Intramolecular Disulfide Bonds between Conserved Cysteines in Wheat Gliadins Control Their Deposition into Protein Bodies*

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Following synthesis, wheat gliadin storage proteins are deposited into protein bodies inside the endomembrane system in a way that enables not only their efficient accumulation and dehydration during seed maturation, but also their rapid rehydration and degradation during germination. In the present report, we studied the mechanism of gliadin deposition and whether it was controlled by the conformation of these proteins. Although gliadins are generally known to be insoluble in aqueous solutions, sucrose gradient analysis showed that a considerable amount of these proteins appeared as relatively soluble monomers in developing grains. In vitro reduction of the intramolecular disulfide bonds that are present in natural monomeric gliadins caused their precipitation into insoluble aggregates. In addition, pulse-chase experiments in the absence or presence of reducing agents showed that formation of intramolecular disulfide bonds also played a major role in folding and deposition of the gliadins in vivo. Our results imply that following sequestration into the endoplasmic reticulum, the gliadins fold into relatively soluble monomers, which are incompetent for rapid aggregation and gradually assemble into protein bodies. This pattern of deposition apparently depends on the conformation of the gliadins, which is stabilized by intramolecular disulfide bonds formed between the conserved cysteines. The contribution of this study to the understanding of the evolution and function of gliadins is discussed.

Wheat storage proteins are co-translationally inserted into the rough endoplasmic reticulum and are then deposited into protein bodies (PB) at various compartments of the endomembrane system (1). In these PB, the storage proteins survive the process of seed desiccation and are then rehydrated again, degraded, and mobilized into the embryo during germination. The major storage proteins in wheat are three closely related classes of alcohol-soluble proteins called sulfur (S)-rich gliadins, S-poor gliadins, and high molecular weight glutenins (HMW-GS). The S-rich gliadins are further subdivided into four subgroups of α, β, γ, and aggregated gliadins. All three protein classes contain a domain that is composed of small amino acid sequence repeats, rich in glutamine and proline (2–4). In the S-poor gliadins, this domain apparently accounts for the majority of the polypeptide. In the HMW-GS, the repetitive domain appears in the center and is flanked by small N- and C-terminal nonrepetitive domains. These nonrepetitive domains contain several cysteine residues that form intermolecular disulfide bonds between the different glutenin subunits resulting in their polymerization (2–4). In the S-rich gliadins, the repetitive domain is present in the N-terminal part of the polypeptide. This domain varies between the different S-rich gliadin subclasses in the number and consensus sequences of the repeats, but all of the repeats are thought to be arranged in a β-turn configuration (3, 4). The C-terminal domain of the S-rich gliadins is apparently arranged predominantly in α-helix and β-sheet configurations and also contains three to four intramolecular disulfide bonds formed between six to eight evolutionary conserved cysteine residues (3, 4).

Despite extensive studies, the fine structure of wheat gliadins and their mechanism of deposition into PB are still not clearly understood. As gliadins and glutenins extracted from mature grains are largely insoluble in aqueous solutions, it was suggested that these proteins spontaneously precipitate and aggregate into insoluble deposits immediately after insertion into the ER (see Ref. 5 for a review). However, in recent years, various lines of evidence suggested that the maturation of wheat storage proteins may not be spontaneous, but rather assisted by molecular chaperones that are present inside the ER (1, 6). Of these molecular chaperones, the binding protein (BiP) was suggested to assist in the general maturation of storage proteins from wheat and other plant species (7–12). A second molecular chaperone, protein-disulfide isomerase (PDI), was suggested to assist in the formation of correct intramolecular disulfide bonds in wheat S-rich gliadins (13). Nevertheless, it is still unclear whether the molecular chaperones assist only in the folding of the storage proteins or also in various steps of their deposition into PB.

Studying the mechanism of wheat storage protein deposition, we have previously expressed wild-type and modified S-rich gliadins in Xenopus oocytes (14). This study demonstrated that, following insertion into the ER, the gliadins could diffuse rather freely within the organelle for a few hours, suggesting that deposition into PB does not occur by their mere precipitation and aggregation. In addition, density gradient analysis showed that not all of the gliadins were present in dense aggregates, suggesting that the deposition occurs by a slow regulated process. In contrast to wild type proteins, mutant gliadins, lacking conserved cysteines in the C-terminal regions, rapidly aggregated into nondiffusible complexes within the ER, suggesting that intramolecular disulfide bonds played a major role in the deposition of the gliadins. Based on these observations (14), we have presently studied the mechanism of depo.
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EXPERIMENTAL PROCEDURES

Plant Material—Developing grains were obtained from hexaploid wheat cultivars Degenat and Atrir.

Protein Extraction—Single grains or thin grain slices, at about 17 days after anthesis, were gently ground by a mortar and pestle in 1 ml of 10 mM Tris-HCl, pH 7.5, 150 mM NaCl (aqueous buffer). In some experiments, as indicated in the text, this buffer was supplemented with 0.5% Triton X-100. The grain extract was then passed through cheesecloth in order to clear it from debris. In experiments depicted in Figs. 2, 3, and 9, the resulting homogenate was further centrifuged for 10 min at 13,000 × g in a bench-top centrifuge. The resulting supernatant was defined as "soluble" proteins.

Purified α and γ gliadins were kindly provided by Dr. J. Mose (15). Freeze-dried purified gliadin was resuspended in "aqueous buffer" at a concentration of 1 mg/ml, and soluble purified gliadin was obtained as described above.

Sucrose Gradient Fractionation—Eight hundred μl from the wheat grain homogenates or soluble proteins were layered onto a 5-2 ml linear density gradient of 5-20% sucrose in aqueous buffer. In some experiments, as described, and then resuspended in protein sample buffer.

SDS-PAGE, Western Blot Analysis, and Radiolabel Detection—Proteins were dissolved in sample buffer and fractionated by SDS-PAGE on 10% polyacrylamide gels as described previously (16). In "nonreducing" SDS-PAGE, β-mercaptoethanol was omitted from the sample buffer. After electrophoresis, gels were either stained overnight with Coomassie Blue or transferred to nitrocellulose membranes and immunoblotted in Western blots with anti-γ-gliadin (17), anti-HMW-GS (17) and anti-α-gliadin sera. These sera, which were used for the general identification of HMW-GS and S-rich gliadins, are not cross-reacting with the anti-HMW-GS serum or anti-γ-gliadin sera, are marked by an asterisk. P, insoluble pellets that sediment to the bottom of the gradient. The location of molecular mass markers in the SDS gel is indicated on the left.

Detection of Reduced Gliadins—Gliadins containing surface thiols were detected by covalent chromatography using activated thiopropyl-Sepharose 6B (Pharmacia Biotech Inc.). Developing wheat grain slices were pulse labeled with the [35S]methionine and cysteine mixture as described above, and then gently extracted in 500 μl of deaerated aqueous buffer, to avoid oxidation of free thiol groups. Extracts were centrifuged and pellets (now deprived of water soluble proteins) were resuspended in 500 μl of deaerated aqueous buffer containing 1% SDS, and resuspended. The supernatant (enriched in gliadins) was added to 10 mg of prewashed thiopropyl-Sepharose 6B beads. Coupling was then performed by incubating at room temperature for 30 min with gentle shaking. The beads were pelleted at 1000 × g for 1 min, and supernatant containing unbound protein was kept. Beads were washed in 1% SDS-containing aqueous buffer, and the bound protein was then eluted in the SDS-containing buffer supplemented with 2% β-mercaptoethanol. Supernatants containing bound and unbound gliadins were trichloroacetic acid-precipitated and washed with acetone and NaOH as described, and then resuspended in protein sample buffer.

RESULTS

Analysis of Storage Protein Complexes in Developing Wheat Grains—In order to study the mechanism of wheat storage protein deposition into PB, we first fractionated homogenates from developing grains on 5-20% sucrose gradients. Individual fractions were then reacted in Western blots with anti-HMW-GS and anti-γ-gliadin sera. These sera, which were used for the general identification of HMW-GS and S-rich gliadins, are not entirely type-specific and also detect other proteins in the size range of 50-60 kDa (apparently S-poor gliadins). In addition, the anti-γ-gliadin serum apparently also detects aggregated gliadins that are similar in size and amino acid sequence to the γ-gliadins. Thus, hereafter, γ-gliadins detected by these antibodies will refer to all γ-type S-rich gliadins. As shown in Fig. 1A, the HMW-GS were entirely present in complexes that sedimented to the bottom of the gradient. A considerable amount of the γ-gliadins also sedimented to the bottom of the sucrose gradients (Fig. 1B), but, in contrast to the HMW-GS, a significant proportion of the gliadins also sedimented in lighter fractions. Although it is difficult to measure the accurate molecular weight of proteins in sucrose gradients, the gliadins sedimenting in the light fractions were apparently monomers, based on the co-sedimentation of protein markers. Thus, for simplicity, these lightly migrating gliadins were defined as "monomeric gliadins" throughout the rest of the study.
Wheat gliadins are generally known to be insoluble in aqueous solutions (3), but whether this is related to the structure of the proteins or to their aggregation state is not clear. We therefore wished to test the solubility of the monomeric gliadins from developing grains in an aqueous solution that is apparently similar to the conditions present inside the ER lumen, where the initial maturation steps of the storage proteins take place. Developing grains were homogenized in aqueous buffer, and, upon centrifugation, soluble (supernatant) and insoluble (pellet) proteins were separated in SDS-PAGE. The gels were then reacted in Western blots with anti-HMW-GS sera. Each fraction of solubility-PD (9) was supplemented with DTT to reduce the intramolecular disulfide bonds. Next, the DTT-treated purified gliadins were dissolved in aqueous buffer and soluble gliadins were obtained by centrifugation. Each fraction of soluble gliadins was divided into two equal portions, and to one portion we added DTT to precipitate gliadins. Bands not related, but cross-reacting with the anti-HMW-GS sera or anti-γ-gliadin sera, are marked by an asterisk. The location of molecular mass markers is indicated on the left.

Reduction of Soluble Gliadins Causes Their Aggregation into Insoluble Deposits in Vitro—We have previously demonstrated that elimination of conserved cysteine residues present in the C-terminal region of a wheat α-gliadin triggered its aggregation into nondiffusible complexes in Xenopus oocytes (14). Therefore, we tested whether the presence of a significant amount of gliadins as soluble monomers was related to their conformation as determined by intramolecular disulfide bond formation. Soluble gliadins, extracted from developing wheat grains, were divided into two equal portions, and to one portion we added DTT to dissociate their disulfide bonds. The homogenates were then fractionated on 5–20% sucrose gradients, and individual fractions were reacted in Western blots with anti-γ-gliadin serum. As shown in Fig. 3, the natural soluble gliadins appeared as monomers (panel A), while reduction of the disulfide bonds in vitro caused their aggregation into complexes that sedimented to the bottom of the gradient (panel B). Notwithstanding, two cross-reacting bands with molecular mass larger than 43 kDa still appeared as monomers upon reduction (Fig. 3A, bands marked by an asterisk). These proteins are bigger than the expected size of the γ-gliadins and may belong to the related fraction of the S-rich gliadins, which do not possess cysteine residues and therefore are not affected by reducing agents (3, 4).

As the soluble extract from developing wheat grains apparently contained a mixture of proteins, including molecular chaperones, it was impossible to deduce from Fig. 3 whether aggregation resulted directly from the conformational change of the gliadins upon reduction, or was assisted by additional factors. To address this, we tested the change in conformation and aggregation of purified α- and γ-gliadins, upon reduction of their intramolecular disulfide bonds. First, purified gliadins were dissolved in SDS sample buffer lacking or containing DTT and the proteins were separated by SDS-PAGE. Reduced gliadins migrate slightly slower in the gel, apparently due to their extended conformation (13). Indeed, as shown in Fig. 4, the DTT-treated purified gliadins migrated slower than the non-treated gliadins, which apparently do not belong to the S-rich gliadins, based on their higher molecular weight, are marked by an asterisk. P, insoluble pellets that sediment to the bottom of the gradient. The location of molecular mass markers is indicated on the left.

![Fig. 2](image-url) **Fig. 2.** Solubility of S-rich gliadins in aqueous solutions. Developing wheat grains were ground in aqueous buffer and supernatant (S) and pellet (P) were separated following centrifugation. Equivalent portions from supernatant and pellet were separated on SDS-PAGE, followed by Western blot analysis using anti-γ-gliadin and anti-HMW-GS sera. Bands not related, but cross-reacting with the anti-HMW-GS sera or anti-γ-gliadin sera, are marked by an asterisk. The location of molecular mass markers is indicated on the left.

![Fig. 3](image-url) **Fig. 3.** In vitro reduction of soluble wheat grain extracts, results in aggregation of S-rich gliadins. Soluble gliadin supernatant was loaded on top of a 5–20% sucrose gradient, either lacking (A) or containing (B) DTT. Following ultracentrifugation, fractions were separated on SDS-PAGE and subjected to Western blot analysis using anti-γ-gliadin sera. Bands not related, but cross-reacting with the anti-γ-gliadin sera that apparently do not belong to the S-rich gliadins, based on their higher molecular weight, are marked by an asterisk. P, insoluble pellets that sediment to the bottom of the gradient. The location of molecular mass markers is indicated on the left.
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**Fig. 4. Reduction of intramolecular disulfide bonds alters the conformation of S-rich gliadins.** A soluble fraction of one γ- and two α-type gliadin groups, were treated with (+) or without (−) 10 mm DTT. Samples were separated on SDS-PAGE and Coomassie-stained. The location of molecular mass markers is indicated on the left.

**Fig. 5. In vitro reduction of purified S-rich gliadins results in their aggregation.** Soluble γ- and two α-type gliadin groups, were loaded on top of 5–20% sucrose gradients, either lacking (A, C, and E) or containing (B, D, and F) DTT. Following ultracentrifugation, fractions were separated on SDS-PAGE and Coomassie-stained. P, insoluble pellets that sediment to the bottom of the gradient. The location of molecular mass markers is indicated on the left side of each gel.

The results of this experiment implied that a significant amount of the newly synthesized gliadins were present as monomers associated with the ER and that their assembly took place later after synthesis. We also analyzed in more detail the time course of gliadin assembly by 5–20% sucrose gradient analysis of Triton X-100-containing homogenates from developing grains that were pulse-labeled with [35S]methionine plus [35S]cysteine for 10 min and chased for 0, 10, and 50 min. As shown in Fig. 7, with the maturation of the gliadins, progressively increasing amount of radioactivity was incorporated both into the top and bottom fractions, while the ratio between monomers and complexes was not altered significantly. This suggested that assembly of the gliadins occurs by a gradual process that continues for hours after their synthesis.

**Fig. 6. Sedimentation of the bulk and newly synthesized gliadins in sucrose gradient.** Developing grains were labeled for either 1 day (A and D) or for 10 min (B and E) with [35S]methionine plus [35S]cysteine. Grains were then gently extracted in an aqueous buffer either not containing (A–C) or containing (D–F) 0.5% Triton X-100. The extracts were then loaded on top of 5–20% sucrose gradients. Following ultracentrifugation and fractionation, gliadin-enriched ethanolic extracts (A, B, D, and E) or total proteins (C and F) were separated on SDS-PAGE. Gels loaded with gliadin-enriched extracts were dried and exposed to film (A, B, D, and E). The other two gels were subjected to Western blot analysis, using simultaneously, both anti-BiP and anti-PDI antibodies (C and F). P, insoluble pellets that sediment to the bottom of the gradient. The location of BiP and PDI is indicated on the right side of panels C and F.
the extensive washing, they clearly did not have any in vitro effect on the aggregation of the gliadins because upon homogenization of each small grain slice in 0.8 ml of aqueous buffer (A) or aqueous buffer containing 100 mM DTT (B). After 30 min, the grains were washed and incubated with [35S]methionine plus [35S]cysteine for 10 additional min. Grains were then extracted in a Triton X-100-containing buffer and layered on top of a 5–20% sucrose gradient. Gliadin enriched fractions were separated by SDS-PAGE, and labeled bands were detected by PhosphorImager. P, insoluble pellets that sediment to the bottom of the gradient. A, 0-min chase; B, 10-min chase; C, 50-min chase.

FIG. 8. Dissociation of gliadins from insoluble precipitates. Developing wheat grain homogenates were centrifuged to obtain supernatant (S, lane 1) and pellet (P, lane 2). The supernatant (1) was either not supplemented (−DTT) or supplemented with 150 mM DTT (+DTT), and centrifuged again to obtain supernatants and pellets (lanes 3 and 4 and lanes 5 and 6, respectively). Pellets from both natural gliadins (lane 2) or reduced gliadins (lane 6) were resuspended in aqueous buffer, vortexed for 10 min at room temperature, and recentrifuged to obtain supernatants and pellets (lanes 7 and 8 and lanes 9 and 10, respectively). Equal proportions of each one of the treatments were subjected to SDS-PAGE and reacted in Western blots with anti-γ-gliadin serum. Bands not related, but cross-reacting with the anti-γ-gliadin sera, are marked by an asterisk. The location of molecular mass markers is indicated on the left.

FIG. 7. Pulse-chase analysis of gliadin maturation in vivo. Developing grains were pulse-labeled with [35S]methionine plus [35S]cysteine for 10 min, and chased with cold aqueous buffer for 0, 10, and 50 additional min. Grain slices were then gently extracted in a Triton X-100-containing aqueous buffer and layered on top of a 5–20% sucrose gradient. Gliadin enriched fractions were separated by SDS-PAGE, and labeled bands were detected by PhosphorImager. P, insoluble pellets that sediment to the bottom of the gradient. A, 0-min chase; B, 10-min chase; C, 50-min chase.
agents or detergents (i.e. potassium thiocyanate, Triton X-100, and SDS) were present in the aqueous buffer (data not shown). Not all of the S-rich gliadins remained as monomers. During maturation, some of the proteins progressively assembled into insoluble complexes that sedimented into the bottom of a 5–20% sucrose gradient (complexes containing at least eight polypeptides based on an average gliadin size of ~30 kDa and on the sedimentation of molecular markers in the gradient, see Fig. 1). The assembly and precipitation of the gliadins may result from increased gliadin concentration within the PB upon grain maturation. It is also possible that gliadin assembly may be furthermore enhanced by a change in the pH, which occurs following their transport from the ER to the vacuoles (1), similar to the case of legume storage proteins (23). Nevertheless, the interactions between the gliadins are presumably relatively weak as these polypeptides can evidently be rapidly rehydrated and degraded upon seed germination.

The present observation that S-rich gliadins are apparently in an equilibrium between monomers and complexes is in agreement with our previous finding that PB containing them possess variable densities, as deduced from sedimentation in metrizamide gradients (17). However, it is impossible to draw a simpler correlation between the density of the PB and the oligomerization state of the gliadins because the dense PB in metrizamide gradients apparently contain both gliadins and HMW-GS (17), while the HMW-GS are sufficient by themselves to form the dense PB (20).

The Intramolecular Disulfide Bonds in S-Rich Gliadins Stabilize Their Conformation and Prevent Their Rapid Aggregation—The high evolutionary conservation of cysteine residues suggests that they are important for the maturation of the S-rich gliadins. However, as the gliadins possess no known function besides storage, the functional role of these cysteines was not clear. In the present report, we showed that the intramolecular disulfide bonds, formed between these cysteines, play a major role in the conformation of the gliadins and in fact also control their deposition by ensuring slow assembly rather than rapid aggregation and precipitation. This was concluded by two lines of evidence: (i) reduction of soluble monomeric gliadins caused their aggregation in vitro, and (ii) prevention of disulfide bond formation in newly synthesized gliadins enhanced their aggregation in vivo. In this regard, we wish to stress that despite of the fact that both the natural insoluble complexes of the gliadins, as well as the ones formed by the aggregation of malfolded gliadins, sedimented to the bottom of the 5–20% sucrose gradients, the properties of each type of these complexes were entirely different due to the following: (i) the natural complexes contained oxidized gliadins, while the aggregates of the malfolded proteins contained reduced polypeptides; and (ii) when the insoluble pellets of the natural and malfolded reduced gliadins were resuspended in the aqueous buffer, only the natural gliadins could be released as monomers (Fig. 9), suggesting that the interactions responsible for the oligomerization of the natural gliadins were much weaker than those causing the aggregation of the reduced malfolded polypeptides. Our conclusion regarding the role of intramolecular disulfide bonds in the deposition of S-rich gliadins is also supported by biophysical studies (24), suggesting that these disulfide bonds play a role in the conformational structure of these proteins.

Functional Evolution of Wheat Storage Proteins—The major function of storage proteins is to fix amino acids into protein-bound forms that can accumulate to high levels in dense protein bodies within the limiting space of the seed storage cells. However, these storage proteins, which are desiccated during seed maturation, are also efficiently rehydrated, degraded, and mobilized into the germinating embryo upon germination. In deed, the composition of amino acids in the coding capacity of the storage proteins is noncommon and generally reflects their composition in the free amino acid pool in the seeds. The noncommon amino acid composition of storage proteins, primarily those of cereal grains, that are highly enriched in glutamine residues, may result from tight aggregation and hence interference with subsequent rehydration and degradation during germination. In the present report, we have shown that the S-rich gliadins have apparently evolved to obtain a specific conformation that enables their folding into forms that are rather incompetent for aggregation despite of their high glutamine content. This appears to be controlled by the formation of intramolecular disulfide bonds between cysteine residues that were highly conserved during gliadin evolution.

Wheat storage proteins also contain a class of HMW-GS that contrarily to the gliadins, efficiently assemble into large insoluble polymers, linked by noncovalent and intramolecular disulfide bonds (Fig. 1) (3, 25). Notwithstanding, in all wheat species, the HMW-GS represent only a minor proportion of about 5% of the total storage proteins and are controlled by loci containing only two genes, in contrast to the gliadins, which appear in multigene families (26). Thus, it is tempting to speculate that the HMW-GS evolved to represent a small fraction of an insoluble core to which the major fraction of the S-rich gliadins can join for the initiation of PB formation. This may have enabled the efficient accumulation of the storage proteins in dense PB within the limiting space of maturing endosperm cells, and their subsequent rapid rehydration and degradation during germination.

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