Role of Individual Disulfide Bonds in the Structural Maturation of a Low Molecular Weight Glutenin Subunit*

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Gliadins and glutenins are the major storage proteins that accumulate in wheat endosperm cells during seed development. Although gliadins are mainly monomeric, glutenins consist of very large disulfide-linked polymers made up of high molecular weight and low molecular weight subunits. These polymers are among the largest protein molecules known in nature and are the most important determinants of the viscoelastic properties of gluten. As a first step toward the elucidation of the folding and assembly pathways that lead to glutenin polymer formation, we have exploited an in vitro system composed of wheat germ extract and bean microsomes to examine the role of disulfide bonds in the structural maturation of a low molecular weight glutenin subunit. When conditions allowing the formation of disulfide bonds were established, the in vitro synthesized low molecular weight glutenin subunit was recovered in monomeric form containing intrachain disulfide bonds. Conversely, synthesis under conditions that did not favor the formation of disulfide bonds led to the production of large aggregates from which the polypeptides could not be rescued by the post-translational generation of a more oxidizing environment. These results indicate that disulfide bond formation is essential for the conformational maturation of the low molecular weight glutenin subunit and suggest that early folding steps may play an important role in this process, allowing the timely pairing of critical cysteine residues. To determine which cysteines were important to maintain the protein in monomeric form, we prepared a set of mutants containing selected cysteine to serine substitutions. Our results show that two conserved cysteine residues form a critical disulfide bond that is essential in preventing the exposure of adhesive domains and the consequent formation of aberrant aggregates.

Gliadins and glutenins are the major storage proteins that accumulate in wheat endosperm cells and are largely responsible for the unique suitability of wheat flour for bread-making. Because of their nutritional and technological importance, these proteins have been the target of a variety of studies concerning their biochemical features, their synthesis and intracellular transport (1).

The polymerization state is a critical feature distinguishing gliadins from glutenins. Although gliadins (which are divided into α, γ, and ω types) are largely recovered in monomeric form, glutenins consist of large polymers whose building blocks are the high molecular weight (HMW) and the low molecular weight (LMW) glutenin subunits. High molecular weight glutenin subunits are constituted by a central repetitive domain flanked by two nonrepetitive regions containing cysteine residues critical for glutenin cross-linking. Two regions can instead be recognized in the primary structure of LMW glutenin subunits; one region is an N-terminal domain largely made up of repeated sequences, and the other is a C-terminal domain of unique sequence, where all the intrachain disulfide bonds are located (Fig. 1). Low molecular weight glutenin subunits are structurally related to monomeric α- and γ-gliadins but critically contain two additional cysteine residues that remain available for the formation of interchain disulfides and that have been proposed to be responsible for the polymeric nature of these proteins (2). One of these residues is located in the N-terminal region of the polypeptide (at two alternative positions, designated Cα and Cαβ (3–5)), whereas the other (Cγ) is located in the C-terminal domain (Fig. 1). Although the detailed structure of glutenin polymers remains unknown, available data indicate that LMW glutenin subunits are linked via disulfide bonds not only to other subunits of the same class but also to HMW glutenin subunits and to polypeptides related to γ-gliadins (2).

Information about the structure of the individual LMW glutenin subunits is also rather sparse, and this is in part due to difficulties in applying x-ray diffraction or NMR analysis to these polypeptides. However, important information is provided by studies on the disulfide bonding pattern of these proteins, as deduced from the analysis of cystine peptides derived from whole glutenin (3, 4, 6). Intrachain and interchain disulfide bonds cannot be unequivocally distinguished by these chemical mapping studies, but the presence of three intrachain disulfide bonds in LMW glutenin subunits can be inferred by analogy with the situation found in monomeric gliadins (6–9) and on the basis of in vitro refolding studies (10). The position of some of the cysteine residues that are involved in these intrachain disulfide bonds is conserved not only in gliadin polypeptides but also in cereal α-amylase/trypsin inhibitors and in 2S albumin storage proteins (11). This suggests that all these proteins may share a common fold in which disulfide bonds play an important and perhaps essential role. Still, direct information about the role of intrachain disulfide bonds in the folding of all these proteins and of LMW glutenin subunits in particular is rather sparse (12, 13).

Many aspects of glutenin polymer assembly, transport, and deposition also remain unclear. Because gliadins and glutenins extracted from mature grains are largely insoluble in aqueous

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Footnotes:
1 The abbreviations used are: HMW, high molecular weight; LMW, low molecular weight; DTT, dithiothreitol; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis.
solutions, it was initially suggested that these proteins spontaneously precipitate and rapidly form insoluble deposits immediately after insertion in the endoplasmic reticulum (ER). However, this hypothesis was difficult to reconcile with the idea that storage proteins must have specific characteristics that allow both their efficient packaging during seed development and their rapid rehydration upon germination. Indeed, more recent studies show that the assembly of the gliadin fraction occurs in a slow and possibly ordered fashion and involves the formation of soluble monomeric intermediates (13). The pathway leading to the formation of the glutenin polymer remains unknown, but it also probably involves the regulated assembly of soluble monomeric subunits. It is also likely that glutenin polymer assembly begins in the ER, where protein folding and assembly can be assisted by molecular chaperons and folding enzymes (14). Most importantly, studies performed in yeast and mammalian cells indicate that this compartment contains an oxidase machinery that plays an essential role in disulfide bond formation and that could therefore also assist the biogenesis of the disulfide-linked glutenin polymer (15). Consistent with this view, immuncytochemical analysis of thin sections from developing wheat seeds indicates that a large fraction of wheat storage proteins assemble inside the ER into so-called “protein bodies,” which are then transported via an autophagic process to the storage vacuole (16).

Given the complex situation found in wheat endosperm cells, wheat storage protein structural maturation can be more easily studied using simplified systems, such as transgenic plants expressing individual subunits (17) or in vitro translation systems. Indeed, this latter kind of approach has been successfully applied to a number of systems obtained from bean cotyledons to investigate the role of specific cysteine residues in the structural maturation of a LMW glutenin subunit (16). In this work, we have exploited an in vitro system constituted of a wheat germ extract and microsomes obtained from bean cotyledons to investigate the role of specific cysteine residues in the structural maturation of a LMW glutenin polypeptide. Our results suggest that formation of soluble monomeric subunits is an early step on the pathway of glutenin polymer assembly and indicate that one intrachain disulfide bond plays a major role in monomer maturation, possibly by maintaining adhesive domains in a buried state and, thus, preventing the precocious aggregation of the newly synthesized polypeptides.

**Experimental Procedures**

**Recombinant DNA Techniques**—Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. Silent mutations creating or eliminating diagnostic restriction sites were introduced to simplify the detection of recombinant clones. The coding sequence of the mutated clones was examined to confirm the introduction of the desired mutations and to exclude the fortuitous insertion of undesired ones.

To prepare a construct (pBSΔSP) coding for a mutant LMW glutenin subunit lacking the putative signal peptide (25), the B11-33-coding sequence was amplified with oligonucleotides CGCAGATCTCTGGAGAC-GTACGAGTGGAGACTGAC and GAATTCAGATCTTTATCAGT-AGGCAACACTTCGGG using pSP64a-B1133 (a kind gift from Gad Galili) as template. The amplification product was digested with BglII and cloned into BglII cut pSP64T (26). A BglII fragment containing the coding sequence was then isolated and cloned into the BamHI site of vector pBluescript II KS+ (Stratagene).

In *Vitro Transcription and Translation*—Plasmid pSP64a-B1133 or derivatives of this plasmid bearing the various cysteine to serine substitutions were linearized with EcoRI and transcribed in *vitro* with SP6 polymerase. Plasmid pBSASP was linearized with XbaI and transcribed using T3 polymerase. In *vitro* transcriptions were performed in the presence of the cap analogue m’5’Gppp’5’G (Amersham Pharmacia Biotech), as previously described (27). The transcripts were visually quantified by comparison to appropriate standards on formaldehyde/agarose gels and stored at −80 °C.

Nuclease-treated microsomes from mid-maturation Phaseolus vulgaris cotyledons were prepared as described (27). Synthetic mRNAs were translated in *vitro* in a reaction containing (for a final volume of 12.5 µl) 6.25 µl of wheat germ extract (Promega, prepared in a buffer containing 10 mM dithiothreitol (DTT), 0.25 µl of 40 units/µl RNaseout (Life Technologies, Inc.), 1 µl of 1 mM amino acid mixture (minus leucine), 0.9 µl of 1 M potassium acetate, 1.2 µl of [3H]leucine (120–190 Ci/mmol), 5 µCi/µl, Amersham Pharmacia Biotech), 1 µl of mRNA solution (100 ng/µl). When indicated, brefeldin A (1–4 µg/ml) and/or oxidized glutathione (from a 12.5 times stock in water, pH 7.8) were included in the translation reaction. In most experiments the amino acid mixture and the [3H]-labeled leucine were dried down in a speed-vac concentrator before assembling the reaction. Translations were performed at 25 °C and were terminated by the addition of RNase A or cycloheximide at a final concentration of 50 µg/ml or 2 mM, respectively. For samples that were separated under nonreducing conditions, iodoacetamide was added to a final concentration of 100 mM. Samples were incubated 10 min on ice, mixed with 6 volumes of 20 mM Tris-HCl pH 8.6, 6% SDS, 8% glycerol, 0.01% bromophenol blue, and then heated for 5 min at 100 °C.

For separation under reducing conditions, samples were mixed with 6 volumes of 200 mM Tris-HCl, pH 8.6, 6% SDS, 8% glycerol, 0.01% bromophenol blue and heated for 5 min at 100 °C. After cooling, iodoacetamide was added to a final concentration of 100 mM, and samples were incubated for 45 min at room temperature. For the protease protection assay, aliquots of the translation reaction were treated as described previously (27), except that incubation was for 15 min at 25 °C with a proteinase K concentration of 50 µg/ml.

**SDS-PAGE** was performed using the system of Laemmli (28), with a separating gel containing 15% acrylamide and an acrylamide/bisacrylamide ratio of 200:1. Gels were treated for fluorography as described by Bonner and Laskey (29).

**Sedimentation Velocity Analysis on Sucrose Gradients**—For sedimentation velocity analysis on sucrose gradients, in *vitro* translations were terminated by adding cycloheximide at a final concentration of 2 mM. N-Ethylmaleimide was then added at a final concentration of 40 mM, and microsomes were recovered by centrifugation at 35,000 rpm for 10 min at 4 °C in a SW 55 Ti rotor (Beckman Instruments) through a high salt/sucrose cushion (250 mM sucrose, 500 mM potassium acetate, 5 mM magnesium acetate, 50 mM Hepes-KOH, pH 7.9) (30). The pellet was resuspended in dilution buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100) and loaded on a 4.8 ml linear sucrose gradient (5% to 25% sucrose in 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Triton X-100). Gradients were centrifuged for 16 h at 45,000 rpm in a SW 55 Ti rotor at 4 °C and fractionated from the top using an AutoDensi-Flow apparatus (Labconco, Kansas City, MO). Material sedimented at the bottom of the gel was recovered (10 min at 80 °C) in 20 mM Tris-HCl, pH 7.6, 0.1% SDS.

Equivalent amounts of gradient fractions and resuspended pellet were dialyzed with half a volume of 600 mM Tris-HCl, pH 8.6, 25 mM Tris-HCl, pH 8.6, 6% SDS, 8% glycerol, 0.01% bromophenol blue and then heated for 5 min at 100 °C. After cooling, iodoacetamide was added to a final concentration of 100 mM, and samples were incubated for 45 min at room temperature. For the protease protection assay, aliquots of the translation reaction were treated as described previously (27), except that incubation was for 15 min at 25 °C with a proteinase K concentration of 50 µg/ml.
In Vitro Folding of a LMW Glutenin Subunit

**RESULTS**

In Vitro Translocation and Processing of B11-33 Polypeptides—Clone B11-33 codes for a LMW glutenin subunit of wheat cultivar Cheyenne (31). When synthetic mRNA corresponding to the full open reading frame of this clone was translated in a wheat germ system in the absence of microsomal membranes, a major polypeptide of M, 41,000 was synthesized (Fig. 2A, lane 1). The mobility of this polypeptide is lower than expected on the basis of the predicted M of the encoded protein (34,213). We have not investigated the reason(s) for this anomalous migration behavior, but it may be due to the presence of the proline-rich repetitive domain. Indeed, other proline-rich proteins have abnormally low mobility on SDS-PAGE (32,33), and in the case of HMW glutenin subunits, this has been directly related to the presence of the proline-rich repetitive domain (34). When *P. vulgaris* microsomes were included in the translation mixture, a second faster-migrating and poorly resolved polypeptide was generated (lane 2). This second polypeptide, likely resulting from removal of the signal peptide, became the major translation product when the amount of microsomes was increased (l use of radioactive amino acids). Although no protected polypeptides were present when translation was performed in the absence of microsomes (Fig. 2B, compare lanes 1 and 2), the faster of the two polypeptides synthesized in the presence of microsomes was found to be protected by protease attack (compare lanes 3 and 4) unless detergent was added to the assay (lane 5). We conclude that B11-33 polypeptides can be efficiently translocated into bean microsomes, where they are processed most likely by the removal of the signal peptide.

In Vitro Disulfide Bond Formation—Translation mixtures used in this study contain 5 mM DTT derived from the wheat germ extract and up to 0.32 mM DTT derived from the bean microsomes preparation. Although optimal for translation, such conditions do not allow the formation of disulfide bonds in the in vitro synthesized proteins. However, the addition of oxidized glutathione (GSSG) has been shown to allow the oxidation of a variety of polypeptides synthesized in the reticulocyte lysate/canine pancreatic microsomes system without drastically affecting translation efficiency (35–38). To assess whether oxidation of B11-33 polypeptides could be similarly achieved in the wheat germ system, translation of B11-33 mRNA was performed in the presence of bean microsomes and increasing GSSG concentrations (Fig. 3A). GSSG addition had a moderate inhibitory effect on protein synthesis. When 5 mM GSSG was included in the translation mixture, a clear mobility downshift was induced in the translocated polypeptides (Fig. 3A, lane 3). Similarly, translation in the presence of 6 mM GSSG caused a mobility downshift of the untranslocated protein (lane 4). The mobility downshifts were very similar, suggesting that the same set of disulfide bonds is formed in translocated and untranslocated polypeptides. Migration remained unchanged when the samples were reduced before SDS-PAGE, confirming that the shifts were indeed due to intrachain disulfide bond formation (Fig. 3A, lanes 7–12).

Although discrete bands corresponding to LMW glutenin oligomers were not detected, accumulation of some labeled protein in the top part of the gel was often observed when samples containing oxidized monomers were run under nonreducing conditions (not shown). Rather than representing bona fide glutenin polymers, this material probably represents misfolded/aggregated polypeptides linked by aberrant disulfide bonds (see “Discussion”). Thus, whereas intrachain disulfide bonds readily form in the in vitro synthesized polypeptides, covalent polymer formation appears to be very inefficient.

Since untranslocated and translocated proteins were oxidized at different GSSG concentrations, we investigated whether the higher threshold for oxidation of the untranslo-
absence or in the presence of 7 mM GSSG. Saturating amounts of tides are indicated.

Figure 3. Effect of GSSG addition on the oxidation state of in vitro synthesized polypeptides. A, mRNA coding for B11-33 polypeptides was translated in vitro for 1 h in the presence of P. vulgaris microsomes (1 μl/12.5 μl of translation) and the indicated concentrations of GSSG. Aliquots of the translation reactions were analyzed by SDS-PAGE under nonreducing or reducing conditions as indicated. Molecular mass markers (kDa) position are shown on the right. B, mRNA coding for B11-33 polypeptides (WT) or for a mutant lacking the putative signal peptide (ΔSP) were translated together in vitro for 1 h in the absence of microsomes and in the presence of the indicated concentrations of GSSG. Aliquots of the translation reactions were analyzed by SDS-PAGE under nonreducing conditions. The positions of reduced (WTred, ΔSPred), and oxidized (WTox, ΔSPox) polypeptides are indicated.

The synthesis of the wild-type protein was due to the presence of the signal peptide. To test this hypothesis, mRNAs coding for the wild-type protein and for mutant B11-33 polypeptides lacking the putative signal peptide were translated together in the absence of microsomes and in the presence of increasing concentrations of GSSG (Fig. 3B). Although the two bands were poorly resolved, it is clear that the response to increasing GSSG concentrations was not sensibly different in mutant and wild-type polypeptides, indicating that other factors such as the folding environment must be responsible for the reduced GSSG requirement for the oxidation of the segregated protein.

Disulfide Bond Formation Prevents Aggregation of in Vitro Synthesized B11-33 Polypeptides—Further information about the assembly state of B11-33 polypeptides synthesized under reducing or oxidizing conditions was then obtained by subjecting the translation products to sedimentation velocity centrifugation on sucrose gradients. mRNA coding for the B11-33 LMW glutenin subunit was translated in vitro either in the absence or in the presence of 7 mM GSSG. Saturating amounts of microsomes were included into the translation reactions to guarantee translocation of most of the in vitro synthesized polypeptides. At the end of the translation, N-ethylmaleimide was added to block free thiols by alkylation. Microsomes were isolated and solubilized, and proteins were then separated by sedimentation velocity centrifugation on sucrose gradients (Fig. 4). B11-33 polypeptides synthesized in the presence or absence of GSSG had a strikingly different sedimentation behavior. Most of the protein synthesized under conditions favoring disulfide bond formation sedimented as monomers, whereas the polypeptides synthesized in the absence of GSSG were extensively aggregated and were recovered at the bottom of the tube. Since incompletely folded polypeptide chains are often found to be poorly soluble and prone to aggregation (39–41), these results strongly suggest that intrachain disulfide bond formation plays an important role in the stabilization of B11-33 polypeptides. In addition, this analysis shows that monomers containing intrachain disulfide bonds are not assembled into stable non-covalent polymers. The correct conformational state of B11-33 polypeptides synthesized under oxidizing condition was further confirmed by the observation that these polypeptides could be readily extracted in 50% 1-propanol (data not shown) and, thus, had the typical solubility properties of LMW glutenin subunits fractions prepared from grain (10).

Role of Individual Disulfide Bonds in the Folding of B11-33 Polypeptides—In the B11-33 protein, the three intrachain disulfide bonds that have been proposed to characterize LMW glutenin subunits would link Cys-134 with Cys-169, Cys-142 with Cys-162, and Cys-170 with Cys-280 (Fig. 1). To investigate the role of the individual disulfide bonds in the structural maturation of B11-33 polypeptides, site-directed mutagenesis was used to generate a set of mutants in which pairs of cysteine residues are substituted with serines. Messenger RNA coding for the wild-type or the mutated polypeptides was translated in vitro in the presence of bean microsomes and different concentrations of GSSG. The in vitro synthesized polypeptides were then separated under non-reducing or reducing conditions (Fig. 5A). Comparison with the pattern obtained when translation was performed in the presence of sub-optimal amounts of microsomes (0.5 μl/12.5 μl reaction) demonstrates that most of the protein synthesized under the conditions of the assay (2 μl/12.5–μl reaction) does indeed correspond to signal processed (translocated) polypeptides.
The most dramatic effect was observed in the case of the 134–169 mutant (Fig. 5A, lanes 1–4). Under conditions that led to the oxidation of the wild-type protein (5 and 7 mM GSSG), most of this mutant was not recovered in a monomeric form containing intrachain disulfide bonds but rather remained fully reduced or entered large disulfide-linked aggregates (as indicated by the difference in signal between reduced and nonreduced samples). These data suggest that in the absence of the 134–169 bridge the formation of the other intrachain disulfides is somehow hampered, whereas covalent aggregation is favored.

Elimination of Cys-142 and Cys-162 (Fig. 5A, lanes 5–8) did not sensibly affect the formation of oxidized monomeric protein, which migrated slightly slower than oxidized wild-type polypeptides (the difference is evident when the mutated and wild-type proteins are run close by, see Fig. 6A). Because the distance between Cys-142 and Cys-162 along the linear sequence is relatively short, it is conceivable that elimination of the bond connecting these two residues does not have a dramatic effect on electrophoretic mobility. The small change in electrophoretic mobility and the efficient recovery of the protein in monomeric form suggest that the two remaining disulfide bonds are normally formed also when the 142–162 bridge is absent.

When Cys-170 and Cys-280 were substituted with serines and the mutant protein was synthesized in the presence of 5 mM GSSG, a doublet likely representing two alternatively disulfide-linked forms was evident in the nonreduced sample (Fig. 5A, lane 11). When a higher GSSG concentration (7 mM) was present during translation, most of the protein was recovered as a single disulfide-bonded form (lane 12, nonreduced sample). Consistent with the elimination of a bridge that locks together two relatively distant regions of the polypeptide, the mobility downshift was much reduced compared with the one observed in the case of the wild-type protein. Still, substitution of Cys-170 and Cys-280 with serines did not preclude the formation of one or more intrachain disulfide bonds and did not cause extensive covalent aggregation of the in vitro synthesized polypeptides.

Mutants in which only one of the cysteine residues involved in each of the three predicted disulfide bonds was substituted with a serine showed a phenotype analogous to the one characterizing the respective double mutants (Fig. 5B). In addition, substitution of Cys-25 or Cys-230 with serines did not have any apparent effect on the mobility of the oxidized polypeptides (compare lanes 4, 16, and 24 in Fig. 5B), indicating that these residues are not involved in the formation of intrachain disulfides.

As a whole, these results are fully compatible with the proposed model of disulfide bond organization deduced from the analysis of cystine peptides isolated from wheat glutenin, thus indicating that the expected set of disulfide bonds is formed in the in vitro system. They also point to the 134–169 bridge as a key element in the structural maturation of B11-33 polypeptides. Still, SDS-PAGE analysis does not rule out the possibility that elimination of other disulfide bonds leads to the formation of noncovalent aggregates that are then dissolved when the sample is heated in the presence of SDS. To address this point, mRNA coding for the wild-type protein or for mutants in which pairs of cysteine residues are substituted to serine, B, analysis of mutants bearing individual cysteine to serine substitutions.

**Fig. 5.** In vitro translation of wild-type and mutated B11-33 polypeptides. mRNA coding for the wild-type B11-33 protein (WT) or for mutants in which the indicated cysteine residues are substituted with serines were translated in vitro in the presence of the indicated GSSG concentrations. 0.5 or 2 μl of bean microsome preparation (PVM) were included in the translation reactions (12.5 μl) as indicated. Samples were analyzed by SDS-PAGE under nonreducing (top panel) or reducing (bottom panel) conditions, A, analysis of mutants in which pairs of cysteine residues are mutated to serine, B, analysis of mutants bearing individual cysteine to serine substitutions.

The most evident difference in mobility observed in the case of the 134–169 mutant (Fig. 5A, lanes 1–4). Under conditions that led to the oxidation of the wild-type protein (5 and 7 mM GSSG), most of this mutant was not recovered in a monomeric form containing intrachain disulfide bonds but rather remained fully reduced or entered large disulfide-linked aggregates (as indicated by the difference in signal between reduced and nonreduced samples). These data suggest that in the absence of the 134–169 bridge the formation of the other intrachain disulfides is somehow hampered, whereas covalent aggregation is favored.

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**Post-translational Oxidation Does Not Allow Rescue of the Aggregated Polypeptides Synthesized under Reducing Conditions**—The results presented so far suggest that disulfide bond formation in B11-33 polypeptides is required to avoid the aggregation of the newly synthesized polypeptides and to allow the accumulation of folded monomers. The aggregates formed when synthesis is conducted under reducing conditions could contain irreversibly misfolded polypeptides or polypeptides that could still be rescued to a folded state if conditions allowing the formation of disulfide bonds are established post-translationally. To discriminate between these possibilities, B11-33 polypeptides were synthesized under reducing conditions, and GSSG was added post-translationally. A control reaction in which GSSG was present during translation was also assem-
indicated on top of the figure. The position of sedimentation markers is equivalent fraction of the material recovered from the bottom of the fraction was analyzed by SDS-PAGE under reducing conditions. An aliquot of each gradient was further incubated for 1 h at 25 °C. As expected, synthesis in the presence of GSSG led to the accumulation of covalent aggregates containing aberrant disulfide bonds. The in vitro synthesized polypeptides were efficiently translocated into bean microsomes and proteolytically processed, most likely by the removal of the signal peptide. The translocated polypeptides were found to be extensively aggregated unless conditions allowing the formation of disulfide bonds were established by including GSSG in the translation mixture. Under such oxidizing conditions the folding of the B11-33 protein was very efficient; the untranslocated B11-33 polypeptides were not aggregated, formed the three intrachain disulfide bonds that characterize LMW subunits present in glutenin polymers, and had the expected solubility properties.

The efficient folding and oxidation of the translocated B11-33 polypeptides is likely due to the assistance provided by chaperones and oxidoreductases present within the microsomes (14, 15). Still, when sufficient GSSG was added to the system, formation of intrachain disulfide bonds was rather efficiently achieved even in the absence of translocation. The oxidation of untranslocated B11-33 polypeptides is clearly an artifact of the in vitro system but raises the possibility that cytosolic oxidoreductases present in the wheat germ extract are able to surrogate the role of microsomal ones when appropriate redox conditions are artificially generated. For instance, it has been shown that bacterial thioredoxin (homologues of which are present in the cytosol of plant cells (42, 43)) can under certain conditions serve as a catalyst of disulfide bond formation (44).

**DISCUSSION**

In Vitro Folding of a LMW Glutenin Subunit—The data presented in this work show that an in vitro system based on plant components can be a useful tool in investigating the initial stages of LMW subunit folding by monitoring the formation of intrachain disulfide bonds. The in vitro synthesized polypeptides were efficiently translocated into bean microsomes and proteolytically processed, most likely by the removal of the signal peptide. The translocated polypeptides were found to be extensively aggregated unless conditions allowing the formation of disulfide bonds were established by including GSSG in the translation mixture. Under such oxidizing conditions the folding of the B11-33 protein was very efficient; the untranslocated B11-33 polypeptides were not aggregated, formed the three intrachain disulfide bonds that characterize LMW subunits present in glutenin polymers, and had the expected solubility properties.

**FIG. 6.** Sedimentation velocity analysis of wild-type and mutated B11-33 polypeptides. mRNA coding for the wild-type (WT) B11-33 protein or for mutants in which the indicated cysteine residues are substituted with serines were translated in vitro in the presence of 7 mM GSSG. A, total translation products were separated under nonreducing or reducing conditions, as indicated. B, microsomes were isolated from an aliquot of the translation reactions and solubilized with detergent, and the associated proteins were separated by sedimentation velocity centrifugation on sucrose gradient. An aliquot of each gradient fraction was analyzed by SDS-PAGE under reducing conditions. An equivalent fraction of the material recovered from the bottom of the tube was also analyzed (P). The position of sedimentation markers is indicated on top of the figure. Cyt, cytochrome C; Ov, ovalbumin; BSA, bovine serum albumin; Cat, catalase; Fe, ferritin.

**FIG. 7.** Post-translational oxidation leads to the formation of aberrant interchain disulfide bonds. mRNA coding for wild-type B11-33 polypeptides (WT) or for a mutant in which Cys-25 and Cys-230 are substituted with serines (25–230) were translated in vitro in the presence of P. vulgaris microsomes (2 μl/12.5 μl of reaction). Translations were terminated by the addition of 2 mM cycloheximide. GSSG was added at the indicated final concentrations either at the beginning (Ct) or at the end (Pt) of the translation period. The samples which received GSSG post-translationally (and the relative controls) were further incubated for 1 h at 25 °C. Ag, covalently aggregated B11-33 polypeptides. Analysis was by SDS-PAGE under nonreducing (top panel) or reducing (bottom panel) conditions.
Although folded monomers were readily produced in the in vitro system, assembly into native polymers was very inefficient. A small amount of covalent polymers could often be detected at the top of the stacking gel, but their presence was not dependent on Cys-25 and Cys-230, indicating that LMW glutenin subunits present in these complexes were not cross-linked via native interchain bonds. 2 It is perhaps interesting to note that an analogous lack of polymer formation was observed when a HMW glutenin subunit was synthesized in a rabbit reticulocyte/canine microsome system competent for forming disulfide bonds (23). The reason why in vitro assembly of glutenin subunits is so inefficient remains unclear. Although it is possible that B11-33 assembly into polymers can only occur with the participation of other LMW and/or HMW glutenin subunits, one conceivable explanation may also come from the observation that assembly is a concentration-dependent process and is influenced by the level of expression of a given protein (21, 45–47). If the level of expression is sufficiently low, the assembly can be virtually blocked (46, 47). The relatively small amount of protein that is synthesized in the cell-free system may be therefore insufficient to raise the concentration inside individual microsomes to a level compatible with the formation of detectable amounts of native-like polymers.

With regard to the in vivo situation, it may be interesting to note that, although a monomeric γ-gliadin was efficiently secreted by Xenopus oocytes, B11-33 polypeptides were retained intracellularly and that this different behavior was largely reproduced when the N-terminal, repetitive domain was deleted from the two proteins (12). It is therefore possible that some intrinsic characteristics of the C-terminal domain of LMW glutenin subunits is responsible for their retention in the ER and that this retention is essential to raise the concentration of LMW glutenin monomers in this compartment to a level compatible with efficient polymerization.

Role of Individual Disulfide Bonds—The current model of LMW glutenin subunit disulfide structure is based on the analysis of cystine peptides derived from a whole glutenin fraction. According to this model, the individual LMW subunits are stabilized by three intrachain disulfide bonds, whereas two other specific cysteine residues mediate polymer formation (2). The results of our mutagenesis analysis provide evidence for the formation of three intrachain disulfide bonds in an in vitro synthesized LMW glutenin subunit and are also fully compatible with the general disulfide bonding pattern proposed on the basis of chemical mapping studies (2, 3). In addition, our data confirm that Cys-25 and Cys-230 are not involved in the formation of intrachain disulfide bonds. Since the overall disulfide bonding pattern of B11-33 polypeptides does not appear to be affected in mutants lacking Cys-25 and/or Cys-230, we can also conclude that formation of any transient disulfide involving these residues is not required for the successful folding of the C-terminal domain of the protein.

The three intrachain disulfide bonds appear to play different roles in the structural maturation of B11-33 polypeptides. The elimination of Cys-134 and Cys-169 is sufficient to cause extensive covalent protein aggregation, suggesting that adhesive domains that would get buried in the wild-type protein become exposed in this mutant, thus leading to aberrant interactions that can be stabilized by the formation of interchain disulfides. Although we have not determined which cysteines are actually involved in the formation of the covalent aggregates, it is possible that residues that are normally engaged in the formation of intrachain bonds become available for the formation of interchain ones as a consequence of protein misfolding. Indeed, the majority of the protein that did not enter the covalent aggregates migrated like the fully reduced protein, and only a small fraction could be recovered in a disulfide-bonded monomeric form (not evident in Fig. 5A, but see lane 1 in Fig. 6A).

The role of the second bridge, linking Cys-142 to Cys-162, is less clear. The fact that the mutated protein is still recovered in monomeric form and the small effect on polypeptide migration suggest that the two other bridges can form normally even in the absence of Cys-142 and Cys-162. This disulfide bond is therefore likely to have a role in the stabilization of a local structure rather than on the overall folding of the protein. In this regard, it is interesting to note that cysteine residues corresponding to Cys-142 and Cys-162 in the sequence of α-gliadins (2) are not present in the sequence of γ-gliadins (2). The most C-terminal disulfide (linking Cys-170 with Cys-280) is involved in the interaction between two relatively distant regions in the nonrepetitive domain, but also the elimination of this bridge does not appear to be sufficient to expose adhesive domains able to drive protein aggregation.

Thus, although the 134–169 disulfide bond is indeed necessary to prevent aggregation of the B11-33 protein, the two other bridges may have a different function, possibly linked to polymer assembly and/or packaging into protein bodies. Indeed, a cysteine to serine substitution at position C24 in a γ-gliadin resulted in the generation of protein bodies with altered sedimentation properties when the mutant protein was expressed in Xenopus oocytes (12).

All together, these data suggest that folding and aggregation are competing phenomena in the biosynthesis of B11-33 polypeptides and that formation of a critical disulfide is required to maintain the protein on a productive folding pathway. If the critical disulfide is not formed, either because the relevant cysteine residues have been mutagenized or because the synthesis occurs under reducing conditions, the newly synthesized polypeptides end up into large aggregates. Since (at least in the environment provided by the microsomes) post-translational oxidation could not rescue the aggregated polypeptides into a soluble monomeric form, it is unlikely that aggregates containing the reduced protein can be an intermediate on a pathway that can eventually lead to the production of correctly folded monomers.

Our results also raise interesting questions about the dynamics of disulfide bond formation in B11-33 polypeptides. Since disulfides can form rapidly upon exposure of the growing chains to the ER lumen, it has been suggested that early disulfide-associated folding steps may have the immediate function of suppressing nonspecific interactions between polypeptides (40). In addition, cotranslational folding has been proposed to be required for the successful structural maturation of certain modular proteins in eukaryotes (48). Our data indicate that disulfide bond formation must be either a cotranslational or an early posttranslational event in the synthesis of B11-33 polypeptides. It is therefore tempting to speculate that cotranslational folding could play an important role in the biosynthesis of this protein, first allowing the independent folding of the N-terminal domain and then directing the establishment of critical intrachain interactions that must be stabilized by the formation of the 134–169 disulfide bond.

The finding that one of the disulfide bonds plays such a crucial role in the structural maturation of a LMW glutenin polypeptide may have more general implications that extend to other classes of proteins found in evolutionarily distant members of the plant kingdom. Cysteine residues corresponding to cysteine 134 and cysteine 169 can be identified in plant proteins sharing little sequence homology, such as sunflower SFA8, lupin conglutin δ, and cereal α-amylase/trypsin inhibi-

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2 A. Orsi, F. Sparvoli, and A. Ceriotti, our unpublished observation.
tors (11). It would be now interesting to check whether the specific role played by these residues has been conserved throughout the evolution of seed proteins.

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Role of Individual Disulfide Bonds in the Structural Maturation of a Low Molecular Weight Glutenin Subunit
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