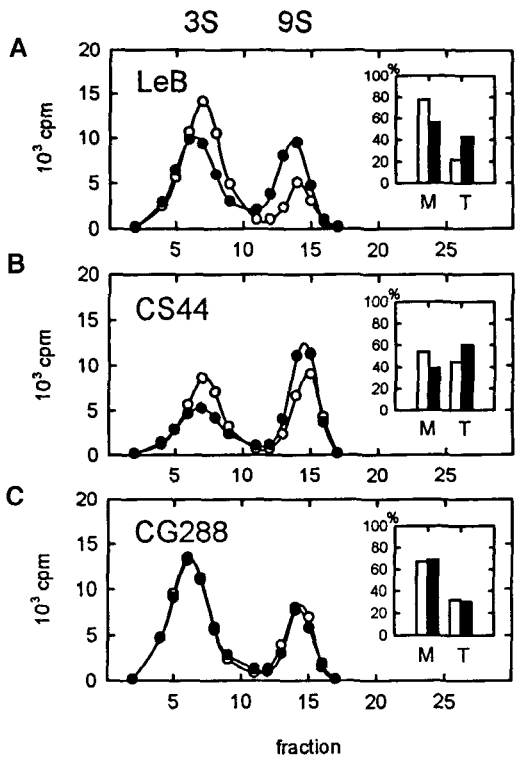

Figure 3. Effect of GSSG on in Vitro Assembly of LeB and Cysteine Mutant Subunits.

In vitro translation of mRNAs encoding LeB and cysteine mutant proproteins was performed in the absence (open circles and bars) or presence (black circles and bars) of 5 mM GSSG. After translation, the reticulocyte lysate mixtures were incubated for 6 hr for assembly before separation in sucrose density gradients. Fractions recovered from the gradients were assayed for incorporated radioactivity. The hydrogen-3 counts associated with the 3S or 9S fractions were combined, and the percentage distribution in monomers (M) and trimers (T) was calculated (inserted graphs).

(A) Assembly of LeB.

(B) Assembly of CS44 with a disrupted intrachain disulfide bond.

(C) Assembly of CG288 with a disrupted disulfide bond.

time point (2 hr after assembly) and a later point after assembly (24 hr) supported these results (data not shown). However, the total amounts of trimers formed in the mutants were similar to those seen in unmodified prolegumins after prolonged incubation. This finding suggests that formation of correct disulfide bonds is not required for folding of an individual prolegumin subunit. Nevertheless, the stimulating effect of the oxidative redox state on the kinetics of trimer assembly appears to be mediated principally by formation of the disulfide bond between Cys-87 and Cys-288, which becomes the interchain disulfide bridge in the mature subunit.

Effect of an Oxidative Redox State on Processing and 11S Hexamer Formation in Vitro

Cleavage of 11S proglobulins at a conserved Asn–Gly bond by an asparaginyl endopeptidase is a prerequisite for the transition of trimers into hexamers, both in vivo and in vitro (R. Jung, M.P. Scott, Y.W. Nam, T.W. Beaman, R. Bassüner, I. Saalbach, K. Müntz, and N.C. Nielsen, submitted manuscript). To study the effect of disulfide bonds on the transition to hexamers, we produced trimers of unmodified and mutant prolegumins in the absence of GSSG and purified them by sucrose gradient centrifugation. The purified trimers were subjected to cleavage by asparaginyl endopeptidase in the absence or presence of GSSG. In the latter case, the molecules were preincubated in GSSG before digestion. Figure 4A shows that cleavage products from trimers consisting either of unmodified LeB or of each of the mutant subunit polypeptides were cleaved into acidic and basic chains with the expected electrophoretic mobilities. In each of these cases, cleavage of the subunits into acidic and basic chains was not affected to a detectable extent by the addition of GSSG. These observations indicated that mutations at Cys-44 and Cys-288 in the subunits did not impede specific cleavage of the subunits after they were assembled into trimers.

To analyze the capacity of prolegumin subunits in trimers to be assembled into hexamers, we used the reassembly assay described by Dickinson et al. (1989). As shown in Figure 4B, the shift from a reduced to an oxidative redox environment, elicited by the addition of 10 mM GSSG, had a marked impact on the incorporation of unmodified LeB subunits into hexamers. When reassembly in the presence of different concentrations of GSSG was tested, GSSG concentrations of 5 to 25 mM were effective in stimulating hexamer formation (data not shown). Despite being specifically processed, the subunits containing the cysteine mutations were impaired markedly in their ability to assimilate into mature hexamers (Figures 4C and 4D). The decreased formation of 11S complexes, as compared with unmodified subunits, was most pronounced with the interchain disulfide mutant CG288 (Figure 4D). The incorporation of this mutant into 11S hexamer complexes was virtually undetectable, whereas incorporation of the CS44 mutant occurred to a small degree. Consistent results were obtained from repeated performance of these experiments. Therefore, we concluded that correctly formed disulfide bonds play an important role in the assembly of 11S globulin hexamers in vitro.

Transformation and in Vivo Expression of Mutant Legumin B Genes

Cysteine Mutants of LeB Are Processed into Acidic and Basic Chains

The coding regions of unmodified LeB and its cysteine mutants CS44 and CG288 (Figure 1) were placed under the con-

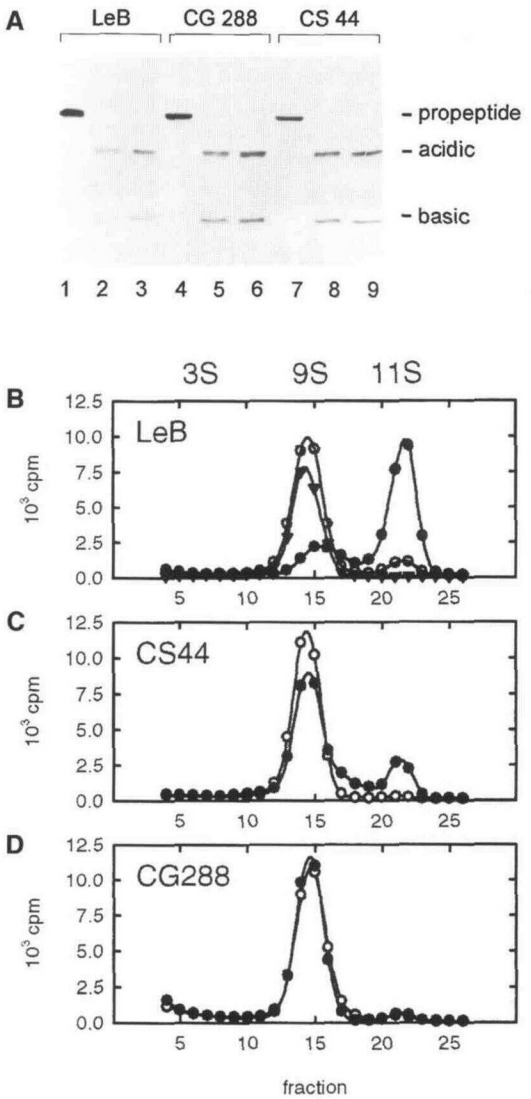

Figure 4. Effect of Disruption of Disulfide Bonds on in Vitro Processing and Assembly of LeB Hexamers.

(A) Effects on proteolytic processing. Purified ^{3}H-leucine–labeled trimers of unmodified LeB (lanes 1 to 3) as well as those of the cysteine mutants CG288 (lanes 4 to 6) and CS44 (lanes 7 to 9) were digested with soybean asparaginyl endopeptidase in the absence (lanes 2, 5, and 8) or presence (lanes 3, 6, and 9) of 10 mM GSSG. The cleavage products were separated in a 12% SDS–polyacrylamide gel and analyzed by fluorography. Lanes 1, 4, and 7 show undigested controls of each proprotein, whose trimers were treated with heat-inactivated asparaginyl endopeptidase.

(B) to **(D)** Effects on reassembly into hexamers. Products from the digestion reactions shown in **(A)** were analyzed by the reassembly assay (Dickinson et al., 1989) in the absence (open circles) or presence (black circles) of 10 mM GSSG. The assay mixtures were separated in sucrose gradients, and the radioactivity in each fraction was determined. Triangles **(B)** show reassembly of undigested LeB trimers as a control. Whereas mutation of both the intrachain **(C)** and interchain **(D)** disulfide bonds affects the formation of 11S hexamers, disruption of the interchain disulfide bond **(D)** virtually abolishes hexamer assembly.

trol of the strong seed-specific legumin B promoter (Bäumlein et al., 1986). The resulting constructs were then introduced into tobacco via Agrobacterium-mediated transformation. DNA gel blot analysis of the regenerated tobacco plants confirmed that six independent transformation events were obtained with the CG288 construction—12 with CS44 and nine with unmodified LeB. Plants containing single-copy insertions were analyzed further (data not shown). The amount of seed-specific gene expression in developing T_1 seed was monitored by RNA gel blot analysis. As is evident from the selected examples shown in Figure 5A, transgenic tobacco lines CG288-1, CS44-11, and LeB-4 displayed the highest level of gene expression among the transformants obtained with each of the individual constructs. Mature seeds from these plants were used for protein analysis. Proteins in seed extracts were separated by SDS-PAGE under either reducing or nonreducing conditions. Blots prepared from these gels were probed with legumin B–specific antibodies (Figures 5B and 5C). The antibodies used for this purpose selected the acidic chains in the precursors and mature subunits. The data in Figure 5B show that mutant subunits CG288 and CS44 were each processed into acidic and basic chains. Differences in the quantity of mutant and unmodified polypeptides correlated roughly with the legumin-specific mRNA levels in developing seed of the corresponding transformants (data not shown). These observations indicate that the differences were not caused by post-translational events during seed development.

The product from the deletion mutant LeB-P1ΔN281 (ΔN) (R. Jung, M.P. Scott, Y.W. Nam, T.W. Beaman, R. Bassüner, I. Saalbach, K. Müntz, and N.C. Nielsen, submitted manuscript) served as a size standard in the experiments shown in Figures 5B and 5C. Asn-281, which is missing in the prolegumin encoded by this mutant, is located at the conserved 11S globulin cleavage site (Hara-Nishimura et al., 1995). Consequently, the cleavage site mutant was unable to form the acidic and basic chains that are typical of mature subunits. In SDS–polyacrylamide gels (Figures 5B and 5C), the product migrated to the same position that would be occupied by mature acidic and basic chains that remain disulfide-bonded together.

Cysteine Mutants of Prolegumin Are Targeted into Protein Storage Vacuoles

To determine whether the mutant legumin polypeptides were deposited in protein storage vacuoles, we homogenized seeds from the transgenic tobacco plants in water-free glycerol. The homogenates were then fractionated into four fractions in potassium iodide–glycerol step gradients as described by Sturm et al. (1987). The fractions were collected and analyzed for protein content and for activity of the vacuolar marker enzyme α-mannosidase. The highest specific α-mannosidase activity was located in fraction 4—an observation consistent with previous reports (Sturm et al., 1987; Saalbach et al., 1991; R. Jung, M.P. Scott, Y.W. Nam, T.W. Beaman, R. Bassüner, I. Saalbach, K. Müntz, and

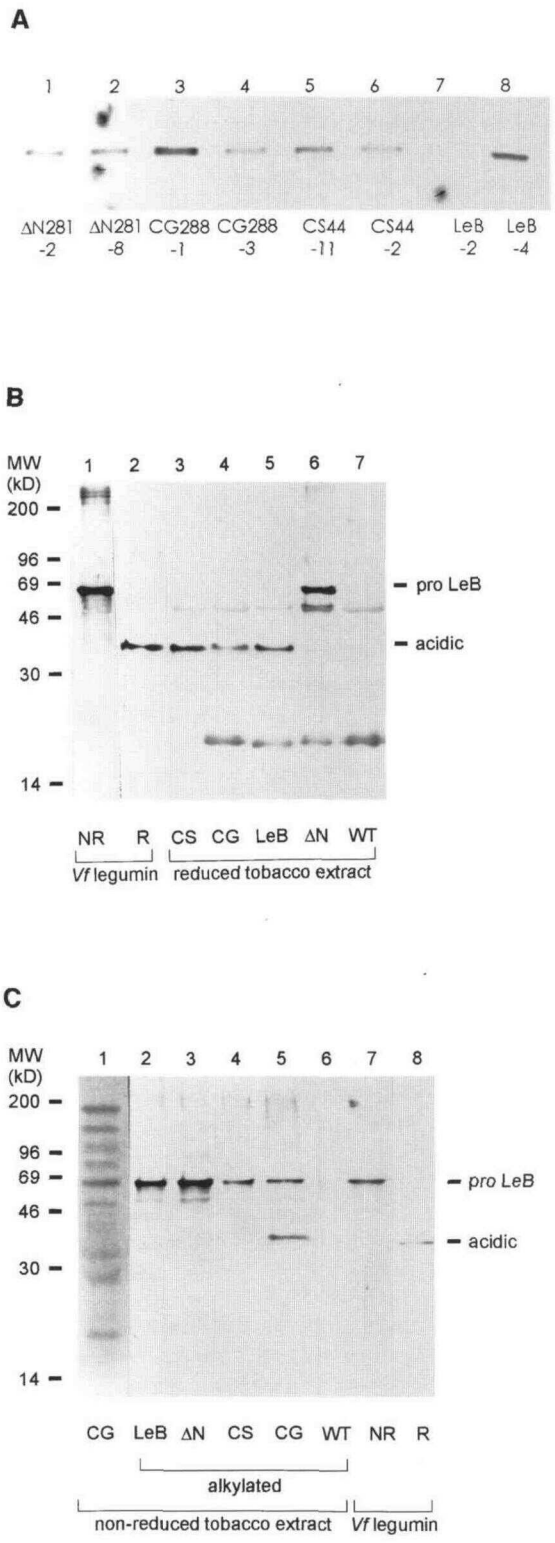

Figure 5. Expression of LeB Cysteine Mutant Genes and Accumulation of the Polypeptides in Transgenic Tobacco Seeds.

N.C. Nielsen, submitted manuscript). This result indicates that protein storage vacuoles are enriched in this subcellular fraction. Equal amounts of protein from each fraction of the gradient were used to perform the protein gel blot analysis (Figure 6). Legumin polypeptides were detected mainly in fraction 4 in transgenic tobacco seeds that contained both the mutant and wild-type genes. The coincidence of vacuolar marker localization signals in the protein gel blots and α-mannosidase enzymatic activity in fraction 4 indicated that the mutant LeB polypeptides were deposited in protein storage vacuoles.

Are Mixed Subunits Formed from Legumin and 11S Tobacco Globulin Chains?

Figure 5C shows a protein gel blot of globulins from tobacco seed extracts whose disulfide bonds were not reduced by treatment with 2-mercaptoethanol. The blot contained proteins of both unmodified LeB and the mutants CS44 and CG288. Because the mutant CS44 and unmodified LeB subunits contained Cys-288, they were both expected to form the interchain disulfide bond and to migrate in SDS-PAGE, as does the unprocessed legumin precursor under nonreducing conditions (cf. lanes 3 and 4 with lane 7 in Figure 5C). The CG288 mutant, on the other hand, lacked Cys-288, the only cysteine residue in the basic chain. The basic chain in this mutant therefore lacked a cysteine residue and would be unable to form the normal disulfide bond to the acidic chain. Consequently, we expected that compared with the acidic chains of LeB and CS44, the acidic chain

(A) Gel blot of total RNA isolated from developing seeds of transgenic tobacco. Each lane contains RNA from independent T_1 transformants of unmodified LeB, a cleavage site deletion mutant ΔN281 (R. Jung, M.P. Scott, Y.W. Nam, T.W. Beaman, R. Bassüner, I. Saalbach, K. Müntz, and N.C. Nielsen, submitted manuscript), and the two cysteine mutants CG288 and CS44. The numbers below each construct (e.g., −2, −8, and −1) designate individual transgenic plants examined. Because of the elevated expression levels, seeds from transformants LeB-4, CG288-1, and CS44-11 were chosen for subsequent experiments.

(B) and **(C)** Protein gel blot analysis of extracts from transgenic tobacco seeds after separation by SDS-PAGE under reducing **(B)** or nonreducing conditions **(C)**. The LeB proteins were identified by use of legumin-specific polyclonal antibodies. *Vf* legumin, legumin controls isolated from fava bean seed that was reduced (R) or not reduced (NR); WT, untransformed tobacco control; CS, CS44; CG, CG288; ΔN, LeB Asn-281 deletion mutant; pro LeB, migration position of LeB propeptide; acidic, migration position of LeB acidic chain. Except for the sample shown in lane 1 of **(C)**, tobacco seed extracts were alkylated with iodoacetic acid during extraction to prevent disulfide bond formation between free sulfhydryl groups. Position of molecular weight (MW) markers is shown at left.

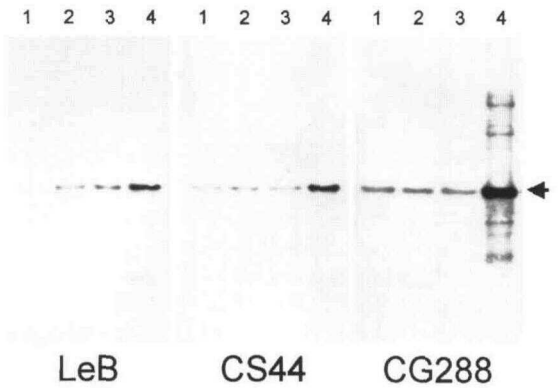

Figure 6. *Localization of* LeB Cysteine Mutant Polypeptides in Protein Bodies of Transgenic Tobacco Seeds.

Transgenic tobacco seeds were homogenized in the presence of water-free glycerol, and the homogenate was centrifuged in a three-step potassium iodide gradient (Sturm et al., 1987). Equal amounts of protein from each of the two top layers (lanes 1 and 3) and from the two interfaces (lanes 2 and 4) were analyzed in gel blots by using legumin-specific polyclonal antibodies that select the acidic chain. The migration position of the acidic chain is indicated by an arrowhead. In plants transformed with either mutant (CS44 or CG288) or unmodified LeB, the lower interface fractions (lane 4) contain the highest α-mannosidase specific activity and are therefore considered to be enriched with protein storage vacuoles. These fractions also contain the greatest amount of legumin-specific polypeptides.

polypeptide from this mutant would migrate at a different position under nonreducing SDS-PAGE conditions.

Surprisingly, however, two acidic chain–specific signals were visible (Figure 5C, lane 5). One was where an acidic chain would go and the second was where the disulfide-linked acidic and basic chains would migrate. Analysis of each of the six independent tobacco transformants expressing mutant CG288 gave the same result. Because this phenomenon was not caused by incomplete processing (see Figure 5B), the most reasonable explanation was that disulfide exchange reactions had caused some acidic legumin chains to become associated with basic chains of tobacco 11S globulins such that mixed subunits formed. The apparent ability of the free thiol group of Cys-87 in the acidic chains of CG288 to interact with thiol groups of other proteins became even more pronounced when iodoacetic acid, a thiol-blocking agent, was omitted from the globulin extraction medium and additional legumin-specific signals were detected in immunoblots (Figure 5C, lane 1). Legumin-specific antibodies were able to cross-react to some extent with 11S tobacco globulin subunits (see Figures 5B and 5C); therefore, attempts to immunoprecipitate in vivo–radiolabeled complexes of the CG288 acidic chain with tobacco polypeptides resulted in ambiguous results (data not shown).

Cysteine Mutants of Legumin Assemble into Hexamers in Vivo

To determine whether disruption of disulfide bonds in legumin mutant polypeptides interfered with the ability of the mutant proteins to be incorporated into 11S hexameric complexes (as observed during the in vitro experiments), we fractionated tobacco seed globulin extracts by centrifugation in sucrose density gradients. Figure 7 shows results obtained when aliquots from the fractions were analyzed in immunoblots after separation by SDS-PAGE. In contrast to the results from the in vitro experiments (see Figure 4), the majority of CS44 (Figure 7A) and CG288 (Figure 7B) mutant polypeptides were associated with 11S hexamers.

Effect of Mixed Assembly of Unmodified and Mutant Globulins

To estimate the amount of legumin accumulated in transgenic tobacco seed, we measured the signal observed in immunoblots after the addition of varying amounts of the authentic protein from fava bean. This calibration permitted an estimation of the fava bean legumin/tobacco 11S globulin ratio in seeds of the transgenic plants to be between 1:100 and 1:500 (data not shown). Because of the prevalence of the tobacco subunits, we considered the possibility that the fava bean legumin and tobacco 11S globulins became incorporated together into mixed trimers and hexamers in situ. This reaction is conceivable because genes coding for different subunits of globulins are expressed exclusively during seed embryogenesis (Goldberg et al., 1989), and subunits that enter the ER cotranslationally could encounter and interact with one another during assembly into trimers. Mutant subunits that are aberrantly folded due to the absence of a disulfide bond might be accommodated into mixed complexes, despite the fact that they would be unable to form homogeneous hexamers (Figure 4).

To test this hypothesis, we performed in vitro reassembly experiments with mixed trimers containing both unmodified and mutant LeB subunits. A model shown in Figure 8A represents the concept and the expected consequences of such an experiment. Trimers containing subunits of either the unmodified LeB or the interchain disulfide mutant CG288 are produced in vitro. The mixed complexes are processed and tested for their ability to interact with excess amount of dissociated 11S globulins during reassembly into hexamers.

For the experiment shown in Figure 8B, LeB and CG288 subunits were differentially radiolabeled with carbon-14 and hydrogen-3, respectively, so that their fate during reassembly into mixed complexes could be followed. The two separate in vitro translation reactions were terminated with cycloheximide to prevent further incorporation of radioactively labeled amino acids into the growing polypeptide chains. Immediately after termination of synthesis, equal

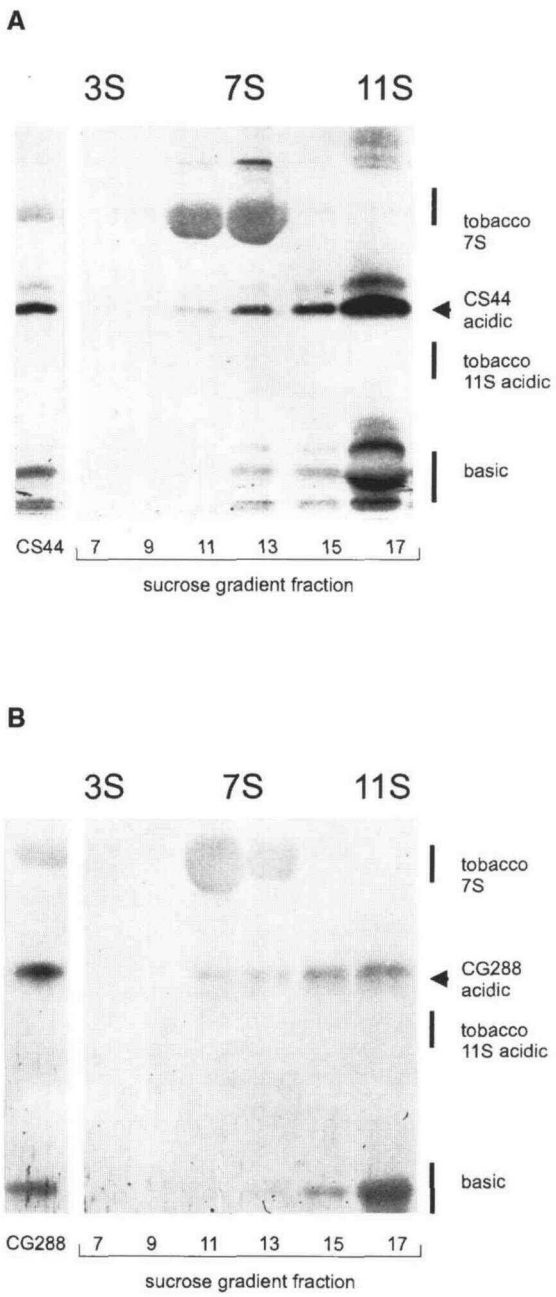

Figure 7. Sedimentation Analysis of LeB Cysteine Mutant Subunits That Assembled in Transgenic Tobacco Seeds.

Globulins from transgenic tobacco seeds were separated in sucrose gradients, and fractions from the gradient were analyzed by SDS-PAGE and immunoblotting by using legumin-specific polyclonal antibodies. The positions of sedimentation markers (3S [globin], 7S [vicilin], and 11S [legumin]) are indicated above the lanes, whereas fraction numbers from the sucrose gradient are shown at the bottom. The leftmost lanes contain samples of extracts before separation in sucrose gradients. The legumin acidic chain immunoreactive signals (arrowheads) are associated mainly with the 11S globulins (fraction 17). Bars at right indicate the positions to which the tobacco 7S and 11S polypeptides migrate after electrophoresis.

(A) In vivo assembly of CS44 with a disrupted intrachain disulfide bond.

(B) In vivo assembly of CG288 with a disrupted interchain disulfide bond.

amounts of ^{14}C-labeled LeB and ^{3}H-labeled CG288 subunits were mixed and incubated to permit assembly of heterogeneous trimers. Because the assembly of proglobulin trimers normally takes a long period of incubation (Figure 2) and the translation of these two subunits lasted for only 30 min in the absence of GSSG, most subunits present at the time of mixing probably had little chance to engage in intermolecular association for assembly into trimers. Analysis of aliquots taken from the mixture at various time intervals during the assembly confirmed that translation was indeed terminated (data not shown). The heterogeneous trimers produced in this manner were then isolated by sucrose density gradient centrifugation. The purified trimers were digested with the asparaginyl endopeptidase, and the reassembly assay was performed either in the absence or presence of GSSG.

Products from the reassembly experiment were separated in sucrose gradients, and the hydrogen-3 and carbon-14 contents of fractions from the gradient were measured. This procedure permitted assessment of the distribution of both types of subunits between the 9S (trimer) and the 11S (hexamer) fractions of the gradient. In the absence of GSSG (Figure 8B), neither mutant CG288 (Figure 8B, right) nor the unmodified LeB subunits (Figure 8B, left) were capable of efficient assembly into hexamers. In the presence of GSSG (Figure 8B), however, both carbon-14 due to unmodified LeB subunits (Figure 8B, left) and hydrogen-3 due to mutant CG288 subunits (Figure 8B, right) were found in the hexamer fractions. Thus, unlike trimers that contain only mutant CG288 subunits, which cannot assemble into hexamers after subunit processing (Figure 4D), mixed trimers that contain both modified and unmodified subunits are capable of assembly into hexamers (see Discussion).

DISCUSSION

Assembly of Proglobulin Trimers Is Stimulated by an Oxidative Redox State

The effect of modifications to the redox state in rabbit reticulocyte lysates was monitored to determine the extent to which it affected the assembly of 11S storage globulins. The reticulocyte lysate is considered to be in a cytosolic condition under which a GSH/GSSG ratio ranges from 30:1 to 100:1 (Hwang et al., 1992). Although the precise redox state of the lysates used for this study has not been determined, it

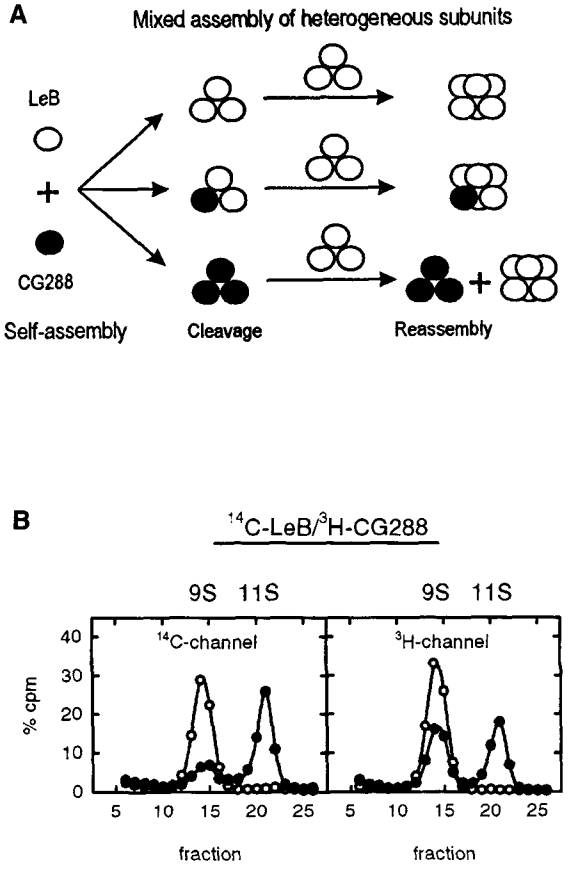

Figure 8. Assembly of Heterogeneous Complexes Composed of Unmodified and Cysteine Mutant LeB Subunits.

(A) Model depicting heterogeneous subunit assembly in vitro (see text). Open circles, LeB; black circles, CG288 interchain disulfide mutant.

(B) Reassembly of mixed trimers containing differentially labeled LeB and CG288 subunits into hexamers. Equal volumes of translation mixtures containing either ^{14}C-LeB or ^{3}H-CG288 were combined and incubated to form mixed trimers. Subunits in the mixed trimers were isolated by sucrose density gradient centrifugation and digested with soybean asparaginyl endopeptidase. The processed subunits were then reassembled in the absence (open circles) or presence (black circles) of 10 mM GSSG, according to Dickinson et al. (1989). After separation of reassembly products in sucrose gradients, radioactivity due to either ^{3}H-CG288 or ^{14}C-LeB in each gradient fraction was determined. Unlike homogeneous trimers that contain only CG288 subunits and cannot assemble into hexamers (Figure 4D), mixed trimers consisting of LeB and CG288 subunits are capable of hexamer assembly to a substantial extent.

is likely to be highly reducing because according to the manufacturer (Promega) it contains 2 mM DTT. A reducing condition is required for efficient translation in the lysate (Scheele and Jacoby, 1982; Kaderbhai and Austen, 1985; Jagus, 1987).

We demonstrated that the addition of GSSG in concentrations up to 10 mM to the lysate to shift the mixture to a more oxidized state stimulated the rate of assembly of proglobulin trimers. The same addition did not affect protein synthesis. These results are in accordance with previously reported cases in which supplementation of lysates with exogenous GSSG at concentrations of 2 to 5 mM facilitated correct disulfide formation of prolactin (Kaderbhai and Austen, 1985) and pancreatic exocrine proteins (Scheele and Jacoby, 1982). The threshold concentration of added GSSG needed for maximal effect of the assembly of proglobulin trimers was 1.0 mM (Table 1). The physiological concentration of total glutathione is estimated to be 1 to 10 mM (Hwang et al., 1992). Because part of the added GSSG would rapidly be turned to GSH as a result of existing thiols in DTT, the addition of 1 mM GSSG probably changed the GSH/GSSG ratio to less than 10:1. The latter ratio is believed to be that necessary for efficient thiol–disulfide exchange reactions (Saxena and Wetlaufer, 1970).

Disulfide bond formation and folding of secretory proteins normally begin on the nascent polypeptide chain (Bergman and Kuehl, 1979; Braakman et al., 1991), and it is likely that the same thing happens during the folding process in vitro. The observation that an oxidized redox environment stimulates trimer assembly indicates that disulfide bond formation is important if proglobulin subunits are to be folded into the conformations required for an efficient assembly of trimers. In this regard, thiol–disulfide interchange reactions have been suggested as a rate-determining step in the folding of many proteins that contain disulfide bonds (Creighton, 1986). Structural differences among various globulin subunits might affect the rate of trimer assembly with respect to this process. For example, the G4 subunits from soybean assemble much more slowly in the absence of GSSG than do LeB subunits from broad bean. In an oxidizing redox environment, however, they both assemble to a similar extent after 6 hr (Table 1). It is possible that the two extra cysteine residues present in G4, compared with LeB, contributed to the slower folding in the absence of GSSG. In vitro, at least, disulfide bond formation is more rate limiting for the assembly of G4 subunits than for LeB subunits.

We have been unable to demonstrate that the stimulation of proglobulin trimer assembly by an oxidizing redox condition is mediated by the protein-folding catalyst PDI. It has been shown that thiol–disulfide interchange reactions of some proteins are achieved without catalysis by PDI (Wetlaufer, 1984). The correct three-dimensional structure of lysozyme is attained by nonenzymatic reactivation of the reduced protein in mixtures of GSSG and GSH (Saxena and Wetlaufer, 1970). Also, the rate of the oxidative renaturation of some proteins is strongly dependent on the composition of the redox buffer. During renaturation of ribonuclease A, the catalytic effectiveness of PDI is diminished under more oxidizing conditions (Lyles and Gilbert, 1991).

The reticulocyte lysate is a complex milieu containing many unknown factors, and it might be difficult to predict a

precise redox condition that would permit optimal activity of PDI. It has been suggested that different molecular chaperones can exert complementary effects of folding and assembly of newly translocated polypeptides in the lumen of the ER (Gething and Sambrook, 1992; Vitale et al., 1993). In this regard, we showed that the folding and/or assembly of proglobulin subunits in reticulocyte lysate is most likely mediated by its endogenous chaperone system that operates with the Hsp70 and TRiC chaperones (Nam et al., 1997). It remains to be determined whether supplementation of the reticulocyte with other molecular chaperones, such as BiP and Hsp70, would cause PDI to have an observable effect on trimer assembly.

It is interesting that the formation of disulfide bonds in the proglobulins is not necessarily required for trimer formation. To demonstrate this, mutations were generated that eliminated cysteine residues involved either in the formation of the conserved interchain bond between acidic and basic chains or of an intrachain disulfide bond in the acidic chain. Although the lack of the interchain disulfide decreased the rate of assembly in vitro, lack of the intrachain bond did not detectably influence the assembly process. Nonetheless, because assembly occurred, it was clear that the subunits could achieve a conformation that permitted assembly despite the absence of the interchain disulfide bond. Apparently, the extent of aberrant folding that occurred after translation of the interchain disulfide mutant protein was not sufficiently deleterious to hinder the subsequent intermolecular association during trimer assembly. The mutant subunits were also accumulated in protein bodies in seeds from transgenic tobacco. Because proper folding and oligomerization of secretory proteins in the ER are considered prerequisite to their further transport along the secretory pathway (Hurtley and Helenius, 1989; Pelham, 1989; Chrispeels, 1991; Vitale et al., 1993), the mutant legumin subunits must have become incorporated into trimers despite the lack of disulfide bonds. Although the data suggest that disulfide bonds are not required to achieve a protein conformation suitable for oligomer formation, the presence of disulfide bonds probably helps to stabilize those conformations and thereby stimulates oligomerization. These results are in accordance with the results of Utsumi et al. (1993), who found that cysteine mutants of proglycinin G3 failed to prevent assembly of trimers in *E. coli*.

The Interchain Disulfide Bond Is Essential for the Assembly of 11S Globulin Hexamers

The availability of a purified asparaginyl endopeptidase capable of correctly processing proglobulins into acidic and basic chains (Scott et al., 1992) permitted an assessment of the role of disulfide bonds during assembly of trimers into hexamers. Cleavage into acidic and basic chains is a prerequisite for this assembly process (Dickinson et al., 1989; R. Jung, M.P. Scott, Y.W. Nam, T.W. Beaman, R. Bassüner, I. Saalbach, K. Müntz, and N.C. Nielsen, submitted manuscript). In this article, we demonstrate not only that the presence of an oxidizing redox environment stimulates hexamer formation but that disruption of the interchain disulfide bond linking the acidic and basic chain leads to a complete lack of assembly of trimers into hexamers. The inability of the mutant CG288 subunits to assemble into hexamers occurred despite the fact that these subunits were correctly processed into acidic and basic chains. The latter observation is of considerable interest because it has previously been demonstrated that conformational changes in the subunits can lead to complete degradation of the subunits (Jung et al., 1993). Therefore, it is unlikely that the Cys-to-Gly mutation to LeB altered the shape of the molecule to the extent that additional asparagine residues became exposed, as would be required for subunit degradation. In this regard, cleavage of the proglobulin into acidic and basic chains has been suggested to elicit a conformational change that facilitates the trimer-to-hexamer transition (Dickinson et al., 1989). The fact that trimers composed of mutant legumin CG288 are unable to form hexamers suggests that the interchain disulfide bond plays a critical role in the adjustment to a three-dimensional structure that is suited for association in mature hexamers.

Mixed Assembly of Different 11S Globulin Subunits Enables the Disulfide Mutant Subunits to Enter into Hexamers

An intermingling of acidic and basic chains from tobacco and fava bean apparently occurred in the 11S complexes isolated from tobacco transformed with mutant legumin genes. The disulfide bonds that stabilized these hybrid subunits could have formed during assembly of the complexes in the seed, or they could have resulted from disulfide exchange reactions that took place during purification of the complexes. The interchain disulfide bonds are labile during purification of soybean glycinin, and it is necessary to inhibit disulfide exchange reactions to purify subunits composed of disulfide-bonded acidic and basic chains (Staswick et al., 1984). These interactions can be minimized by alkylation of free sulfhydryl groups before isolation of the subunits. Prevention of disulfide exchange reactions between the CG288 mutant and other seed proteins by alkylation with iodoacetic acid may have stabilized noncovalent interactions between the mutant acidic and basic chains, in addition to preventing the formation of the extraneous disulfide bonds as described in Figure 5C. Although such a consideration would support the suggestion that the hybrid subunits form in vivo, formation of hybrid disulfide-bonded 11S subunits in vitro has also been described. Mori et al. (1979) reported that disulfide bonds formed in vitro when subunits composed of soybean and sesame acidic and basic chains were reassembled, and Dickinson et al. (1989) showed that purified radiolabeled acidic chains were incorporated into trimers

and hexamers during hexamer reassembly in vitro. Therefore, the available evidence does not permit distinction between these two possibilities.

Although the legumin CG288 subunits were incapable of assembly into hexamers in vitro, the mutant subunits nevertheless accumulated as hexamers in the seeds of transgenic tobacco. This apparent discrepancy arose because the trimers used in vitro were composed of only mutant subunits, whereas those formed in situ probably contained a heterogeneous mixture of mutant and unmodified subunits. To demonstrate this, mixtures of mutant and unmodified subunits were formed in vitro; in the presence of GSSG, the resulting trimers were able to form hexamers. However, as shown in Figure 8B, the extent to which each of the two differentially radiolabeled subunits was incorporated into hexamers varied. Whereas more than two-thirds of the carbon-14 due to unmodified LeB subunits was incorporated into hexamers (Figure 8B, left), only approximately half of the hydrogen-3 due to the mutant CG288 subunits ended up in the hexamer fractions of the sucrose gradients (Figure 8B, right). This observation is likely due to the presence of homogeneous complexes containing only LeB or the mutant subunits in the reassembly mixture. These homogeneous complexes have been produced concurrently during the assembly of mixed trimers and would comprise a significant proportion of the total trimer population if the intermolecular association among individual subunits during trimer assembly had taken place at random (Figure 8A). Consequently, the carbon-14 or hydrogen-3 label determined after reassembly would include not only the same label originating from the heterogeneous complexes but also that contributed by the homogeneous counterparts.

The explanation outlined above accounts for the relatively unchanged distribution pattern of the carbon-14 label due to LeB (Figure 8B, left) compared with the pattern observed when the homogeneous LeB trimers were used for reassembly (Figure 4B). Likewise, hydrogen-3 due to the mutant CG288 would include the label from the homogeneous CG288 complexes, which were unable to assemble into hexamers (Figure 4D) and therefore remained as trimers (Figure 8B, right). From these lines of reasoning, it is clear that the substantial level of the hydrogen-3 label that was found to be incorporated into the hexamer fractions is indeed due to the CG288 subunits comprising the heterogeneous trimers (Figure 8B, right). Similar results were obtained when reciprocally labeled mutant and unmodified subunits were used to produce mixed trimers (data not shown). Although it is also possible to interpret these data as showing that aberrantly folded mutant CG288 subunits had a negative impact on assembly when present in mixed trimers, the results do support our conclusion that mixed 11S globulin complexes are capable of assembly into hexamers.

The results from the mixed assembly experiments indicate that some structural variation among subunits can be tolerated within the trimers before hexamer assembly is prevented. The ability to compensate for minor structural variation was anticipated because heterogeneity exists among the family of subunits able to interact in oligomers. This feature is common to both the 11S and 7S families of seed proteins. Without more precise structural information about the shape of the molecules and an identification of the parts of the molecules critical for assembly, it will be difficult to predict the extent of structural variability tolerated before assembly is compromised. Nonetheless, this tolerance raises the interesting possibility of cotransporting through the secretory system other molecules that are capable of interacting with the 11S and 7S seed storage proteins.

METHODS

Materials

Glutathione (GSH), glutathione disulfide (GSSG), and diamide (diazenedicarboxylic acid *bis*[*N*,*N*-dimethylamide]) were purchased from Sigma. Protein disulfide isomerase (PDI) was a gift from R. Noiva (University of South Dakota, Vermillion; Noiva and Lennarz, 1992). Asparaginyl endopeptidase was purified from developing soybean cotyledons, according to R. Jung, M.P. Scott, Y.W. Nam, T.W. Beaman, R. Bassüner, I. Saalbach, K. Müntz, and N.C. Nielsen (submitted manuscript). Dissociated, native glycinin preparations were prepared as described by Dickinson et al. (1989).

Plasmids

Plasmids pSP65/248 (Dickinson et al., 1987) and pLeMS (Scott et al., 1992) have been described previously. Plasmids pLeBCG288 and pLeBCS44 are the mutagenized derivatives of pLeMS in which codons for Cys-288 and Cys-44 were replaced with those for glycine and serine, respectively. After oligonucleotide-directed in vitro mutagenesis (Kunkel et al., 1987), these constructs were sequenced (Sanger et al., 1977) to ensure that only the desired mutation occurred. Before use as templates for in vitro transcription, the plasmids were linearized with either PvuII (pSP65/248) or HindIII (pLeMS and its derivatives).

In Vitro Synthesis and Assembly of Proglobulins with Glutathione

mRNAs transcribed in vitro from each plasmid were used for translation in a rabbit reticulocyte lysate (nuclease treated; Promega) essentially as described by Dickinson et al. (1987). Typically, reaction mixture with a total volume of 25 μL was prepared; the mixture included 0.8 μM ^{3}H-leucine (150 Ci/mmol; Amersham) and 5 mM GSSG. For the experiments shown in Table 1, GSH, GSSG, or GSH/GSSG was added to the designated final concentrations at the beginning of or immediately after the completion of translation. Translation was performed at 30°C for 1 hr and terminated by the addition of 0.5 mM cycloheximide. Then, the synthesized polypeptides were permitted to self-assemble for 6 hr. The assembly mixtures were analyzed by separation in sucrose density gradients. The radioactivity

in each gradient fraction was determined by scintillation counting after precipitation with trichloroacetic acid (Dickinson et al., 1987).

Mixed Assembly of Proglobulin Subunits

Subunits of LeB and CG288 were synthesized in reticulocyte lysates from their respective expression plasmids in the presence of ^{14}C-leucine (30 mCi/mmol; Amersham) or ^{3}H-leucine (150 Ci/mmol; Amersham), respectively. The ^{14}C-leucine received from the manufacturer was concentrated 50-fold in a Speedvac concentrator (Savant, Holbrook, NY) before use. Translation reactions were terminated after 30 min by addition of 0.5 mM cycloheximide, and mixtures containing differentially labeled subunits were immediately combined by mixing equal-volume aliquots of each subunit. These mixtures were incubated for 24 hr to permit assembly. After assembly, the mixtures, which contained both monomers and trimers, were separated in sucrose density gradients, and the 9S trimer fractions were purified, concentrated, and used for in vitro processing and reassembly experiments.

Treatment of Proglobulin Trimers with Soybean Asparaginyl Endopeptidase

Labeled trimers of LeB and its cysteine mutants CS44 and CG288 were isolated from in vitro assembly mixtures by sucrose density gradient fractionation. Pooled fractions containing these trimers were concentrated to ~50,000 cpm/μL with a Centricon-30 microconcentrator (Amicon) pretreated with acetylated BSA. During concentration, the buffer was changed to 35 mM phosphate, pH 7.6, 0.4 M NaCl. Purified and concentrated trimers were digested with an active asparaginyl endopeptidase preparation isolated from developing soybean cotyledons (R. Jung, M.P. Scott, Y.W. Nam, T.W. Beaman, R. Bassüner, I. Saalbach, K. Müntz, and N.C. Nielsen, submitted manuscript) in a citrate phosphate buffer (62.4 mM Na_2PO_4, 18.8 mM citric acid, 100 mM sodium acetate, 10 mM DTT), pH 5.8. Typically, 1 μL of the processing enzyme was added to trimers of ~100,000 cpm (hydrogen-3) in the presence of 10 mM GSSG, and 25 μL of total reaction mixture was incubated at 25°C for 16 hr. Trimers were preincubated on ice in 35 mM phosphate, pH 7.6, that contained 0.4 M NaCl and 10 mM GSSG for 30 min before digestion. After digestion, the products were separated by SDS-PAGE (Laemmli, 1970) and analyzed by fluorography as described by Scott et al. (1992).

Reassembly Procedure

To 25 μL of digestion mixture that contained trimers cleaved with soybean asparaginyl endopeptidase, 10 μL of dissociated native glycinin (5.0 mg/mL) was added. This step was quickly followed by addition of 2.5 μL of 175 mM phosphate, pH 7.6, and 2.0 M NaCl; the mixture was then vortexed. The addition of dissociated glycinin and the buffer was repeated once more, and the final volume was adjusted to 100 μL with 35 mM phosphate, pH 7.6, and 0.4 M NaCl. The GSSG concentration was maintained at 10 mM during reassembly. Reaction mixtures were incubated at 25°C for 8 hr and analyzed after separation in sucrose density gradients. Of each fraction from the gradients, 100 μL was assayed for protein concentration (Bradford, 1976), and 200 μL was analyzed for direct radioactivity measurement. The distribution of hydrogen-3 or carbon-14 labels in trimers and hexamers was determined by scintillation counting in appropriate channels.

Plant Transformation

The transformation of tobacco with DNA coding for LeB and its cleavage site (LeB-P1ΔN281) and disulfide mutants (CS44 and CG288) was performed by *Agrobacterium tumefaciens*–mediated leaf disc infection, as described by Bäumlein et al. (1987). Transformation and expression of the genes were verified by DNA and RNA gel blotting. Mature seeds from transgenic plants (T_1 generation) were harvested and used for the analyses reported in this study.

Analysis of Extracts from Tobacco Seeds

Globulin extracts were prepared from transgenic tobacco seeds essentially as described by Jung et al. (1993), except that iodoacetic acid (10 mM) was generally included in protein extraction buffers to alkylate-free thiol groups. Concentrated globulin preparations were separated in SDS gels (Laemmli, 1970) in the absence or presence of 2-mercaptoethanol. Protein samples in the gel were transferred to nitrocellulose (Towbin et al., 1979), and the blot was treated with antibody preparations raised against legumin B (Saalbach et al., 1991) and analyzed subsequently (Jung et al., 1993). Alternatively, the globulin extracts were centrifuged in 6 to 22% linear sucrose density gradients. Fractionated samples after centrifugation were analyzed by SDS-PAGE and protein gel blotting, as described previously (Jung et al., 1993). Tissue homogenates, which were prepared from transgenic tobacco seeds in water-free glycerol, were fractionated by potassium iodide–glycerol step gradient centrifugation according to Sturm et al. (1987).

ACKNOWLEDGMENTS

We thank Dr. Robert Noiva for supplying protein disulfide isomerase. We also thank Drs. Larry Dunkle and Mark Hermodson for critical reading of the manuscript and Jean Galbraith for editing it. This work was supported in part by U.S. Department of Agriculture National Research Initiative Competitive Grant No. 91-37307-6517 and American Soybean Association Grant No. SPR-2305 to N.C.N.

Received May 16, 1997; accepted September 17, 1997.

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