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Role of the Amino- and Carboxy-Terminal Regions in the Folding and Oligomerization of Wheat High Molecular Weight Glutenin Subunits[1]

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ABSTRACT

The high molecular weight glutenin subunits are considered one of the most important components of wheat (*Triticum aestivum*) gluten, but their structure and interactions with other gluten proteins are still unknown. Understanding the role of these proteins in gluten formation may be aided by analyses of the conformation and interactions of individual wild-type and modified subunits expressed in heterologous systems. In the present report, the bacterium *Escherichia coli* was used to synthesize four naturally occurring X- and Y-type wheat high molecular weight glutenin subunits of the *Glu-1D* locus, as well as four bipartite chimeras of these proteins. Naturally occurring subunits synthesized in the bacteria exhibited sodium dodecyl sulfate-polyacrylamide gel electrophoresis migration properties identical to those of high molecular weight glutenin subunits extracted from wheat grains. Wild-type and chimeric subunits migrated in sodium dodecyl sulfate gels differently than expected based on their molecular weights due to conformational properties of their N- and C-terminal regions. Results from cycles of reductive cleavage and oxidative reformation were consistent with the formation of both inter- and intramolecular disulfide bonds in patterns and proportions that differed among specific high molecular weight glutenin species. Comparison of the chimeric and wild-type proteins indicated that the two C-terminal cysteines of the Y-type subunits are linked by intramolecular disulfide bonds, suggesting that the role of these cysteines in glutenin polymerization may be limited.

The storage proteins of wheat grains (*Triticum aestivum*, genome AABBDD) are synthesized in the endosperm and accumulate in protein bodies inside vacuoles. Following synthesis, the storage proteins interact with each other and become incorporated into intermolecular disulfide-bonded aggregates (5). Although wheat storage proteins have been extensively studied, very little is known about their structure and interactions during protein body formation. It was shown, however, that the physiochemical properties of the various storage proteins as well as the interactions among them play important roles in the viscoelasic characteristics of the gluten formed upon hydration of the storage protein aggregates (20, 23).

Wheat storage proteins were originally classified on the basis of their solubility and electrophoretic mobilities. Proteins extractable from flour with aqueous alcohol were termed gliadins (17) and are monomeric subunits (30–60 kD); those that require pH extremes or denaturing solvents for extraction were termed glutenins and associate by noncovalent interactions and disulfide bonds into aggregates of 10^4 kD or more (4). Glutenin aggregates as described above are too large to enter polyacrylamide gels, but reveal monomeric components with an apparent size of 45 to 125 kD by SDS-PAGE upon reduction of disulfide bonds. These subunits have been divided into classes of HMW-GS[2] and LMW-GS. Analysis of coding sequences by *in vitro* mRNA translation (12) and nucleotide sequence determination (26) indicated that all wheat seed storage protein synthesis occurs on membrane-bound polysomes and is accompanied by cleavage of a signal peptide from precursor molecules with subsequent sequestration into the RER and deposition in protein bodies (5).

Both the gliadin and glutenin subunits participate in the formation of the storage protein aggregates (18). However, the HMW-GS are probably the most important building blocks, because variation among wheat cultivars in bread-making quality from gluten formed upon hydration of these aggregates is correlated most clearly with allelic variation of HMW-GS genes and especially with those encoded by the D genome (23). It has been suggested that the HMW-GS associate via noncovalent interactions and intermolecular disulfide bond formations to form a protein matrix that provides the backbone of the gluten aggregate (20).

In hexaploid wheat, six multiallelic HMW-GS genes have been identified, two at each of the homeologous loci *Glu-1* located on group 1 chromosomes (22). Alleles of each of the six HMW-GS genes have been cloned and sequenced (1, 8, 15, 28). The HMW-GS proteins coded at each *Glu-1* locus form two general classes, which were originally termed X-

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[2] Abbreviations: HMW-GS, high mol wt glutenin subunit; LMW-GS, low mol wt glutenin subunit; β-ME, β-mercaptoethanol; C.S., Chinese Spring; Ab, antibodies.

and Y-types on the basis of mobility by SDS-PAGE (22). The genes are thought to have diverged from a common progenitor (15).

All known HMW-GS exhibit anomalously slow SDS-PAGE mobility relative to their actual mol wt (13). Accurate mol wt estimates of HMW-GS have been obtained by extrapolating relative SDS-PAGE mobilities to 0% (w/v) polyacrylamide via Ferguson plots (16), which indicates that the general anomalous mobility of HMW-GS is a function of the conformation of their complexes with SDS. HMW-GS genes encode a three-domain protein consisting of unique sequence N- and C-terminal domains and a highly repetitive central domain (26). In both X- and Y-types, the terminal domains contain most of the HMW-GS cysteine residues; however, there are differences between them in the number and exact locations of these residues. It has been suggested that the repetitive regions of HMW-GS possess high proportion of β-turn configuration that may form higher-order structures of β-spirals, similar to those suggested for the protein elastin (6). The putative β-spiral structure has been suggested to play a role in the elastic properties of wheat gluten, and the associated amino acid sequence may play a role in the anomalous SDS-PAGE mobility of HMW-GS.

Although the HMW-GS have been characterized in detail, their structure, folding, and interactions have not been characterized. Studies on the structure of HMW-GS and their interaction in wheat grains suffered from the difficulty of isolating single subunits from the complex mixture of closely related gluten proteins present in wheat endosperm. One way to overcome this problem is by the synthesis of individual wild-type and modified HMW-GS in heterologous systems. In the present report, we have expressed in *Escherichia coli* four natural HMW-GS genes as well as chimeric constructs derived from four pairwise combinations between different genes. We show differences among HMW-GS in patterns of folding and oligomeric assembly via disulfide bond formations. We also show that SDS complexes of the N- and C-terminal regions of X- and Y-type subunits possess different conformations as measured by anomalous migration by SDS-PAGE.

MATERIALS AND METHODS

Plasmid Constructions

The plasmid pET-3a-Glu-1Dx2 (9) was the basic construct for the cloning of DNA sequences coding for 1Dy12, 1Dx5, and 1Dy10 HMW-GS into the expression vector pET-3a (24). All the genes contain a conserved *Stu*I restriction site 12 nucleotides downstream from the first nucleotide of the mature protein NH_2-terminal codon. These 12 nucleotides code for a conserved amino acid sequence. The genes also contain a *Nsi*I restriction site downstream from the stop codon (Fig. 1A). Constructions of the natural sequences were accomplished by replacing the *Stu*I to *Nsi*I fragment of *Glu-1Dx2* with analogous fragments from the other genes (our manuscript in preparation). The resulting expression plasmids were designated pET-3a-Glu-1Dy12, pET-3a-Glu-1Dx5, and pET-3a-Glu-1Dy10, respectively. The chimeric constructs were generated by reciprocal exchanges of coding region segments

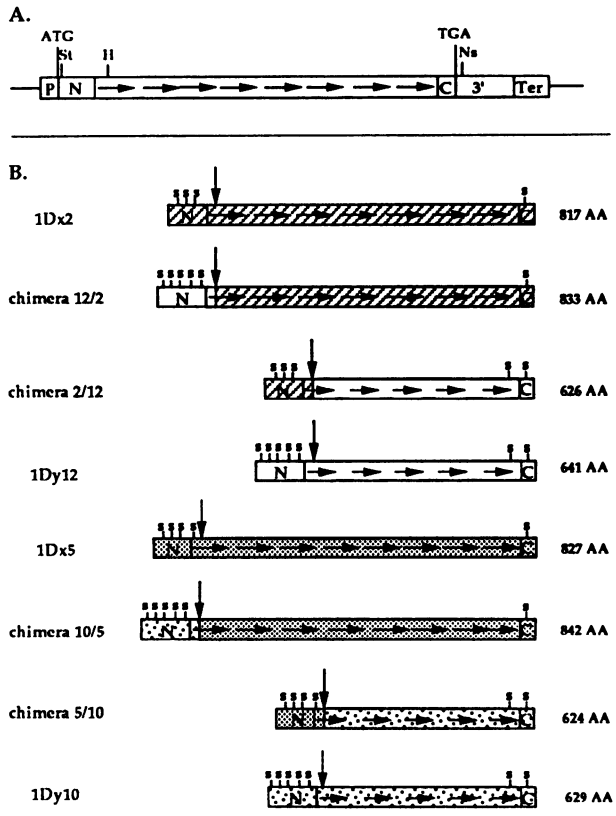

Figure 1. A, A general schematic diagram of the pET-3a-HMW-GS plasmid constructs. The DNA sequences coding for the mature 1Dx5, 1Dy12, and 1Dy10 HMW-GS were subcloned into the pET-3a, by replacing fragments bordered by the conserved restriction sites St and Ns, with a similar fragment in pET-3a-1Dx2 (9). Chimeric HMW-GS, containing mixed X- and Y-type sequences, were constructed by replacing DNA fragments bordered by the conserved restriction sites H and Ns between different pET-3a-HMW-GS plasmids. B, Schematic diagrams of wild type and chimeric HMW-GS produced in *E. coli* from the plasmid constructs discribed in A. ATG, translation initiation sites; TGA, translation termination sites; P, promoter DNA; Ter, terminator DNA; 3′, 3′-nontranslated DNA; N, DNA or protein sequences of the NH_2-terminal unique domain; C, DNA or protein sequences of the carboxy-terminal unique domain; St, *Stu*I; H, *Hind*III; Ns, *Nsi*I; S, cysteine residue; tandem horizontal arrows represent DNA or protein sequences of the central repetitive domain. Vertical arrows in B represent positions of fusion between X- and Y-type proteins.

at a conserved *Hind*III restriction site located close to the border of the DNA coding for the unique N-terminal region and the repetitive region (Fig. 1A) (our manuscript in preparation). Resulting chimeric constructs were designated pET-3a-2/12, pET-3a-12/2, pET-3a-5/10, and pET-3a-10/5, respectively (Fig. 1B).

Heterologous Expression in *Escherichia coli*

Newly transformed colonies of the *E. coli* strain BL21 (DE3) pLysS (27), harboring a pET-3a-glutenin plasmid, were grown into ZY (9) medium/0.4% (w/v) glucose to $A_{600} = 0.5$ and then induced by addition of isopropyl β-D-thiogalactoside to 0.4 mM. The cells were harvested 5 to 7 h after induction.

Purification of HMW-GS

Proteins were purified from bacteria as follows. Bacterial pellets were resuspended in 0.1 M Tris-HCl (pH 7.5), 1 mM PMSF, and then treated for 30 min on ice with 1 mg/mL lysozyme. The suspension was then sonicated until its viscosity became markedly reduced and then shaken on ice for additional 30 min. After centrifugation at 25,000*g* for 15 min, the pellet was resuspended in 8 M urea, 0.1 M acetic acid, 1% (v/v) β-ME[2], and 1 mM PMSF using a glass-tissue homogenizer. The solution was shaken at ambient temperature for 30 min, subjected to additional centrifugation, and the supernatant dialyzed against 0.1 M NaCl, 0.1 mM EDTA for 20 h at 4°C. The dialyzed HMW-GS precipitate was spun at 25,000*g* for 15 min and the pellet stored at −70°C.

To purify the natural 1Dx2 and 1Dy12 HMW-GS from wheat, grains from an aneuploid line of the cv C.S. lacking the long arms of chromosomes 1B were used (this aneuploid produces only HMW-GS 1Dx2 and 1Dy12). The grains were milled, and protein fractions separated by gel filtration followed by ion-exchange chromatography as described (25), with the following modifications: the gel filtration matrix was Sephacryl S-200 (Pharmacia, Uppsala, Sweden) and the eluting solvent was 3 M urea, 0.01 M acetic acid, and 0.2% (v/v) β-ME. The purified protein pellet was stored at −70°C.

In Vitro Reduction and Reoxidation of HMW-GS Disulfide Bonds

Proteins purified either from bacteria or wheat grains were reduced in 3 M urea, 0.1 M acetic acid, and 1% (v/v) β-ME at protein concentrations of 0.1 to 0.2 mg/mL, and then allowed to reoxidize during dialysis against 0.01 M acetic acid, 0.001% (v/v) β-ME, for 20 h at 4°C.

Fractionation on SDS-PAGE

Proteins were dissolved in SDS sample buffer (9) containing or lacking 5% (v/v) β-ME and then separated by SDS-PAGE on 10% (w/v) or 7.5% (w/v) polyacrylamide gels (19).

Detection of Proteins in the Gels

Proteins were detected in gels, either by Coomassie blue staining as described previously (10), or by immunological reaction in Western blots essentially according to Towbin *et al.* (30) as described by Galili (9). Anti X-type HMW-GS Ab were kindly provided by P.R. Shewry (University of Bristol, Long Ashton, Bristol, United Kingdom), and anti-Y-type HMW-GS Ab were kindly provided by R. Rubin (R. Rubin, G. Galili, unpublished observations).

RESULTS

Expression of HMW-GS Genes in *E. coli*

Plasmids for expression of mature wheat HMW-GS in *E. coli* were constructed as described in "Materials and Methods" and are represented in the general scheme in Figure 1A. *E. coli* cells harboring the pET-3a-derived plasmids with the coding sequences for HMW-GS 1Dx2, 1Dx5, 1Dy12, and 1Dy10 were induced with isopropyl-β-D-thiogalactoside and the purified subunits were analyzed by SDS-PAGE (Fig. 2, lanes c–f). In each case, a major band appeared that comigrated with the corresponding natural subunit derived from the cv C.S. (Fig. 2, lane b, subunits 1Dx2 and 1Dy12) and the intervarietal substitution line C.S. (Chy-1D) (Fig. 2, lane a, subunits 1Dx5 and 1Dy10). Identification of the proteins produced in *E. coli* with the natural wheat HMW-GS was confirmed by Western blots using Ab raised against purified wheat X- and Y-type HMW-GS (data not shown). Occasionally, several faint bands migrating slightly more rapidly than the intact HMW-GS were detected mainly in the X-type subunits (see for example Fig. 2, lanes e and f). These bands presumably represent degradation products of the HMW-GS.

In Vitro Folding and Assembly of HMW-GS

To study the folding and assembly of the individual *E. coli*-derived wheat HMW-GS *in vitro,* the subunits were denatured and reduced in either 7 M urea, 0.1 M acetic acid, 1% (v/v) β-ME, or 3 M urea, 0.1 M acetic acid, 1% (v/v) β-ME and allowed to renature/reoxidize during dialysis or dilution into solutions containing 3 M urea, 0.1 M acetic acid, 0.001% (v/v) β-ME, or 0.01 M acetic acid, 0.001% (v/v) β-ME. In some initial experiments, we also replaced the β-ME in the renatur-

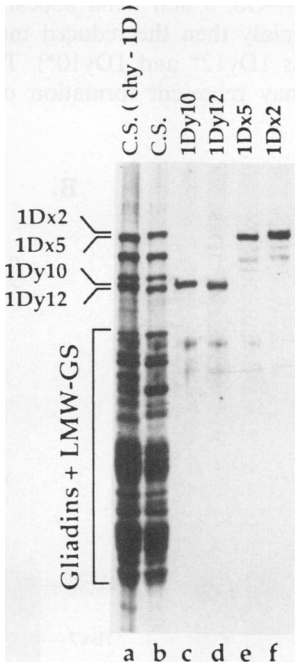

Figure 2. SDS-PAGE analysis of wild-type HMW-GS purified from *E. coli*. Bacteria harboring one of the plasmid constructs pET-3a-1Dy10, pET-3a-1Dy12, pET-3a-1Dx5, or pET-3a-1Dx2 (lanes c–f, respectively), were induced with isopropyl-β-D-thiogalactoside. HMW-GS were then purified and fractionated on 10% (w/v) SDS-PAGE under reducing conditions. The gel was stained with Coomassie blue. Lane a, Total storage proteins from the intervarietal substitution line C.S. (chy-1D) possessing the HMW-GS 1Dx5 and 1Dy10. Lane b, Total storage proteins from the wheat line C.S. possessing the HMW-GS 1Dx2 and 1Dy12. Specific HMW-GS and location of gliadins and LMW-GS are indicated on the left.

ation solutions with reduced and oxidized glutathione at the respective concentrations of 1 mM/0.1 mM or 10 mM/1 mM. All of these renaturation and reoxidation conditions yielded similar results (data not shown). Therefore, in further experiments we denatured and reduced the proteins in 3 M urea, 0.1 M acetic acid, 1% (v/v) β-ME and then dialyzed them in excess of 0.01 M acetic acid, 0.001% (v/v) β-ME. The denaturation midpoint of typical globular proteins in urea occurs in a concentration range from 4 to 7 M (14), so the 3 M urea concentration employed in these experiments serves primarily to dissolve the proteins and probably only partially to denature them. However, we shall use the term renature to describe changes that occur during urea removal.

Renaturation/reoxidation *in vitro* of the X-type subunits 1Dx2 and 1Dx5 resulted in the formation of four slower migrating disulfide bonded oligomers that could be detected either by staining with Coomassie blue or by immunological reactivity with X-type HMW-GS-specific Ab (Fig. 3, A and B, see arrows; see also ref. 9). Formation of disulfide bonded oligomers upon renaturation and reoxidation of the Y-type subunits 1Dy10 and 1Dy12 was much less efficient than in the X-type subunits and in some experiments was not even detected. However, when more protein was loaded on the gels, its presence was clearly confirmed by Coomassie blue staining and Western blot analysis using anti-1Dy12 Ab (Fig. 4, A and B, see arrows). Upon renaturation/reoxidation of the Y-type HMW-GS, a new band appeared that migrated slightly more rapidly then the reduced monomers (Fig. 4, bands marked as 1Dy12* and 1Dy10*). These rapidly migrating bands may represent formation of intramolecular disulfide bonds in these subunits. The SDS-PAGE mobility of the putative intramolecular disulfide-bonded form of HMW-GS 1Dy10 was greater than that of 1Dy12 (1Dy12* and 1Dy10* in Fig. 4). These relative mobilities are consistent with the relative mol wts of these subunits, but reversed from the mobilities of their completely reduced forms (1Dy10 and 1Dy12 in Fig. 4).

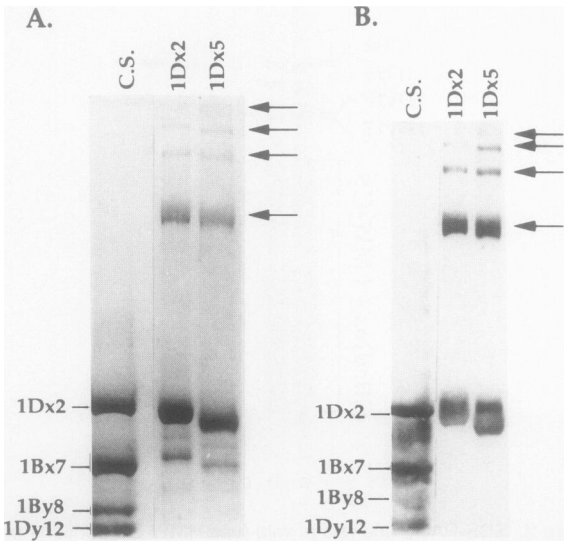

Figure 3. SDS-PAGE analysis of renatured/reoxidized X-type HMW-GS. The HMW-GS 1Dx2 and 1Dx5 HMW-GS, purified from *E. coli*, were reoxidized by dialysis and samples fractionated on a 10% (w/v) SDS-PAGE in the absence of reducing agents. Reduced native endosperm proteins of the wheat line C.S. were also included. The gel was either stained with Coomassie blue (A) or submitted to a Western blot with antibodies raised against 1Dx2 (B). The migration positions of the monomeric subunits are indicated by bars; arrows indicate the migration positions of oligomeric X-type subunits.

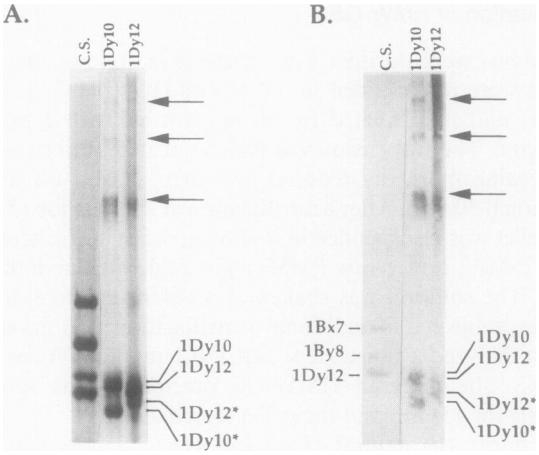

Figure 4. Fractionation of reoxidized Y-type HMW-GS. Reduced 1Dy10 and 1Dy12 HMW-GS, produced in *E. coli*, were reoxidized by dialysis and samples fractionated on a 10% (w/v) SDS-PAGE in the absence of reducing agents. Reduced native endosperm proteins of the wheat line C.S. were also included. The gel was either stained with Coomassie blue (A) or submitted to a Western blot with antibodies raised against 1Dy12 (B). The migration position of oligomeric Y-type subunits are indicated by arrows. Bars indicate the migration positions of the monomeric subunits, and the faster migrating monomeric subunits are marked with an asterisk.

Formation of rapidly migrating monomers in the X-type subunits was not detected in 10% (w/v) SDS-PAGE analysis (Fig. 3A). To assess this more accurately, the X-type subunits were subjected to extensive fractionation on 7.5% (w/v) SDS-PAGE. In this gel, a rapidly migrating monomeric band was clearly evident in subunit 1Dx5 (Fig. 5, band 1Dx5*). Although the quality of separation of this rapidly migrating subunit from the reduced form of 1Dx5 varied between different separations, we could not detect a rapidly migrating form for subunit 1Dx2 (Fig. 5).

The sensitivity of the *in vitro* formed inter- and intrachain disulfide bonds to reduction by β-ME is shown in Figure 6, using subunits 1Dx2 and 1Dy12 as examples. The putative intermolecular disulfide-bonded oligomeric forms of 1Dx2 could not be detected if renatured/reoxidized samples were adjusted to 0.1% (v/v) β-ME before SDS-PAGE. Likewise, adjustment of renatured/reoxidized samples to 1% (v/v) β-ME was needed to dramatically reduce the amount of the apparent dimeric band (Fig. 6A). In contrast, as low as 0.01% (v/v) β-ME was needed to dramatically reduce the putative intramolecular disulfide-bonded 1Dy12 monomeric forms (Fig. 6B, band marked as 1Dy12*). This indicates that these forms are not artifacts of reduction/reoxidation. The apparently greater stability of the intermolecular disulfide-bonded forms may involve a contribution from noncovalent protein-protein association forces.

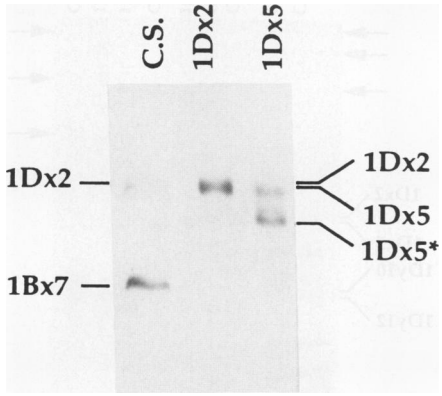

Figure 5. Formation of a faster migrating band by reoxidized 1Dx5 HMW-GS. Reduced 1Dx2 and 1Dx5 HMW-GS, purified from *E. coli*, were reoxidized by dialysis and samples fractionated on a 7.5% (w/v) SDS-PAGE in the absence of reducing agents. Reduced native endosperm proteins of the wheat line C.S. were also included. The gel was stained with Coomassie blue. The migration positions of the monomeric subunits are indicated by bars, and the faster migrating folded form of the 1Dx5 subunit is marked with an asterisk.

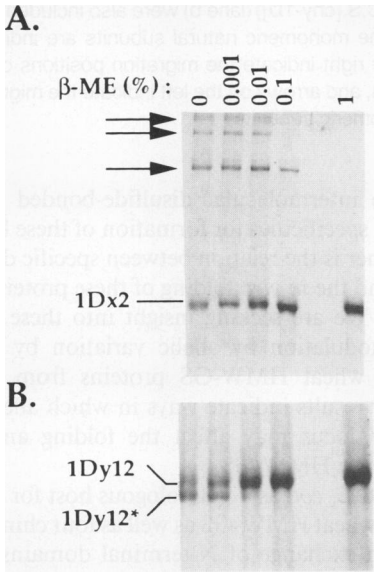

Figure 6. Fractionation of reoxidized 1Dx2 and 1Dy12 HMW-GS under increasing concentrations of reducing agents. Reduced 1Dx2 (A) and 1Dy12 (B) HMW-GS, purified from *E. coli*, were reoxidized by dialysis. Proteins were then dissolved in sample buffers containing increasing concentrations of β-ME and fractionated on a 10% (w/v) SDS-PAGE. The gel was stained with Coomassie blue. The migration positions of the monomeric subunits are indicated by bars. Arrows indicate the migration position of oligomeric X-type subunits, and the 1Dy12 subunit faster migrating folded forms are marked with an asterisk.

To eliminate the possibility that the formation of intra- and intermolecular disulfide bonds in the HMW-GS purified from *E. coli* were due to possible artifacts derived from the expression system, we compared the renaturation/reoxidation profile of the *E. coli*-derived subunits 1Dx2 and 1Dy12 with the corresponding natural subunits. The subunits were purified from the aneuploid C.S. line ditelosome $1B_L$, lacking the long arms of chromosomes 1B. The renaturation/reoxidation profiles of subunits 1Dx2 and 1Dy12 derived from both origins were identical (Fig. 7). In both cases, formation of disulfide bonded oligomers was much less efficient for the 1Dy12 then for the 1Dx2 subunits. Moreover, in both 1Dy12, but not 1Dx2 subunits, a rapidly migrating monomeric subunit was detected resulting from intramolecular disulfide bond formation (Fig. 7, bands 1Dy12*).

Expression of Chimeric HMW-GS in *E. coli*

HMW-GS migrate in SDS-PAGE anomalously slower than expected based on their actual M_r (22). To identify specific domains that are responsible for this anomalous migration, we constructed chimeric coding DNA sequences of the HMW-GS in which the N-terminal unique domains were substituted between the X- and Y-type subunits (Fig. 1B). Chimeric proteins produced in *E. coli* from these chimeric coding sequences were then analyzed by SDS-PAGE under reducing conditions. Although the N-terminal domain of 1Dy12 is larger in size than that of 1Dx2 (Fig. 1B), the reduced chimeric subunit 12/2, that is 1Dx2 in which the N-terminal sequence

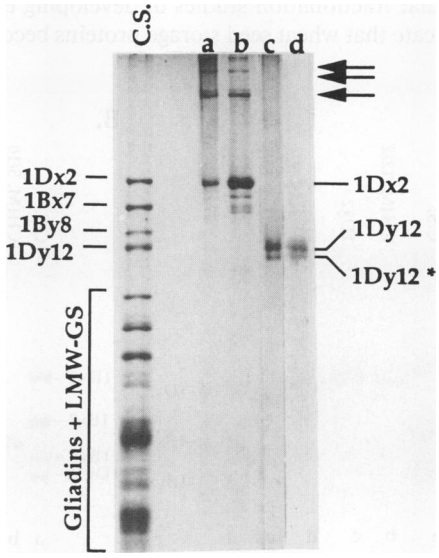

Figure 7. Fractionation of reoxidized HMW-GS purified from *E. coli* and from wheat seeds. Reduced 1Dx2 and 1Dy12 HMW-GS made in bacteria (lanes b and c) or purified from wheat seeds (lanes a and d), were reoxidized by dialysis and samples fractionated on a 10% (w/v) SDS-PAGE in the absence of reducing agents. Reduced, native endosperm proteins of the wheat line C.S. were also included. The gel was stained with Coomassie blue. Arrows indicate the migration positions of oligomeric X-type subunits. The migration positions of the monomeric subunits are indicated by bars, and the 1Dy12 subunit faster migrating folded form is marked with an asterisk.

was substituted with that of 1Dy12, migrated faster than 1Dx2 (Fig. 8A, *cf.* lanes b and c). Similarly, although the N-terminal domain of 1Dy10 is larger in size than that of 1Dx5 (Fig. 1B), the reduced chimeric subunit 10/5 migrated faster by SDS-PAGE than 1Dx5 (Fig. 8A, *cf.* lanes d and e). Similar results were also obtained (our manuscript in preparation). When the N-termini of the Y-type subunits were substituted with those of the X-type subunits, unexpected results were also obtained. Although smaller in M_r, the reduced chimeric subunits 5/10 and 2/12 migrated slower by SDS-PAGE than subunits 1Dy10 and 1Dy12, respectively (Fig. 8B, *cf.* lanes b and e, and c and d). These results clearly show that the N-terminal domains of the HMW-GS contributed significantly to their anomalous migration.

To identify the domains responsible for the intramolecular disulfide bond formation within the Y-type HMW-GS, the chimeric proteins were subjected to a renaturation/reoxidation experiment. The rapidly migrating monomeric subunit was detected in the chimeric subunit 2/12 but not in 12/2 (Fig. 9, *cf.* lanes i and c), showing that the intramolecular disulfide bond in 1Dy12 was determined by its carboxy-terminal domain. Similarly to 12/2, no rapidly migrating monomeric form was evident for the chimeric subunit 10/5 (Fig. 9, lane f). Unfortunately, detection of a possible rapidly migrating monomer in the chimeric subunit 5/10 was difficult, since two faint contaminants migrating slightly faster than the reduced monomer were always present (Fig. 8B, lane b).

DISCUSSION

Subcellular fractionation studies of developing endosperm tissue indicate that wheat seed storage proteins become incorporated into intermolecular disulfide-bonded aggregates *in vivo* (5). The specificities for formation of these bonds are not known; neither is the relation between specific disulfide bond formation and the *in vivo* folding of these proteins as they are synthesized. We are seeking insight into these relationships and their modulation by allelic variation by studying the behavior of wheat HMW-GS proteins from recombinant sources. Our results indicate ways in which allelic variations at the *Glu-1* locus may affect the folding and interactive behaviors of the HMW-GS.

We utilized *E. coli* as a heterologous host for expression of four mature wheat HMW-GS as well as four chimeric proteins derived from exchange of N-terminal domains between X- and Y-type subunits. Each of the four wild-type HMW-GS produced in *E. coli* migrated by SDS-PAGE identical to their corresponding natural subunits extracted from wheat grains (Fig. 2). Wheat HMW-GS migrate by SDS-PAGE more slowly than expected based on their actual M_r. This is likely to be due to specific conformations of β-turn and β-spiral that exist in the central repetitive region (6, 11) and are not completely denatured in SDS. Therefore, the identical migration by SDS-PAGE indicates that the subunits produced in *E. coli* possess structures similar if not identical to those of the natural subunits. The similarity in structure between the natural and *E. coli*-derived subunits was further supported by the fact that subunits 1Dx2 and 1Dy12 derived from wheat grains or from

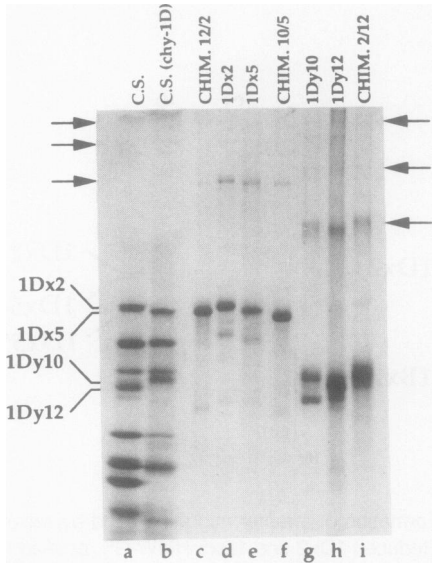

Figure 9. Fractionation of reoxidized, natural, and chimeric HMW-GS purified from *E. coli*. Samples of natural 1Dx2, 1Dx5, 1Dy10 and 1Dy12 HMW-GS (lanes d, e, g, and h, respectively) and the chimeric proteins 12/2, 10/5, and 2/12, (lanes c, f, and i, respectively) were fractionated on a 10% (w/v) SDS-PAGE in the absence of reducing agents. The gel was stained with Coomassie blue. Lanes containing native endosperm proteins of the wheat line C.S. (lane a) and an intervarietal substitution line of C.S., harboring the 1D chromosomes of cheyenne (C.S.[chy-1D]) (lane b) were also included. The migration positions of the monomeric natural subunits are indicated by bars. Arrows on the right indicate the migration positions of Y-type oligomeric subunits, and arrows on the left indicate the migration positions of X-type oligomeric subunits.

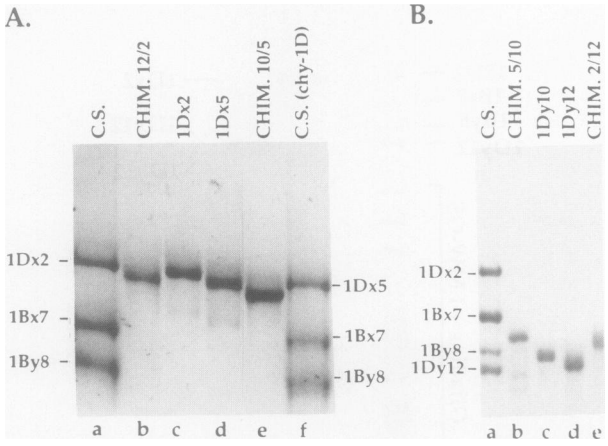

Figure 8. Fractionation of natural and chimeric HMW-GS purified from *E. coli*. Samples of reduced 1Dx2, 1Dx5, and the chimera 12/2, 10/5 HMW-GS (A) as well as reduced 1Dy10, 1Dy12, and the chimera 2/12, 5/10 HMW-GS (B), were purified from *E. coli* and fractionated on a 10% (w/v) SDS-PAGE. The gel was stained with Coomassie blue. Lanes containing native endosperm proteins of the wheat line C.S. and an intervarietal substitution line of C.S., harboring the 1D chromosomes of cheyenne (C.S.[chy-1D]), were also included. The migration positions of the monomeric natural subunits are indicated by bars.

E. coli showed identical patterns of folding and assembly after renaturation/reoxidation (Fig. 7).

Following renaturation/reoxidation, new, slowly migrating bands that cross-reacted with the HMW-GS-specific Ab were detected. These bands are sensitive to reduction by β-ME and are therefore considered oligomers linked by intermolecular disulfide bonds (Fig. 6). We previously proved that they are disulfide-bonded oligomers by two-dimensional SDS-PAGE in which the first dimension was performed in the absence of reducing agents and the second in their presence (9).

Not only oligomeric bands were formed after the renaturation/reoxidation process. New bands that migrated slightly more rapidly than the reduced monomeric subunits were detected in all of the HMW-GS, except for subunit 1Dx2. These were apparently generated by the formation of intramolecular disulfide bonds as was previously shown for a variety of other proteins including wheat gliadins (2). Interestingly, the disulfide bonded monomer of 1Dy12 migrated slower by SDS-PAGE than that of 1Dy10, in accordance with the actual M_r of the two proteins (Fig. 4A). This is in contrast with the fact that the reduced subunit 1Dy12 migrates faster by SDS-PAGE than the reduced subunit 1Dy10, although its actual size is larger. It was previously shown (11) that this anomalous SDS-PAGE migration of the reduced 1Dy12 relative to the reduced 1Dy10 is determined by one to six amino acid differences in the C-terminal end of the repetitive region (see Fig. 10A). These authors observed that the relative migration anomalies of HMW-GS 1Dy10 and 1Dy12, which occurred in SDS-PAGE, were eliminated in the presence of 4 M urea. Our results indicate that the formation of intramolecular disulfide bonds within these subunits eliminates or counterbalances the specific conformational differences that cause the anomalous SDS-PAGE migration (Fig. 4, bands 1Dy10* and 1Dy12*). The SDS-PAGE analysis after reoxidation/renaturation also revealed a large proportion of molecules with mobility characteristic of the reduced monomeric state (Fig. 4, bands 1Dy10 and 1Dy12). It is not clear whether this most nearly reflects a sulfhydryl/disulfide equilibrium position reached during the reoxidation or during the analysis. An experiment designed to alkylate (50 mM iodoacetamide) free sulfhydryls remaining after reoxidation resulted in elimination of the high mobility monomeric form (data not shown). Additional experimental work will also be required to determine whether the urea-resident protein conformational similarities or the SDS-PAGE observed differences are more representative of the Y-type HMW-GS native state.

Possible locations of the cysteine residues involved in the 1Dy-type HMW-GS intramolecular disulfide bond may be inferred from the fact that the environments of the N-terminal cysteines are identical in 1Dy10 and 1Dy12. As a result, cysteines in this region would be expected to behave similarly in the two proteins, whether in SDS-PAGE or in urea. Thus, whereas intramolecular disulfide bonds could, hypothetically, be formed in the N-terminal regions, they could not differentiate the two proteins. At least one of the cysteine residues participating in this intramolecular disulfide bond must come from the C-terminal region of the protein. This is also supported by the fact that out of the two chimeras of 1Dx2 and 1Dy12 examined, only the 1Dx2/1Dy12 species, which contains the 1Dy12 C-terminal cysteines, formed a faster moving

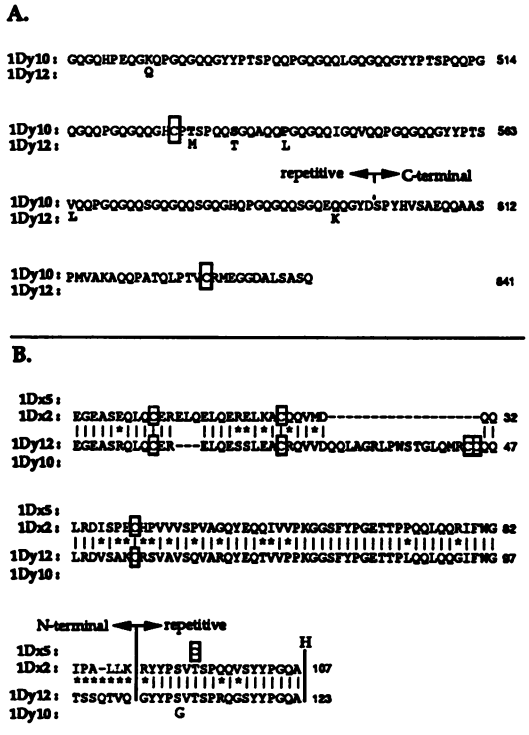

Figure 10. Amino acid sequence comparison of the C- and N-terminal regions of different HMW-GS. A, Comparison of the C-terminal amino acid sequences of subunits 1Dy10 and 1Dy12. In subunit 1Dy12, only amino acids that are not identical with Dy10 are indicated. B, Amino acid comparison of the N-terminal unique region plus the first 19 residues of the repetitive domain of subunits 1Dx2, 1Dx5, 1Dy12, and 1Dy10. In subunits 1Dx5 and 1Dy10, only amino acids that are not identical with 1Dx2 or with 1Dy12, respectively, are indicated. Identical amino acids between the HMW-GS 1Dx2 and 1Dy12 are indicated by vertical lines and different amino acids are indicated by asterisks. Cysteine residues are enclosed in squares. Dashed lines indicate amino acid deletions. H represents the cleavage point of *Hin*dIII at the DNA level.

SDS-PAGE band upon reoxidation/renaturation (Fig. 9, *cf.* lanes c and i). Consideration of both the rod-like shape, which native HMW-GS are proposed to assume (6), and of end-to-end radial distribution functions for polymer chains in random-walk configuration (7), which completely denatured HMW-GS would be expected to assume, indicate that the N- and C-terminal region groups of cysteines would be remote from each other. Thus, although we cannot exclude the possibility that the intramolecular disulfide bonds within the Y-type subunits may resume from association of C- and N-terminal cysteines, we conclude that the highest probability for intra-chain disulfide bond formation in the 1Dy-type HMW-GS exist through cysteine pairing within rather than between the N- and C-terminal groups. An example of such domain-limited interchain disulfide bond formation that occurs *in vivo* is the biosynthesis of the pro-α chain of type I procollagen (3). We suggest that disulfide bonds are formed between the two proximate C-terminal region cysteines of each subunit to form a 103-amino acid polypeptide loop that contains five of the six amino acids, implicated by Golds-

brough *et al.* (11), in the SDS-PAGE mobility difference between 1Dy10 and 1Dy12 HMW-GS (Fig. 10A). No analogous situation exists for the X-type subunits, inasmuch as they possess only one C-terminal region cysteine (Fig. 1B). Our indications for the presence of an intramolecular disulfide bond between the two C-terminal cysteines of the Y-type subunits is of particular importance inasmuch as these cysteines were previously speculated to participate in intermolecular bonding and to play an important role in glutenin assembly (13, 15, 26, 29). Thus, the ability of the two C-terminal cysteines in Y-type subunits to form an intramolecular disulfide bond may reduce their ability to form intermolecular disulfide bonds during glutenin polymerization. Indeed, whereas both X- and Y-type subunits could self-assemble into oligomers linked by intermolecular disulfide bonds, the efficiency of oligomer formation was much greater for the X-type than for the Y-type subunits (see Fig. 7).

The detection of a high-mobility monomeric form of HMW-GS 1Dx5, but not 1Dx2, after reoxidation (Fig. 5) is consistent with formation of an intramolecular disulfide bond involving cysteine-97 of 1Dx5, to generate a polypeptide loop of 58 to 81 amino acids. Cysteine-97 is unique among sequences characterized as X-type HMW-GS genes and is present in 1Dx5 in addition to the other cysteines (see Fig. 1B). Subunit 1Dx2, which lacks cysteine-97, possesses no disulfide bonding potentials equivalent to those that require this residue. We are continuing the investigation of this possibility.

The generally slower SDS-PAGE mobility of X-type, as compared with Y-type, HMW-GS is determined by the nature of their combined repetitive and C-terminal domains, but may be modulated in unexpected ways by the nature of the N-terminal regions (Fig. 8; our manuscript in preparation). Although the N-terminal unique domains of Y-type subunits are larger than those of X-types, chimeric Y/X subunits migrated in SDS-PAGE anomalously faster than the corresponding X-type subunit, and X/Y chimeras migrated anomalously slower than the corresponding Y-type subunit (Fig. 8). Since the effects are reciprocal, these are probably isolated functions of the N-terminal regions. Further analysis of the basis of these anomalies requires additional information. A single amino acid substitution has been shown to cause large differences in the SDS-PAGE mobilities of wild-type and mutant histidine-transport proteins (21); though the N-terminal regions of 1Dx and 1Dy HMW-GS are homologous, they show extensive divergence, differing by 29 amino acid substitutions in addition to two insertion/deletion events (Fig. 10B). The possible role of these differences in the observed migration anomalies and in the physiochemical properties of the HMW-GS is a continuing subject of study in our laboratories.

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