The transcription and translation *in vitro* of individual cereal storage-protein genes from wheat (*Triticum aestivum*, cv. Chinese Spring)

Evidence for translocation of the translation products and disulphide-bond formation

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Genes coding for the high- M_r ['high-molecular-weight' (HMW)] glutenin subunit 12 and for a γ -gliadin from wheat (*Triticum aestivum*, cv. Chinese Spring) were subcloned into transcription–translation vectors. In each case transcription *in vitro* yielded a RNA transcript which when added to a rabbit reticulocyte cell-free translation system directed the synthesis of a polypeptide of appropriate M_r by SDS/polyacrylamide-gel electrophoresis (SDS/PAGE). When dog pancreatic microsomal vesicles were added to the translation system, translocation of the newly synthesized polypeptides occurred, as judged by protection from proteolysis. When translation and translocation of the γ -gliadin was carried out under conditions favouring the formation of disulphide bonds, a polypeptide was synthesized which had a faster mobility on SDS/PAGE carried out under non-reducing conditions than under reducing conditions. This suggests that the processed and translocated γ -gliadin forms an intramolecular disulphide bond or bonds during synthesis *in vitro*.

INTRODUCTION

Wheat (*Triticum aestivum*) cereal storage proteins are synthesized on the endoplasmic reticulum of the developing endosperm (Greene, 1981; Donovan *et al.*, 1982) and are deposited in the cell as high- M_r ['high-molecular-weight' (HMW)] aggregates (protein bodies) (Graham *et al.*, 1962). In flour, these proteins are constituents of gluten which forms a visco-elastic complex important in determining the baking quality of doughs (Payne, 1986). The protein components of gluten can be classified into two types, those soluble in water/alkanol solvent mixtures, namely the gliadins, and those that are soluble in such solvents only after being reduced, namely the glutenins. The gliadins are thought to give extensibility to doughs and do not form oligomers, whereas the glutenins are thought to give elasticity to doughs by forming disulphide-linked oligomers (Payne, 1986; Ewart, 1972, 1979). Information on the structure of gluten and on the formation of disulphide bonds in these proteins is limited because of the insolubility of the complex and the difficulty in purification of the individual proteins. A number of the HMW-glutenin-subunit genes and gliadin genes have, however, been isolated and sequenced (Kasarda *et al.*, 1984; Rafalski *et al.*, 1984; Forde *et al.*, 1985; Sugiyama *et al.*, 1985; Thompson *et al.*, 1985; Bartels *et al.*, 1986; Halford *et al.*, 1987).

The deduced amino acid sequences reveal that the gliadins contain even numbers of cysteine residues and are likely to form intramolecular disulphide bonds. The HMW glutenins, however, contain between four and seven cysteine residues, with most of the cysteine residues occurring at the end of the protein. It may be possible,

therefore, that these polypeptides form linear polymers joined by intermolecular disulphide bonds. In order to understand more about the contribution of individual cereal storage proteins to the structural and physical properties of gluten, a model system is required to study the formation of disulphide bonds during protein synthesis.

In order to study this process in individual proteins, we have subcloned a HMW-glutenin gene and a γ -gliadin gene into a transcription–translation vector. The transcribed RNA from each gene has been used to direct the synthesis of the polypeptide coded by that gene. We have shown that, when microsomal vesicles are included in the '*in vitro*' translation system, co-translational translocation and signal-peptide cleavage occur. Also we have shown in the case of the γ -gliadin that, when translation is carried out under conditions favouring disulphide-bond formation, a polypeptide is synthesized which has a greater mobility during SDS/PAGE carried out under non-reducing conditions than under reducing conditions, consistent with the formation of an intramolecular disulphide bond or bonds.

EXPERIMENTAL

Materials

The HMW-glutenin gene, γ -gliadin gene and *Escherichia coli* (strain ED8800) were generously provided by Dr. R. D. Thompson (Thompson *et al.*, 1985; Bartels *et al.*, 1986). Restriction enzyme, phage- T_4 DNA ligase, phage- T_7 RNA polymerase, RNAase inhibitor, nucleotides and 7-methylguanosylguanosine triphosphate

Abbreviations used: HMW glutenin, high- M_r ('high-molecular-weight') glutenin; PAGE, polyacrylamide-gel electrophoresis; 7-mGpppG, 7-methylguanosylguanosine triphosphate; DTT, dithiothreitol; poly(A)⁺, polyadenylated.

(7-mGpppG) were purchased from Boehringer-Mannheim (Lewes, East Sussex, U.K.), L-[³⁵S]methionine and Amplify were purchased from Amersham International (Amersham, Bucks., U.K.). RNAase-free DNAase was purchased from Promega Biotech (P & S Biochemicals, Liverpool, U.K.), and the transcription-translation vector 'Bluescribe' was from Stratagene (Northumbria Biologicals, Cramlington, Northumberland, U.K.).

Dog pancreatic microsomal membranes were a gift from Dr. B. M. Austen (Austen *et al.*, 1984). The authentic prolamins were extracted from milled wheat (cv. Chinese Spring) (Miflin *et al.*, 1980) and ¹⁴C-labelled by reductive methylation of lysine residues (Dottavio-Martin & Ravel, 1978). Pituitary poly(A)⁺ RNA was isolated from bovine pituitary glands by standard procedures (Maniatis *et al.*, 1982).

Construction of recombinant plasmids

The entire coding sequence of the HMW-glutenin gene, including some 3' and 5' flanking sequences, was supplied as a 2.7 kb fragment sub-cloned into pUC9. This plasmid (pSBB17) was digested with restriction endonuclease *Sma*I, followed by *Sa*II to yield two fragments of equal size (2.7 kb). Bluescribe plasmid was digested with *Sma*I and *Sa*II and the resulting 3.2 kb fragment purified by electroelution after electrophoresis through agarose (Maniatis *et al.*, 1982). The two fragments from the digestion of pSBB17 were mixed and ligated with this vector. Competent cells were prepared by CaCl₂ treatment and were transformed with the recombinant plasmids. The recombinants containing the HMW-glutenin-gene sequence were identified after digestion with *Hind*III and designated 'pUKC1000' (Fig. 1). The γ -gliadin DNA was supplied as a 1.4 kb fragment subcloned into pUC9. The coding sequence was isolated by digesting this plasmid (pTag1436) with *Hind*III, followed by *Hpa*I. This fragment was sub-cloned into the *Sma*I/*Hind*III site of Bluescribe and designated 'pUKC1001' (Fig. 1). Sequencing of this clone showed that it differed from the published sequence (Bartels *et al.*, 1986) by lacking 159 bp, giving an in-frame deletion of 53 residues in the proline- and glutamine-rich repeat region, which does not contain cysteine and forms a distinct domain from the disulphide-bonded C-terminal portion of the molecule. Plasmid DNA was purified by the method of Birnboim & Doly (1979) as described by Maniatis *et al.* (1982).

Cell-free transcription

Transcription was carried out essentially as described by Krieg & Melton (1984). Plasmid DNA (2 μ g) was linearized with *Sa*II (pUKC1000) or with *Hind*III (pUKC1001) and transcribed in a total volume of 100 μ l containing 6 mM-MgCl₂, 2 mM-spermidine, 5 mM-NaCl, 10 mM-dithiothreitol (DTT), 0.5 mM -ATP, -UTP, -CTP and -7 mGpppG, 0.1 mM-GTP, and 40 mM-Tris/HCl, pH 7.5 at 37 °C, with 100 units of human placental RNAase inhibitor and 30 units of phage-T₇ RNA polymerase. Transcription was carried out at 37 °C for 1 h, after which template DNA was removed by incubating with 2 units of DNAase at 37 °C for 15 min. The mixture was extracted with phenol, followed by two extractions with chloroform/3-methylbutan-1-ol (24:1, v/v). The RNA was recovered by adding ammonium acetate, pH 5.0, to 2.5 M and precipitating with 2.5 vol. of ethanol at -20 °C for 16 h. The precipitate was recovered

by centrifugation and the pellet washed twice with 75 % (v/v) ethanol and dried under vacuum. The RNA was resuspended in 20 μ l of diethyl pyrocarbonate-treated water.

Cell-free translation

Synthesized RNA was translated in a micrococcal-nuclease-treated rabbit reticulocyte lysate prepared by the method of Pelham & Jackson (1976). The lysate was either supplemented with dithiothreitol (DTT) to 2 mM or no reductant was added. The translation mixture contained, in a final volume of 20 μ l, 10 μ l of reticulocyte lysate, 1 μ l of transcribed RNA or 1 μ g of pituitary poly(A)⁺ mRNA, 1 μ l of amino acids (1 mM) without methionine, 50 mM-potassium acetate, 0.75 mM-magnesium acetate, 25 mM-creatine phosphate, creatine phosphokinase (25 μ g·ml⁻¹), 1 mM-ATP, 0.2 mM-GTP, 0.5 mM-glucose 6-phosphate, 10–15 μ Ci of L-[³⁵S]methionine and, where indicated, micrococcal-nuclease-treated dog pancreatic microsomal membranes (0.85 mg·ml⁻¹).

Translations were carried out for 60 min at 30 °C. Sample preparation for electrophoresis was as described under 'SDS/PAGE' below.

Post-translational proteolysis

Translation mixtures were assayed for translocation by treating post-translationally with proteinase K (250 μ g·ml⁻¹) for 25 min on ice, in either the absence or presence of Triton X-100 (1 %, v/v). Phenylmethanesulphonyl fluoride was then added to 2 mg·ml⁻¹ and the mixture incubated for a further 5 min on ice. SDS/PAGE sample buffer was added and proteins separated as described under 'SDS/PAGE'.

SDS/PAGE

For samples which were separated under non-reducing conditions, iodoacetamide was added to a final concentration of 100 mM. Samples were then incubated on ice for 10 min, mixed with 5 vol. of SDS/PAGE sample buffer [0.02 M-Tris/HCl (pH 8.0)/0.002 M-EDTA/6 % (w/v) SDS/20 % (w/v) sucrose/0.003 % (w/v) Bromophenol Blue] and boiled for 4 min.

For samples separated under reducing conditions, DTT was added to a final concentration of 50 mM, along with 5 vol. of SDS/PAGE sample buffer and boiled for 4 min. After cooling, iodoacetamide was added to a final concentration of 100 mM, and the samples were incubated at 22 °C for 45 min. Electrophoresis was carried out in the presence of SDS by the method of Laemmli (1970).

RESULTS

A genomic clone containing the uninterrupted coding sequence for the HMW-glutenin subunit 12 and a cDNA clone containing part of the coding sequence for a γ -gliadin from wheat (cv. Chinese Spring) were subcloned into the transcription-translation vector 'Bluescribe'. The resulting plasmids pUKC1000 and pUKC1001 are shown in Fig. 1. Transcription of the cloned genes was brought about by linearizing pUKC1000 with *Sa*II and pUKC1001 with *Hind*III and transcribing with T₇ RNA polymerase. The integrity of the mRNA was checked by 'native' electrophoresis through agarose.

The RNA obtained from transcription of pUKC1000 was translated in a rabbit reticulocyte lysate, in either the presence or absence of dog pancreatic microsomal

Fig. 1. Construct of pUKC1000 and pUKC1001

The genes coding for the HMW-glutenin subunit 12 and a γ -gliadin were subcloned into the *SmaI*/*SaI* and *SmaI*/*HindIII* sites of Bluescribe respectively. The direction of transcription is indicated with an arrow.

vesicles (Figs. 2 and 3). The major polypeptide synthesized had a mobility on SDS/PAGE similar to that of the authentic protein (Fig. 2, tracks 1 and 3). A small difference in mobility on SDS/PAGE was observed between translation products synthesized in the presence and absence of microsomal vesicles (Fig. 2, tracks 2 and 3; Fig. 3, tracks 1 and 2). The susceptibility of translation product to proteolysis was investigated to ascertain whether this difference in mobility was due to translocation of the synthesized protein to the interior of the microsomal vesicle and associated signal-peptide cleavage. Translation was carried out either in the presence or absence of microsomal vesicles, and translation products were subjected to proteolysis with proteinase K. As can be seen (Fig. 3, track 3) the translation products synthesized in the absence of microsomal vesicles were degraded, whereas protection from proteo-

lysis was observed for translation products synthesized in the presence of vesicles (track 4). When samples were pre-treated with detergent to solubilize microsomal vesicles before proteinase K treatment, translation products were degraded (track 5). Thus the products of translation in the presence of microsomal vesicles are translocated across the membrane and segregated in the vesicle interior. Similar evidence for co-translational translocation and segregation was observed on translation of the transcribed mRNA for γ -gliadin (see below).

To investigate the formation of disulphide bonds *in vitro* during translation, a rabbit reticulocyte lysate was prepared to which no DTT was added. The poly(A)⁺ RNA from bovine pituitary gland, which contains high levels of prolactin mRNA, was used to characterize this lysate preparation for the formation of disulphide-bonded proteins. Translations were carried out in the absence or presence of microsomal vesicles. The trans-

Fig. 2. Cell-free translation of HMW-glutenin subunit 12 mRNA in the presence and absence of dog pancreatic microsomal vesicles

Translation was carried out in a DTT-supplemented rabbit reticulocyte lysate and translation products resolved by SDS/PAGE on a 11% (w/v) polyacrylamide gel and detected by fluorography as described in the Experimental section. Lane 1, alkylated authentic ¹⁴C-radiolabelled prolamins; lane 2, products of translation in the absence of microsomal vesicles; lane 3, products of translation in the presence of microsomal vesicles. The HMW-glutenin subunits are numbered as described by Payne *et al.* (1980).

Lane...	1	2	3	4	5
Microsomes...	—	+	—	+	+
Proteinase K...	—	—	+	+	+
Triton X-100...	—	—	—	—	+

Fig. 3. Cell-free translation of HMW-glutenin subunit 12 mRNA: protection from proteolysis in the presence of microsomal vesicles

Translation was carried out in a DTT-supplemented rabbit reticulocyte lysate, in either the presence or absence of microsomal vesicles. Lanes 1 and 3, products of translation in the absence of microsomal vesicles; lanes 2, 4 and 5, products of translation in the presence of microsomal vesicles; lanes 3 and 4, translation products were treated with proteinase K; lane 5, translation products were treated with Triton X-100 (1%, v/v) and proteinase K.

Fig. 4. Cell-free translation of pituitary poly(A)⁺ RNA under conditions favouring the formation of disulphide bonds

Cell-free translation was carried out in a rabbit reticulocyte lysate prepared without the addition of DTT. Translation products were separated by SDS/PAGE on a 11 %-(w/v)-polyacrylamide gel after either being reduced with 50 mM-DTT and carboxamidomethylated (lanes 1 and 2) or after being carboxamidomethylated only (lanes 3 and 4). Lanes 1 and 3, products of translation in the absence of microsomal vesicles; lanes 2 and 4, products of translation in the presence of microsomal vesicles.

lation products were separated by SDS/PAGE after being either *S*-carboxamidomethylated in the absence of DTT, or reduced with DTT and then *S*-carboxamidomethylated. The results are presented in Fig. 4. In the absence of microsomal vesicles there is no difference in mobility between prolactin separated under reducing or non-reducing conditions (tracks 1 and 3). In the presence of microsomal vesicles, signal-peptide cleavage occurs (tracks 1 and 2), and a clear difference is observed between the prolactin separated under reducing and non-reducing conditions (tracks 2 and 4). Thus, under conditions which preserve pre-existing disulphide bonds the prolactin synthesized *in vitro* has a greater mobility, demonstrating the formation of intramolecular disulphide bonds. *S*-Carboxymethylation of purified prolactin did not alter its mobility during SDS/PAGE run under reducing or non-reducing conditions (results not shown). Inclusion of oxidized glutathione (GSSG) in the translation mixture inhibited translation and did not increase the yield of disulphide-bonded prolactin (results not shown).

This lysate preparation was used to investigate the formation of disulphide bonds during the synthesis of the γ -gliadin. The RNA from transcription of pUKC1001 was translated in either the presence or absence of microsomal vesicles, and the translation products were separated by SDS/PAGE (Fig. 5). In the absence of microsomes, several polypeptides were synthesized (track 1), which were sensitive to proteinase K (track 5). However, in the presence of microsomal vesicles one major translation product was synthesized (track 2) which was resistant to proteolysis with proteinase K (track 3). When samples were pre-treated with detergent, this translation product was degraded (track 6). Thus the γ -gliadin synthesized is translocated into the vesicle interior. When the products from translation in the presence of microsomal vesicles were treated with proteinase K and separated under reducing and non-

Fig. 5. Cell-free translation of γ -gliadin mRNA under conditions favouring the formation of disulphide bonds

Translation was carried out in a rabbit reticulocyte lysate prepared as described in the legend to Fig. 4. Translation products were separated by SDS/PAGE on an 11 %-(w/v)-polyacrylamide gel under reducing conditions (lanes 1, 2, 3, 5 and 6) and under non-reducing conditions (lane 4). Lanes 1 and 5, products of translation in the absence of microsomal vesicles; lanes 3, 4 and 6, products of translation in the presence of microsomal vesicles; lanes 3 and 4, translation products were treated with proteinase K; lane 6, translation products were treated with 1 % (v/v) Triton X-100 and proteinase K.

reducing conditions, a clear difference in mobility was observed (tracks 3 and 4). Thus, under conditions which preserve disulphide bonds, the γ -gliadin synthesized *in vitro* has a greater mobility, demonstrating the formation of intramolecular disulphide bonds during the translation incubation.

DISCUSSION

Study of the details of the biosynthesis and assembly of cereal storage proteins has been limited, in part because of the complexity of products obtained on translation *in vitro* of mRNA isolated from cereal endosperm. When wheat endosperm poly(A)⁺ mRNA is translated in a rabbit reticulocyte-lysate system, some of the products formed co-migrate with '*in vivo*'-labelled storage proteins (Forde & Mifflin, 1983). However, because of the complexity of the bands obtained after SDS/PAGE, it is difficult to detect changes in the electrophoretic mobility of individual translation products, such as may occur after signal-peptide cleavage or disulphide-bond formation.

We have described here a system whereby the synthesis of individual wheat cereal storage proteins can be studied by using a cell-free system. The DNA sequence of interest is subcloned into a transcription-translation vector and mRNA synthesized *in vitro* by using a viral RNA polymerase. The resulting transcript can then be used in a cell-free system to direct the synthesis of the

protein coded by the original gene. We have shown that, in this case, inclusion of microsomal vesicles in the translation mixture allows co-translational translocation of the nascent polypeptide chain and signal-peptide cleavage. This result is consistent with earlier results reported for the synthesis of isolated wheat gliadin mRNA (Greene, 1981; Donovan *et al.*, 1982). In that work, the products translated *in vitro* using a wheat-germ translation system had M_r values higher than those of products synthesized *in vivo*. Our results now demonstrate that the synthesis of individual wheat storage proteins can be studied *in vitro* by the use of a heterologous cell-free system.

In the case of the HMW-glutenin subunit 12, the product obtained *in vitro* after translation in the presence of microsomal vesicles had a mobility identical with that of the authentic subunit 12 observed in extracts from the mature endosperm. For the γ -gliadin translation product this positive identification was not possible. In wheat endosperm the sulphur-rich prolamins (α -, β - and γ -gliadins and low- M_r glutenin subunits) are a highly heterogeneous group of apparent $M_r \sim 30000$, and individual γ -gliadins are not well resolved in SDS/PAGE of total extracted wheat endosperm storage proteins. Furthermore, the γ -gliadin clone has a deletion of 53 residues in the repeat region and hence does not correspond precisely to any authentic γ -gliadin; however, the apparent M_r of the translation product corresponds to that deduced from the cDNA sequence. Since the 'repeat' domain and the 'cysteine-rich' domain of S-rich prolamines appear to function as independent folding units (Shewry *et al.*, 1987), this modified γ -gliadin remains a satisfactory model for studying co-translational disulphide-bond formation in these proteins.

As a first step towards understanding the complex processes involved in protein-body formation *in vivo*, we have attempted to study the formation of disulphide bonds during cereal-storage-protein synthesis *in vitro*. To this end we prepared a rabbit reticulocyte lysate that was active in synthesis but did not inhibit the formation of disulphide bonds. Under normal conditions, DTT is added to freshly prepared reticulocyte lysates to prevent oxidation of initiation factors (Jackson *et al.*, 1983) and to maintain the endogenous ribonuclease inhibitor (Scheele, 1983). We prepared a rabbit reticulocyte lysate using standard procedures, but did not add any exogenous reducing agent. The lysate prepared in this fashion was characterized for disulphide-bond formation by using pituitary poly(A)⁺ RNA which codes predominantly for the hormone prolactin. As reported previously (Kaderbhai & Austen, 1985), the formation of disulphide bonds in prolactin can be monitored by performing SDS/PAGE under non-reducing conditions. The disulphide-bonded product has a greater mobility than the reduced protein, owing to the formation of a more compact structure, which decreases its hydrodynamic volume (Goldenberg & Creighton, 1984). Previous work has demonstrated the formation of disulphide bonds *in vitro* with either gel-filtered (Scheele & Jacoby, 1982) or non-gel-filtered (Kaderbhai & Austen, 1985) lysates. In each case reductant was added to the lysate during preparation, and GSSG was added before initiation of translation in order to obtain a lysate which was competent in forming disulphide bonds in newly synthesized proteins. We also prepared similar DTT-

supplemented non-gel-filtered lysates, but found that the high concentrations of GSSG required for disulphide-bond formation (4–5 mM) drastically inhibited synthesis (results not shown). Thus we chose to use a lysate which did not contain any added reductant or oxidant, in order to study the formation of disulphide bonds in cereal storage proteins.

The results obtained from this experiment showed that the γ -gliadin can form intramolecular disulphide bonds *in vitro*. This is consistent with the physical properties of gliadins in that they are monomeric globular proteins. Translation of the HMW-glutenin mRNA in a lysate competent in forming disulphide bonds in newly synthesized proteins resulted in the synthesis of a protein with a slightly greater mobility on SDS/PAGE carried out under non-reducing conditions (results not shown). Whether this is due to the formation of disulphide bonds is unclear. Under the conditions used, no well-resolved oligomeric structures were observed from the translation of HMW glutenin *in vitro*, but we cannot rule out the possibility that this protein could form intermolecular disulphide bonds *in vivo*. Both the time course over which translation *in vitro* is performed and the ill-defined concentration of translation products within the microsomal vesicles make it difficult to extrapolate to the situation *in vivo*. In order to understand more about the formation of disulphide bonds during biosynthesis and aggregation of these proteins one needs to study the synthesis of other cereal storage proteins, both individually and in combination.

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