Plant Physiol. (1983) 73, 949–955
0032-0889/83/73/0949/07/$00.50/0

Role of Endoplasmic Reticulum in Biosynthesis of Oat Globulin Precursors[1]

Received for publication May 25, 1983 and in revised form August 12, 1983

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ABSTRACT

Oat (*Avena sativa* L.) groats were labeled with radioactive leucine and salt-soluble proteins were extracted and analyzed. Polyacrylamide gel electrophoresis followed by fluorography indicated two radioactive polypeptides with molecular weight 58 to 62 kilodaltons which were similar in size to unreduced globulin α-β dimers. The role of endoplasmic reticulum in the synthesis of these globulin polypeptides was investigated by *in vivo* and *in vitro* protein synthesis studies. Labeled tissue was fractionated by centrifugation and rough endoplasmic reticulum was isolated. Two polypeptides which had molecular weights of 58 to 62 kilodaltons and were immunoprecipitable with antiglobulin immunoglobulin G were found to be transiently associated with the endoplasmic reticulum. Rough endoplasmic reticulum, as well as membrane-bound polysomes, directed the *in vitro* synthesis of two polypeptides with molecular weight 58 to 62 kilodaltons corresponding in size to unreduced α-β dimers and could be immunoprecipitated with antiglobulin immunoglobulin G. The translation products of free polysomes did not show this. In pulse-labeling, globulin polypeptides with molecular weight 58 to 62 kilodaltons, as well as the α + β subunits, were labeled in protein bodies.

The data suggest that oat globulin polypeptides are synthesized as higher molecular weight precursors on ER-associated polysomes. These precursors are probably transported into protein bodies and cleaved into smaller α and β subunits.

Oat seeds begin to synthesize a group of salt-soluble globulin polypeptides during development from 9 daf;[2] thereafter, these globulins are predominant in the seeds among the four Osborne fractions (18, 21). At maturity, the globulin fraction constitutes up to 75% of total oat protein in the groat (18, 20). This is a unique characteristic of oat since other cereals such as wheat, barley, rye, and maize contain alcohol-soluble prolamins as the major storage protein fraction (14). Oat globulin is found in mature grain as a 12S multimeric molecule with an average mol wt of 348,000 (17). This molecule consists of 6 dimers, each dimer containing a 35 (α) and a 22 (β) kD subunit (17). The α and β subunits are apparently linked through disulfide bonds to form a dimer molecule with average mol wt 58,000 (3, 12, 21). Oat seed polysomes, when translated *in vitro*, give rise to two globulin polypeptides (60–62 kD) which are immunoprecipitable with globulin antibodies (4, 12, 23, 26). This suggests that post-translational processing of 60 to 62 kD precursors occurs to yield the α and β subunits. *In vivo* labeling studies have also shown the appearance of 58 to 62 kD polypeptides after short-period labeling of oat caryopses (4).

RER is thought to be the site of synthesis for legume reserve proteins (2). Recent evidence indicates that pea legumin is synthesized on the RER and transported into protein bodies, where it is processed into smaller acidic and basic subunits (6). Soybean glycinin (25) and rice glutelin (27) have also been shown to be synthesized on membrane-bound polyribosomes. In cereals, however, the process of storage protein biosynthesis is not as clear. Miflin *et al.* (15) have discussed several hypotheses and suggested that the storage proteins of starchy endosperm are synthesized on the RER, and aggregate into clumps within it; these clumps either break away from the RER to form protein bodies (wheat, barley) or aggregate into spherical deposits which accumulate within the RER which completely surrounds them (maize). Little is known about the pathway by which globulin polypeptides are synthesized, processed, and deposited in developing oat endosperm. Using electron microscopy, Saigo *et al.* (24) observed the development and formation of protein bodies in developing oat seeds and they have suggested that in the cells of the subaleurone layer (starchy endosperm), proteins are synthesized on the RER, inserted into the RER cisternae, and later transported into vacuoles. This process was not observed in the aleurone layer. Such observations suggest that the RER may be the site of storage protein biosynthesis in developing oat endosperm. We have found that oat globulin precursors are synthesized on RER. Our data suggest that these precursors are later processed in protein bodies.

MATERIALS AND METHODS

Plant Material. Oat (*Avena sativa* L. cv Hinoat) plants were grown at the Ottawa Research Station, Agriculture Canada in the summer of 1982. The developing spikelets were harvested 18 to 20 daf. For *in vivo* labeling experiments and ER isolation, groats were dehulled manually and used immediately. Oat groats were obtained in large scale by mechanical dehulling as described elsewhere (7) and stored at −80°C for polysome and RNA extractions.

Chemicals. Radiochemicals were obtained from New England Nuclear, Montreal, Canada. L-[^{3}H]leucine, L[U-^{14}C]leucine, and L-[^{35}S]methionine had specific radioactivities of 143 Ci/mmol, 340 mCi/mmol, and 400 mCi/mmol, respectively. Reagents for SDS-PAGE of polypeptides were purchased from Bio-Rad Laboratories, Mississauga, Canada. Other chemicals were obtained from Sigma Chemical Company.

***In Vivo* Protein Synthesis.** Developing oat seeds (18–20 daf) were detached and placed on a drop of 20 μl solution containing 2 μCi [^{14}C]leucine, and incubated for 50 min at 20°C. Repeated experiments with detached seeds and the whole spikelets (2–3 cm long) showed no significant difference in the nature of the *in*

[1] This research was supported by an Agriculture Canada Research Contract No. OSU81-00411 and by Natural Science and Engineering Research Council grant A6711.

[2] Abbreviations: kD, kilodalton; IgG, immunoglobulin G; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; daf, days after flowering.

vivo labeled proteins. After 50 min, the seeds absorbed the radioactive solution. In some experiments, the seeds were incubated further in 100-μl distilled H_2O for various periods of time. The labeled seeds were then used for the isolation of ER and protein bodies as described below. Radioactivity incorporated into protein was determined by spotting 10-μl aliquots of samples on a piece of chromatography paper, treating the paper with 10% (w/v) TCA, drying and counting in a liquid scintillation counter. The efficiency of [^{14}C]leucine incorporation was about 21% (950,000 dpm/2 μCi).

Subcellular Isolations. Developing seeds (0.5 g) labeled with [^{14}C]leucine were homogenized in 12% (w/v) sucrose containing 100 mM Tris-HCl, pH 8.6, 30 mM KCl, 1 mM EDTA (medium A). The homogenate was centrifuged at 500*g* for 10 min. The supernatant was applied to a Sepharose 4B column equilibrated in medium A (2) to separate organelles from the soluble proteins and small molecules. The organelles elute in the void volume of the column. The most light scattering fractions were pooled and layered on a linear 16% to 50% (w/v) sucrose gradient made up in medium A (1 mM EDTA). Centrifugation was for 2 h at 4°C at 150,000*g* in a SW 41 rotor of a Beckman ultracentrifuge. The gradients were fractionated and monitored at 280 nm using an ISCO flow cell in a UA5 optical unit. Fractions were tested for enzyme activities and examined for radioactivity as described below.

Fractions rich in ER were isolated by centrifugation on 16% and 35% (w/v) discontinuous sucrose gradients. In this procedure, most of the NADH-Cyt *c* reductase activity could be recovered between the 16% and 35% (w/v) sucrose, which will subsequently be referred to as the 16/35 interface. The labeled seeds were homogenized and prepared for centrifugation as described above. After centrifugation at 150,000*g* for 90 min, the organelles visible at the 16/35 interface were collected and analyzed by SDS-PAGE and fluorography either before or after immunochromatography on globulin-IgG-Sepharose (see below).

Discontinuous and linear sucrose gradients were both used for the isolation of RER. Linear gradients of 16% to 50% (w/v) and step gradients of 20%, 35%, and 50% (w/v) sucrose (in a buffer containing 40 mM Tris-HCl, pH 8.6, 30 mM KCl, and 10 mM Mg acetate) were used. Developing seeds were homogenized in 100 mM Tris-HCl, pH 8.6, 50 mM KCl, 10 mM Mg acetate. Magnesium salt was added in order to retain the ribosomes on ER (19). After a 10-min spin at 1,000*g*, the supernatants were layered on the linear or step gradients and centrifuged at 4°C for 90 min at 32,000*g* in a SW 41 Ti rotor of a Beckman ultracentrifuge. Linear gradients were fractionated and the fractions were tested for NADH-Cyt *c* reductase activity. The fractions with the highest enzyme activity (density of 1.15 g/cm^3) were collected, treated with 1% (w/v) Triton X-100, and used for polysome isolation (see below). In the case of step gradients, organelles at various interfaces (supernatant/20, 20/35, 35/50) and supernatant were collected and used for polysome extractions.

Extraction and Translation of Free and Membrane-Bound Polysomes. Polyribosomes were extracted by a modification of the method described previously for barley endosperms (14). Free and membrane-bound polysomes were suspended in translation buffer (20 mM Hepes, pH 7.6, 100 mM KCl, 4 mM Mg acetate, 1 mM DTT). Both polysome preparations were translated *in vitro* in a wheat germ extract, as described previously (12). *In vitro* translation products were analyzed by SDS-PAGE and fluorography (see below).

Isolation of Poly-Adenylated RNA. Membrane-bound polysomes were dissociated with SDS and poly A$^+$ RNA fraction was isolated by oligo d(T)-cellulose column chromatography as described by Matthews and Miflin (14). The yield of poly A$^+$ RNA was 4 μg/mg polysome.

Analysis of Proteins by SDS-PAGE. SDS-PAGE was performed according to the method of Laemmli (8). Gels were composed of 5% (w/v) stacking and 12% or 14% (w/v) resolving gels. Electrophoresis was at 60 v for 16 h. The gels were fixed and stained with 0.2% Coomassie Blue G-250, in 10% (w/v) TCA, 40% (v/v) methanol. After destaining in 10% acetic acid, 40% (v/v) methanol, the gels were treated with Enhance (New England Nuclear), dried, and exposed to Kodak X-Omat AR5 film. Phosphorylase *b* (94000), albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100), and α-lactalbumin (14400) were used as mol wt standards.

Isolation of Protein Bodies. Protein bodies were isolated as described by Chrispeels *et al.* (6). Pulse-labeled seeds were chopped finely with a razor blade in a Petri dish with 2 ml of 12% sucrose, 2 mM $MgCl_2$, and the extract was filtered through Miracloth. The filtrate was loaded on a 2 ml cushion of 16% sucrose, 2 mM $MgCl_2$, and spun at 1,000*g* for 10 min in a Beckman SW 60 Ti rotor. The pellet contained more than 80% of the total seed protein as determined by the dye-binding assay (Bio-Rad Laboratories).

Immunological Techniques. The preparation of oat globulin antibodies was described elsewhere (12). The immunoprecipitation of *in vitro* synthesized products was conducted according to Roberts and Lord (22). A different technique was used to examine the *in vivo* labeled polypeptides found in the ER. Antibodies raised against mature globulin were covalently coupled to CNBr-activated Sepharose 4B as recommended by Pharmacia. ER fractions isolated with discontinuous sucrose gradients were treated with 1% Triton X-100 and applied to a globulin IgG-Sepharose column. The column was washed extensively with 20 mM NaH_2PO_4-Na_2HPO_4, pH 7.4, 0.15 M NaCl, 2% (v/v) Triton X-100. Bound polypeptides were eluted with 5% SDS at 37°C. The eluant was lyophilized and extracted with acetone, triethylamine, acetic acid, H_2O (85:5:5:5), to remove the excess SDS before analyzing it by SDS-PAGE.

Enzyme Assays. NADH-Cyt *c* reductase was assayed as described by Bollini and Chrispeels (1). Succinate dehydrogenase activity was tested using the method of Pennington (16).

Extraction of Total Globulin Fraction. Salt-soluble globulin was extracted as described previously by Robert *et al.* (21).

RESULTS

***In Vivo* Synthesis of Oat Globulin Polypeptides.** To investigate the biosynthesis of oat globulin in developing oat groats, an *in vivo* radioactive labeling experiment was performed (Fig. 1). Developing groats were labeled with [^{14}C]leucine for 150 min. The salt-soluble globulin was extracted and analyzed by SDS-PAGE under reducing conditions, and compared with authentic globulin (lane A). The stained gel showed the α and β globulin subunits with average mol wt of 38 kD and 21 kD, respectively (Fig. 1, lane B). Some polypeptides with 58 kD were also present which are the unreduced dimer form of the α and β subunits. The fluorograph of the same gel (Fig. 1, lane C) showed that two polypeptides with mol wt of 58 to 62 kD were being synthesized during the pulse period. Those polypeptides corresponded in size to the unreduced α-β dimer. Polypeptides comigrating with the reduced α and β species were not apparently labeled during the 150-min labeling period. However, three polypeptides with mol wt of 34 to 37 kD were labeled. These polypeptides may belong to a separate class of salt-soluble globulins (vicilin-like proteins). These minor protein fractions are currently under investigation (K. Adeli and I. Altosaar, unpublished data).

The data suggest that oat globulin is synthesized *in vivo* primarily as high mol wt precursors (58–62 kD) which cannot be reduced by 2-mercaptoethanol. Each polypeptide is probably composed of the α and β species which are linked together through a peptide bond. These observations compare well with previous studies (4, 12, 23, 26).

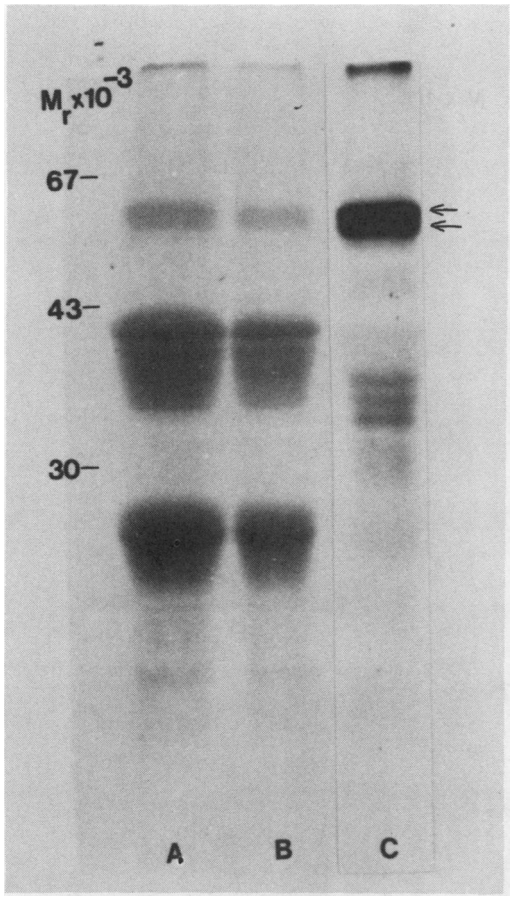

FIG. 1. Pulse labeling of total oat globulin. Four groats were labeled with about 2 μCi [^{14}C]leucine each for 150 min. Salt-soluble globulin was isolated and analyzed by SDS-PAGE and fluorography. Lane A shows a Coomassie Blue-stained track of authentic globulin extracted from mature groats. Lane B is a stained track of total globulin from labeled groats. Lane C is the fluorograph of lane B.

Association of Newly Synthesized Globulin Polypeptides with ER. When intact oat groats were labeled with [^{14}C]leucine for 50 min, homogenized in the presence of EDTA, and fractionated on a sucrose gradient, TCA-precipitable radioactivity could be detected both in the supernatant at the top of the gradient and in a band at a density of 1.10 g/cm^3 (data not shown). On the other hand, when the organelles were separated from soluble proteins by Sepharose 4B chromatography prior to sucrose gradient centrifugation, the peak of radioactivity was located only at the density of 1.10 g/cm^3 coinciding with the position of NADH-Cyt *c* reductase, the ER marker enzyme (Fig. 2). The density of the radioactive membranes with the enzyme activity changed to 1.15 g/cm^3 when labeled seeds were homogenized in a medium containing 5 mM Mg acetate instead of EDTA (data not shown). This is probably due to the retention of ribosomes on the ER membranes (19). The effect of magnesium ions on the density of ER membranes has been known to be a good marker for the detection of these membranes (19). In these conditions, the position of mitochondrial marker enzyme, succinate dehydrogenase, did not change in the gradient (data not shown). As a whole, these results indicate that newly synthesized polypeptides are associated with ER.

To examine the nature of the association between newly synthesized polypeptides and ER, the ER fraction was isolated from [^{14}C]leucine labeled groats. Groats were labeled for 50 min, extracted with medium A, and the extract was fractionated on a 16% and 35% discontinuous sucrose gradient as described above.

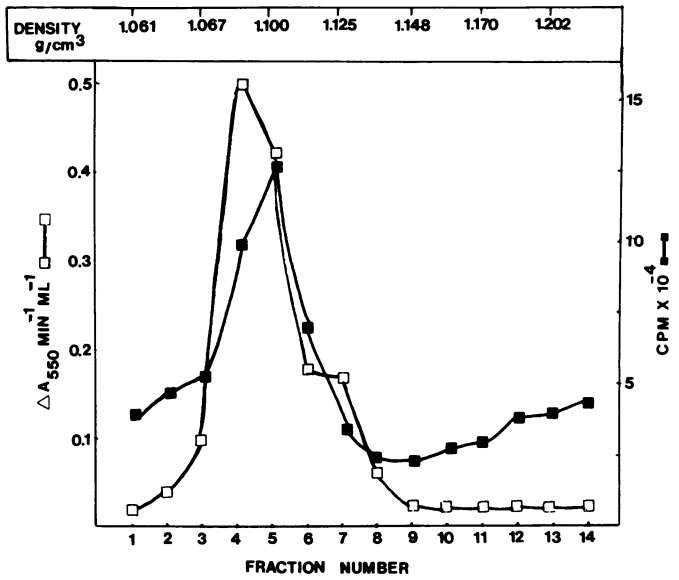

FIG. 2. Distribution of incorporated radioactivity and NADH-Cyt *c* reductase activity from groat extract on a linear sucrose gradient. Ten developing groats (18 daf) were labeled for 50 min with 20 μCi (^{3}H)leucine each. The organelles were separated by a Sepharose 4B column and fractionated on a 15% to 50% sucrose gradient. The gradient was fractionated into 1 ml fractions. TCA-precipitated radioactivity was determined and expressed as cpm $\times 10^{-4}$. NADH-Cyt *c* reductase activity was expressed as ΔA_{550} min^{-1} ml^{-1}.

By this procedure, sufficient quantities of ER membranes could be isolated for the detection of labeled polypeptides. Membrane fractions with the highest NADH-Cyt *c* reductase activity banded at the 16/35 interface and were referred to as ER. This was confirmed by electron microscopic examinations (not shown). The analysis of the isolated ER by SDS-PAGE and fluorography revealed the presence of many radioactive polypeptides (Fig. 3, lane A). Two polypeptides with mol wt of 58 to 62 kD corresponded in size to unreduced α-β globulin dimers. Several other polypeptides were also present which may belong to other storage protein fractions. The same ER fraction was challenged with an affinity gel specific for globulin (globulin-IgG Sepharose). The polypeptides which bound were analyzed by SDS-PAGE and fluorography (Fig. 3, lane B). Labeled globulin polypeptides with mol wt 58 to 62 kD were specifically bound to the affinity column. The fluorograph contained some radioactive background which may be due to nonspecific binding to the affinity gel and the adverse effect of the Enhance.

In a second pulse-labeling experiment, groats were labeled with [^{14}C]leucine for 50 min, then washed, placed in 100-μl distilled H_2O, and incubated for a further 120 min. The ER fraction was isolated, challenged with globulin-IgG Sepharose, and analyzed by SDS-PAGE. The fluorograph of the gel (Fig. 3, lane C) indicated the disappearance of the globulin polypeptides which were present in lane B. Thus, newly synthesized globulin polypeptides may be transiently associated with ER-membranes.

Isolation and Translation of Rough Endoplasmic Reticulum. RER was isolated by both linear and step sucrose gradients. When developing groats were homogenized and fractionated on a linear sucrose gradient (16–50% w/v), the ER marker enzyme, NADH-Cyt *c* reductase formed a broad band with the density of 1.15 g/cm^3. Under these conditions, ER membranes should retain their ribosomes (19). The fractions with the highest enzyme activity were therefore referred to as RER and used for the isolation of polysomes. These fractions were pooled and treated with 1% Triton X-100 to dissociate the ER-bound polysomes which were then sedimented through a sucrose cushion as de-

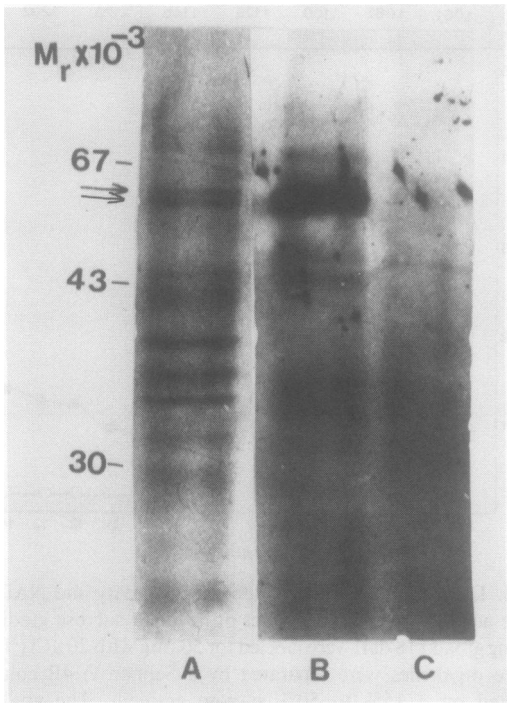

FIG. 3. Analysis of radioactive polypeptides associated with ER. Five developing groats (18 daf) were labeled with 2 μCi [^{14}C]leucine for 50 min. ER fraction was isolated on a step sucrose gradient, challenged with globulin-IgG Sepharose, and analyzed by SDS-PAGE and fluorography. Lane A shows the total radioactive polypeptides associated with ER. Lane B represents the labeled polypeptides which bound to the globulin-IgG Sepharose after 50 min pulse. Lane C shows the ER fraction bound to the globulin-IgG Sepharose from groats labeled for 50 min and incubated for 120 min.

scribed in "Materials and Methods." The polysomes were translated in a wheat germ extract. The translation products are shown in Figure 4A. Lane A is the blank (no template added). As standards, the products of membrane-bound polysomes (lane B) and a poly A$^+$ RNA preparation (lane C) were analyzed on the same gel. The ER-associated polysomes directed the synthesis of two polypeptides with mol wt 58 to 62 kD (lane D) which comigrated with the products made by membrane-bound polysomes (lane B) and poly A$^+$ RNA (lane C). The data suggest that ER-associated polysomes (RER) synthesize polypeptides corresponding in size to globulin dimers *in vitro*.

Discontinuous sucrose gradients were also used to isolate RER as described by Bollini and Chrispeels (1). An extract of developing groats was fractionated on a step gradient of 20%, 35%, and 50% (w/v) sucrose. The homogenization buffer and the gradient contained 10 mM Mg acetate. Under these conditions, most of the NADH-Cyt *c* reductase activity was located at the 20/35 interface (62% of total activity). The membranes at the supernatant/20 interface also had some enzyme activity but to a lesser extent (15% of total activity). Based on these results and electron microscopic observations, the membranes at the 20/35 interface were assumed to be RER. After treatment with 1% Triton X-100, polysomes were isolated from the supernatant and the interface fractions of supernatant/20 and 20/35, and translated in a wheat germ extract. Polysomes from the 20/35 interface (RER) directed the synthesis of polypeptides with mol wt of 58 to 62 kD (Fig. 4B, lane F) which comigrated with polypeptides made by membrane-bound polysomes (lane B) and poly A$^+$ RNA (lane C). The polypeptides which were the translation products of RER polysomes (lane F) were immunoprecipitated with antiglobulin IgG (lane G). Polysomes isolated from super-

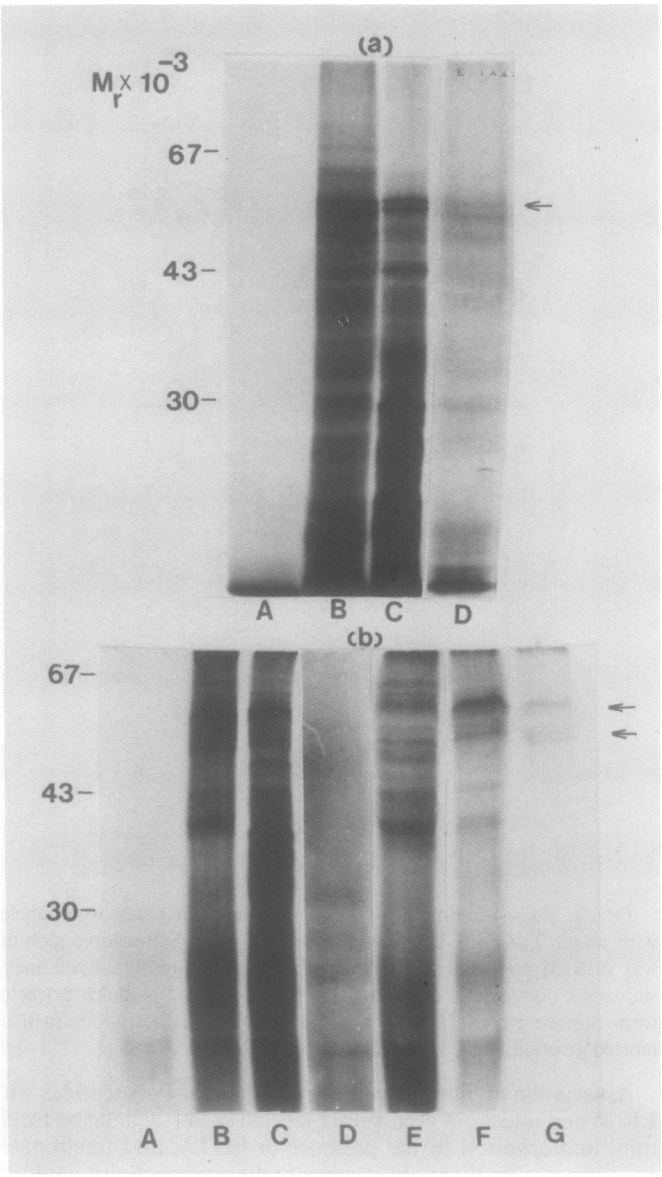

FIG. 4. Isolation and cell-free translation of RER. (a), RER was isolated on a 15% to 50% linear sucrose gradient from developing groats (20 daf). The polysomes bound to the RER fraction were separated and translated in a wheat germ extract. A, blank (no template added); B, products formed by membrane-bound polysomes isolated as described in Fig. 6; C, polypeptides formed by poly A$^+$ RNA (isolated from polysome preparations); D, translation products of polysomes isolated from RER fraction. Equal counts (200,000 cpm) were loaded on each lane. Arrows indicate two polypeptides corresponding in size to globulin dimers. (b), Step sucrose density gradients (20%, 35%, and 50% w/v) were also used to isolate RER membranes. The organelles at the two interfaces of supernatant/20 and 20/35, and the supernatant were treated with Triton X-100 and polysomes were isolated as described elsewhere. A, B, and C, translation products of blank, membrane-bound polysomes, and poly A$^+$ RNA, respectively; D, products formed by polysomes isolated from the supernatant; E and F, products of polysomes attached to the membranes at the supernatant/20 and 20/35 interfaces, respectively; G, immunoprecipitation of translation products from 20/35 interface with antiglobulin IgG. Approximately equal number of counts (150,000 cpm) were analyzed on lanes B to F. Lane G contained 35,000 cpm. Arrows indicate polypeptides immunoprecipitable with antiglobulin IgG.

natant (free polysomes) did not direct the synthesis of the globulin precursors (lane D); however, the supernatant/20 polysomes could form the globulin polypeptides (58–62 kD) but to a lesser extent (lane E). This might be the result of incomplete fractionation of organelles due to the short time of centrifugation. These results also indicate that ER-associated polysomes (RER) can direct the synthesis of oat globulin precursors *in vitro*.

Characterization of Free and Membrane-Bound Polysomes. Free and membrane-bound polysomes were fractionated by differential centrifugation by a modification of the method of Matthews and Miflin (14). The yield of membrane-bound polysomes (8 o.d. units/g groats) was higher than free polysomes (6.4 o.d. units/g groats). Centrifugation on sucrose gradients showed no significant difference between the profiles of membrane-bound and free polysomes (data not shown). These profiles confirmed the integrity of both polysome preparations.

When translated *in vitro* in a wheat germ extract, both free and membrane-bound polysomes directed efficient incorporation of [^{35}S]methionine into oat seed polypeptides. Total incorporation of radioactivity by membrane-bound and free polysomes was 510,000 and 370,000 cpm/o.d. unit, respectively. However, when equal numbers of counts from the translation products of free and membrane-bound polysomes were analyzed by SDS-PAGE and fluorography, the pattern of the *in vitro* synthesized polypeptides was different between the two preparations (Fig. 5). Membrane-bound polysomes directed the synthesis of two polypeptides with mol wt of 58 to 62 kD which corresponded in size to unreduced α-β globulin dimers (lane A) and were immunoprecipitable with antiglobulin IgG (lane B). These polypeptides were not made by free polysomes (lane C) and were not detectable by antiglobulin IgG (lane D). It should

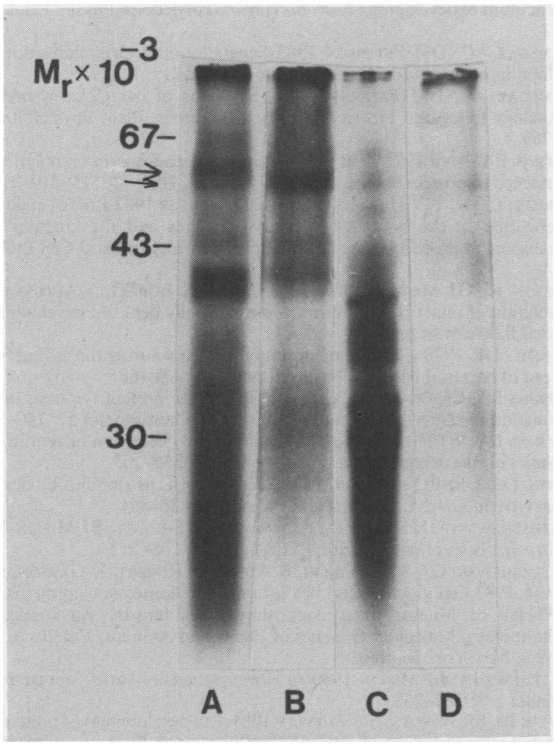

FIG. 5. Comparison of polypeptides made by free and membrane-bound polysomes. Polysomes were translated in a wheat germ extract. Lane A shows the translation products formed by membrane-bound polysomes. Lane B is the immunoprecipitation of lane A with antiglobulin IgG. Lane C represents the polypeptides synthesized by free polysomes; lane D is the immunoprecipitation of the lane C products. Approximately equal amounts of radioactive proteins (200,000 cpm) were loaded on lanes A and C.

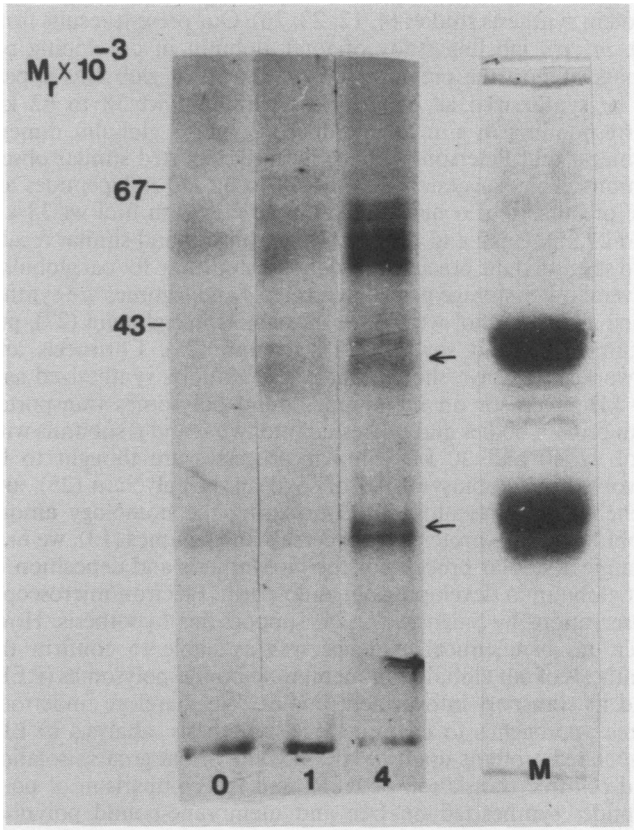

FIG. 6. *In vivo* labeling of globulin polypeptides in protein bodies. Twelve groats (20 daf) were each labeled for 1.5 h with 2 μCi of [^{14}C] leucine, then transferred to nonradioactive distilled H_2O solution, and four groats were harvested at times indicated (0, 1, 4 h). Protein bodies were isolated from the groats and analyzed by SDS-PAGE and fluorography. Lane M shows the SDS-PAGE pattern of oat globulin polypeptides extracted from mature grain protein bodies. Approximately equal amounts of protein were loaded on each lane.

be mentioned that antiglobulin IgG did crossreact with some smaller products of membrane-bound polysomes which may be due to some proteolytic cleavage in the wheat germ system or the result of premature termination. Thus, oat globulin polypeptides are primarily synthesized on membrane-bound polysomes.

Appearance of Newly Synthesized Globulin Polypeptides in Protein Bodies. Oat groats (20 daf) were incubated with [^{14}C] leucine for 1.5 h, transferred into distilled H_2O, and incubated further for 0, 1, and 4 h. Protein bodies were isolated and used to extract salt-soluble globulins. The protein body preparations were fairly pure as judged by electron microscopic investigation and contained more than 85% protein, although starch contaminations occurred. The analysis of SDS-PAGE and fluorography (Fig. 6) showed that several polypeptides with mol wt of 58 to 62 kD were labeled after 1 h incubation time. When the groats were incubated further (4 h), the intensity of these polypeptides increased markedly. This indicated the continuous incorporation of [^{14}C]leucine into high mol wt polypeptides even after the groats were removed from the label and incubated in distilled H_2O. After 4 h incubation, smaller polypeptides with average mol wt 38 kD and 21 kD also appeared (Fig. 6, lane 4). These polypeptides comigrated with mature globulin α and β subunits (lane M). These observations may indicate that globulin precursors are posttranslationally cleaved in protein bodies into the α and β subunits.

DISCUSSION

The synthesis of oat globulin polypeptides as high mol wt precursors has been demonstrated by both *in vitro* and *in vivo*

protein synthesis studies (4, 12, 23, 26). Our present results from the *in vivo* labeling study of total globulin in developing oat groats support the previous observations. Oat globulins appear to be synthesized as polypeptides with mol wt 58 to 62 kD corresponding in size to unreduced α and β globulin dimers. Brinegar and Peterson (4) have recently reported similar observations. They suggested that the 58 to 62 kD polypeptides are the precursors of α and β globulin species with mol wt 38 kD and 21 kD. Rossi and Luthe (23) have also found similar results and suggested the precursor-product relationship for oat globulin. Several other storage proteins in cereals and legumes are synthesized as higher mol wt precursors such as rice glutelin (27), pea legumin (6), and soybean 11S glycinin (25). Chrispeels and coworkers (6) have shown that pea legumin is synthesized as a 60 kD precursor on membrane-bound polysomes transported into protein bodies and processed into two α and β subunits with mol wt 40 and 20 kD. Similar processes are thought to be involved in the biosynthesis of soybean 11S glycinin (25), and french bean phaseolin (2). Considering the homology among globulin storage proteins from cereals and legumes (13), we may assume a similar process for the biosynthesis and deposition of oat globulin in developing oat endosperm. Electron microscopic observations by Saigo *et al.* (24) support this hypothesis. However, no biochemical evidence was available to confirm the synthesis of oat globulin on membrane-bound polysomes (RER) and its transport into protein bodies. We therefore undertook three approaches to examine this hypothesis: analysis of ER-associated proteins upon *in vivo* labeling of oat groats, isolation and cell-free translation of RER, and the comparison of polypeptides synthesized on free and membrane-bound polyribosomes.

Upon labeling of developing groats and fractionation on linear sucrose gradients, the TCA-precipitable radioactivity was associated with ER indicating the association of newly-synthesized polypeptides with this membrane fraction. The analysis of the ER fraction from groats labeled for 50 min showed the presence of two polypeptides with mol wt of 58 to 62 kD which cross-reacted with antiglobulin IgG. These polypeptides disappeared from the ER fraction when the pulsed seeds were incubated for 2 h. These observations suggested the association of oat globulin precursors with the ER in a transient state. Similar observations were made by Chrispeels *et al.* (6) upon *in vivo* labeling of pea cotyledons. Pea legumin and vicilin polypeptides were shown to be transiently associated with ER membranes as unprocessed precursors. Bollini *et al.* (2) reported similar results for french bean phaseolin.

In another approach, RER was isolated and translated *in vitro* in a wheat germ extract. Both step and linear gradients were suitable for the isolation of RER. Polysomes isolated from the RER membrane were able to direct the synthesis of polypeptides with mol wt of 58 to 62 kD. These polypeptides were immunoprecipitable with antiglobulin IgG. These results again support the hypothesis that ER membranes are the site of oat globulin biosynthesis. Bollini *et al.* (1) used similar methods to isolate RER from french bean cotyledons and showed the formation of phaseolin polypeptides by the polysomes attached to the RER membranes.

The third approach was the comparison between free and membrane-bound polysome translation products. Membrane-bound polysomes could direct the synthesis of oat globulin precursors (58–62 kD) in a wheat germ extract as demonstrated earlier (4, 12, 26). Globulin antibodies, however, could not immunoprecipitate any polypeptides from the translation products of free polysomes. In an earlier study, Luthe and Peterson (11) isolated and translated both free and membrane-bound polysomes from developing oat groats. They reported no qualitative difference in the products formed by membrane-bound and free polysomes. However, they did not challenge the translation products with globulin antibodies. Among other cereal storage proteins, zein in maize is thought to be made on both membrane-bound polysomes attached to protein body membrane and on RER (5, 9, 10). In the present study, no detectable NADH-Cyt *c* reductase activity was observed in oat endosperm protein bodies suggesting that ER-membranes are not continuous with protein body membrane in oat endosperm. The electron microscopic studies by Saigo *et al.* (24) support this conclusion.

The translocation of globulin precursors from their site of synthesis into protein bodies was investigated. A pulse-labeling experiment indicated the appearance of newly synthesized globulin precursors as well as the smaller α and β subunits in protein bodies. This suggests that unreduced globulin precursors are probably cleaved into α and β species upon their arrival in protein bodies. A subsequent publication will deal with this aspect of posttranslational modification in more detail.

In conclusion, several lines of evidence suggest that oat globulin polypeptides are synthesized as higher mol wt precursors on membrane-bound polysomes (RER), sequestered in ER, and transported into protein bodies where they are processed into smaller subunits. Very little is known about the mechanics of the transport of newly synthesized proteins between ER and protein bodies. ER-derived vesicles and/or Golgi apparatus may be involved in such protein translocation.

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