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Acidic Phosphoprotein Complex of the 60S Ribosomal Subunit of Maize Seedling Roots[1]

Components and Changes in Response to Flooding

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We determined that ribosomes of seedling roots of maize (*Zea mays* L.) contain the acidic phosphoproteins (P-proteins) known to form a flexible lateral stalk structure of the 60S subunit of eukaryotic ribosomes. The P-protein stalk, composed of P0, P1, and P2, interacts with elongation factors, mRNA, and tRNA during translation. Acidic proteins of 13 to 15.5 kD were released as a complex from ribosomes with 0.4 M NH_4Cl/50% ethanol. Protein and cDNA sequence analysis confirmed that maize ribosomes contain one type of P1, two types of P2, and a fourth and novel P1/P2-type protein. This novel P-protein, designated P3, has the conserved C terminus of P1 and P2. P1, P2, and P3 are similar in deduced mass (11.4–12.2 kD) and isoelectric point (4.1–4.3). A 35.5- to 36-kD acidic protein was released at low levels from ribosomes with 1.0 M NH_4Cl/50% ethanol and identified as P0. Labeling of roots with [^{32}P]inorganic phosphate confirmed the in vivo phosphorylation of the P-proteins. Flooding caused dynamic changes in the P-protein complex, which affected the potential of ribosome-associated kinases and casein kinase II to phosphorylate the P-proteins. We discuss possible alterations of the ribosomal P-protein complex and consider that these changes may be involved in the selective translation of mRNA in flooded roots.

Ribosomes are a two-subunit organelle, and are the site of mRNA translation into protein in all organisms. The large ribosomal subunit is a complex macromolecule that is composed of rRNAs, a large number of basic (high-pI) proteins, and a small number of acidic (low-pI) proteins. Across evolutionary kingdoms and phyla the large ribosomal subunit is variable in size, but possesses a number of morphological features that are universally conserved. For example, a universal feature of the peptidyl transferase region of the large subunit is a complex of acidic proteins that form the body and stalk of a lateral protuberance (Möller, 1990; Liljas, 1991).

In bacteria the acidic protein stalk of the 50S ribosomal subunit is composed of ribosomal protein, L10, and two dimers of L7 and L12 in a $(L7/L12)_2$-L10 pentameric complex (Möller, 1990; Liljas, 1991). L10 is a 17-kD acidic protein that interacts with the 23S rRNA scaffold of the large subunit within the GTPase domain of the rRNA. L7 and L12 are 12-kD acidic proteins that are encoded by a single gene, but differ in that the N-terminal Ser of L7 is post-translationally aminoacetylated. The N-terminal domain of L7 and L12 forms an α-helical structure that is responsible for dimer formation and binding to L10. A central region of acidic residues forms a flexible hinge that allows the dimers to assume an elongated conformation that forms the stalk or a closed conformation in which the C- and N-terminal domains are in close proximity on the body of the 50S ribosomal subunit (Oleinikov et al., 1993; Traut et al., 1993). The C-terminal regions of L7 and L12 are required for binding of elongation factor G, and the subsequent hydrolysis of GTP that occurs in the translocation step of the elongation phase of protein synthesis (Traut et al., 1993). Structural studies have demonstrated that the L7/L12 dimers are highly mobile and contact both the translocation and peptidyl transferase domains of the ribosome (Möller, 1990; Traut et al., 1993).

In the 60S ribosomal subunit of eukaryotes, the acidic protein homologs of L10, L7, and L12 are phosphorylated and are known as the P-proteins P0 (34–36 kD), P1 (11 kD), and P2 (11 kD) (Wool et al., 1991). These proteins have unique N-terminal and central regions, followed by a stretch of acidic residues and a highly conserved C-terminal dodecapeptide. P-proteins are present in the ribosome in an approximate ratio of two P1 and two P2 proteins to one P0 protein in a pentameric complex. Analogous to the bacterial $(L7/L12)_2$-L10 complex, the P-protein complex of eukaryotic ribosomes is required for efficient elongation of translation. The presence of these proteins enhances the binding of aminoacyl-tRNA, elongation, and release factors, as well as eukaryotic elongation factor 2-dependent GTPase activity, release of deacylated tRNA, and movement of mRNA during protein synthesis (Möller, 1990; Liljas, 1991). Unlike most ribosomal proteins, which are targeted to the nucleolus after synthesis and assemble onto preribosomes, P1 and P2 remain cytoplasmic after synthesis, become phosphorylated, and then assemble onto the 60S ribosomal subunit in the cytoplasm. In

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Abbreviations: CK II, casein kinase II; EST, expressed sequence tag; NEpHGE, nonequilibrium pH gel electrophoresis; OD, optical density; P-proteins, phosphoproteins.

addition, these proteins cycle between the ribosome and a cytosolic pool (Zinker and Warner, 1976), with active translation correlated with their phosphorylation and association with the ribosome (Zinker, 1980; Sanchez-Madrid et al., 1981).

A number of studies indicate that the P-protein complex plays a role in regulation of protein synthesis in yeast (*Saccharomyces cerevisiae*). First, biochemical analyses revealed that a greater number of P-proteins per ribosome is found in actively dividing cells than in stationary-phase cells (Saenz-Robles et al., 1990). Second, there are two isoforms of P1 (P1α and P1β) and P2 (P2α and P2β) that are encoded by four single-copy genes (Newton et al., 1990), but neither isoform of P1 or P2 is absolutely required for protein synthesis (Remacha et al., 1990, 1992; Naranda et al., 1993). Third, yeast strains in which all four functional P1 and P2 genes have been disrupted are viable but slow-growing, cold-sensitive, and cannot sporulate (Remacha et al., 1995). Hence, these proteins are not absolutely necessary for accurate protein synthesis but are required for certain aspects of growth and development (Remacha et al., 1995). Both in vivo and in vitro studies demonstrated that ribosomes lacking P1 and P2 selectively translate a different subset of mRNAs than ribosomes containing these proteins (Remacha et al., 1995). Genetic analyses have shown that P0, the more intrinsic protein of the 60S subunit lateral protuberance, is absolutely required for viability of yeast (Santos and Ballesta, 1994).

Little is known about the acidic proteins of plant ribosomes. Scharf and Nover (1987) observed acidic phosphoproteins of the 60S subunit of tomato ribosomes with apparent molecular masses of 38, 13.8, 12.7, and 12.65 kD, and speculated that these proteins may correspond to P0-, P1-, and P2-type proteins of other eukaryotes; heat shock of tomato suspension-cultured cells had no effect on the steady-state phosphorylation of these proteins. Pérez-Méndez et al. (1993) found that acidic phosphoproteins with apparent molecular masses of 37, 16, and 14.5 kD could be extracted from ribosomes of embryo axes of maize (*Zea mays* L.), but the proteins were not identified. Polya et al. (1995) purified a so-called calmodulin-like protein complex from soluble proteins of wheat (*Triticum aestivum*) embryos, and by N-terminal sequence analysis determined that the complex contained P2-like acidic ribosomal proteins. Recently, a three-dimensional reconstruction analysis of wheat ribosomes by cryoelectron microscopy confirmed the presence of a lateral stalk of the 60S ribosomal subunit with morphological homology to the L7/L12 stalk of *Escherichia coli* ribosomes (Verschoor et al., 1996).

To our knowledge, we provide the first comprehensive description of the purification and N-terminal sequencing of P0- and four different P1/P2-type proteins from ribosomes of maize roots. cDNA sequence analysis corroborated the finding that ribosomes of maize roots contain one type of P1, two distinct types of P2, and a fourth, P1/P2-like protein. This novel protein possesses a N-terminal region that is unlike that of P1 or P2 and a C terminus identical to those of P1 and P2. Hence, ribosomes of maize roots have four distinct P1/P2-type proteins. We previously reported that oxygen deprivation (flooding) induces a number of changes in ribosomes of maize seedling roots that include a reduction in the steady-state level of acidic ribosomal proteins (Bailey-Serres and Freeling, 1990). Here we show that oxygen deprivation affects the accessibility of phosphorylation sites on P0- and P1/P2-type proteins of ribosomes from seedling roots. Since selective mRNA translation is an important feature of the response to oxygen deprivation (Fennoy and Bailey-Serres, 1995; Bailey-Serres and Dawe, 1996), we speculate that changes in the P-protein complex of the 60S ribosomal subunit could play a role in, or be reflective of, translational control mechanisms.

MATERIALS AND METHODS

Plant Material, Oxygen-Deprivation Treatment, and Ribosome Isolation

Maize (*Zea mays* L., inbred B73, Pioneer Hi-Bred International, Johnston, IA) kernels were surface-sterilized with 0.25% (v/v) sodium hypochlorite, allowed to imbibe for 24 h, and germinated in the dark for 4 to 5 d at room temperature. Previously described methods were used for oxygen deprivation (flooding) of intact seedlings by submergence in an aqueous solution that was continuously sparged with 99.995% argon (Fennoy and Bailey-Serres, 1995) and in vivo labeling of primary seedling roots with [^{32}P]Pi (Bailey-Serres and Freeling, 1990). Ribosomes were isolated from roots of seedlings from an S-30 extract by centrifugation through a 2 M Suc cushion for 18 to 20 h, and resuspended in a buffer (0.2 M KCl, 40 mM Tris [pH 8.4], 5 mM EGTA, 30 mM $MgCl_2$, 50 mg/mL cycloheximide, and 50 mg/mL chloramphenicol) as described elsewhere (Fennoy and Bailey-Serres, 1995). Ribosomes were fractionated into polyribosomes, monoribosomes, and subunits by centrifugation through 5 mL of 20 to 60% (w/v) Suc-density gradients containing 0.2 M KCl, and 450-μL fractions were collected (Fennoy and Bailey-Serres, 1995).

Purification of Acidic Ribosomal Proteins

The following manipulations were performed at 4°C. Ribosome pellets were resuspended in a buffer containing 0.4 to 1.0 M NH_4Cl (as indicated in the figure legends), 80 mM KCl, 10 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.4), and 1 mM β-mercaptoethanol. Insoluble material was removed from the sample by centrifugation at 16,000g for 5 min. Ethanol was slowly added to 50% (v/v) while the solution was stirred on ice for 15 min. Ribosome cores were pelleted by centrifugation at 16,000g for 15 min. Proteins in the supernatant were precipitated by the addition of 2.5 volumes of acetone and centrifugation at 16,000g for 10 min. Proteins were resuspended in 5 mM $NaPO_4$ (pH 7.0). Protein concentration was determined by the Bradford method using the protein determination reagent (United States Biochemical-Amersham) and BSA as the standard.

In Vitro Labeling of Ribosomal Proteins

Ribosomes (0.5–3 OD A_{260}), resuspended in buffer as previously described (Bailey-Serres and Freeling, 1990),

were diluted with 1 volume of 2× kinase buffer (12 mM Tris-HCl [pH 7.5], 12 mM $MgCl_2$, 100 mM NaCl, and 2 mM dithiothreitol) containing 2 mCi/mL [γ-^{32}P]ATP (3000 Ci/mmol), and incubated at 25°C for 10 min to 1 h. In some experiments phosphorylation of 0.5 OD A_{260} total ribosomes or 3 to 6 mg of purified protein was stimulated by addition of 1 unit of rabbit reticulocyte CK II (kindly provided by J. Traugh, University of California, Riverside) per 50-μL reaction.

Gel Electrophoresis

Analysis by two-dimensional NEpHGE (pH 3.5–10)/14% SDS-PAGE was as described previously (Bailey-Serres and Freeling, 1990). Following in vitro phosphorylation, MgOAc was added to 1 mM followed by the slow addition of acetic acid to 67% (v/v). RNA was removed by centrifugation at 16,000*g* for 10 min, and ribosomal proteins were concentrated by precipitation with 2.5 volumes of ice-cold acetone. For analysis by one-dimensional SDS-PAGE following in vitro phosphorylation, reactions were terminated by the addition of 2 volumes of ice-cold ethanol and concentrated by centrifugation at 16,000*g* for 10 min. Proteins were fractionated in 14% SDS-polyacrylamide gels (14% [w/v] acrylamide, 0.45% [w/v] *N,N'*-methylenebisacrylamide, 0.075- × 9- × 16-cm slab gel [Laemmli, 1970]) by electrophoresis for 4 h at 250 V, and visualized by silver-staining. Phosphorylated proteins were visualized by autoradiographic exposure to Hyperfilm (United States Biochemical-Amersham). Molecular mass standards were purchased from Sigma.

Protein Solubilization and HPLC Analysis

Protein extracted from ribosomes with 0.4 M NH_4Cl/50% ethanol was solubilized using a number of techniques before HPLC fractionation. Proteins (15–25 mg) were solubilized in 5 μL of 3 M guanidine-HCl and 100 mM β-mercaptoethanol by heating at 100°C for 10 min. Insoluble material was removed by centrifugation at 16,000*g* for 10 min. Alternatively, proteins were solubilized in 100 μL of SDS sample buffer (50 mM Tris-HCl [pH 6.8], 5% [v/v] glycerol, and 2% [w/v] SDS), diluted to 333 μL with water, and precipitated with 2.5 volumes of ice-cold acetone. Protein was then either loaded directly onto the column, after solubilization in 5 mM $NaPO_4$ (pH 7.0), or digested with alkaline phosphatase. For digestion, protein was resuspended in 100 μL of alkaline phosphatase buffer (20 mM Tris-HCl [pH 8.8], 25 mM KCl, 1.5 mM $MgCl_2$, and 5 mM β-mercaptoethanol), and insoluble material was removed by centrifugation at 16,000*g* for 5 min. Agarose-bound alkaline phosphatase (Sigma) (0.1 unit/mg protein) was added and the sample was incubated at 37°C for 90 min. Agarose-bound alkaline phosphatase was removed from the supernatant by centrifugation at 4,000*g*, and protein was reprecipitated with acetone. Protein pellets were resuspended directly in column buffer (0.05% [v/v] trifluoroacetic acid), and protein solutions were diluted with 10 volumes of the column buffer. Samples were injected onto a C-18 column (1 mm × 150 mm; Vydac, Hesperia, CA) and eluted with a linear gradient of 0 to 60% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid at a constant flow rate of 0.5 mL/min. Samples were vacuum-dried before analysis by gel electrophoresis.

Protein Sequencing Analysis

Proteins from HPLC fractions or bound to PVDF membrane were microsequenced directly by Edman degradation using an analyzer system (model 477A/model 120A PTH, Applied Biosystems). Transfer of proteins from SDS-polyacrylamide gels to PVDF membrane (TransBlot, Bio-Rad) was with 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11) as transfer buffer, for 90 min, at a current proportional to 0.8 mA times the area (cm^2) of the membrane.

Gene Characterization

A cDNA library (gift of B. Veit; Schmidt et al., 1993) from maize immature ear mRNA (Pioneer Hi-Bred, maize inbred B73) in the λZap vector (Stratagene) was screened with rice cDNAs that putatively encode ribosomal protein P1 or P2 (GenBank accession numbers D16092, D15754, and D23542; gift of Y. Nagamura and T. Sasaki, STAFF Institute, Ibaraki, Japan) and a maize cDNA that putatively encodes P2 (GenBank accession number T18290; see also Vangala and Bailey-Serres, 1995) using standard recombinant DNA procedures. Approximately 500,000 recombinant phage were screened with each cDNA probe. After three rounds of screening, positive clones were sequenced by the dideoxy chain-termination technique using Sequenase (United States Biochemical) or by the cycle-sequencing technique using *Taq* polymerase (Promega-Fisher) according to the manufacturers' protocols. cDNAs encoding full-length, putative P1- and P2-type proteins were fully sequenced using custom primers. cDNA and deduced polypeptide features were determined using the University of Wisconsin Genetics Computer Group (Madison, WI) software.

RESULTS

Purification and in Vivo Phosphorylation of the Acidic Ribosomal Proteins from Seedling Roots

On the basis of the highly conserved nature of eukaryotic ribosomes, we predicted that the acidic phosphoproteins P0, P1, and P2 are present in the ribosomes of maize roots. Low-molecular-mass acidic (low-pI) proteins were released from ribosomes by use of a high-salt/ethanol procedure described for the purification of P1 and P2 from yeast ribosomes (Sanchez-Madrid et al., 1981). Ribosomes from untreated maize roots were resuspended in a buffer containing 0.3 to 1.0 M NH_4Cl, and the ribosome cores were removed by precipitation with 50% (v/v) ethanol. Proteins that eluted from the ribosome were concentrated by the addition of acetone, fractionated by SDS-PAGE, and visu-

alized by silver-staining. Proteins with the approximate molecular mass of 13 to 15.5 kD were efficiently eluted with 0.3 and 0.4 M NH_4Cl (Fig. 1A). Increasing amounts of proteins with apparent molecular masses of 28, 20, 18, and 17 kD were eluted with increasing concentrations of NH_4Cl. NEpHGE/SDS-PAGE analysis revealed that the 13- to 15.5-kD proteins were acidic proteins, whereas the 28-, 20-, 18-, and 17-kD proteins were basic in charge (data not shown). A small quantity of acidic proteins of 36.5 and 36 kD, the expected size of P0, was eluted from ribosomes at NH_4Cl concentrations of 0.8 M or greater (Fig. 1A).

In a previous report we showed that acidic ribosomal proteins of 36 and approximately 10 kD are phosphorylated in aerobic and oxygen-deprived roots by in vivo labeling with $[^{32}P]Pi$ (Bailey-Serres and Freeling, 1990). To confirm that the small, acidic ribosomal proteins that were released from ribosomes with high salt are phosphorylated in vivo, maize roots were incubated with $[^{32}P]Pi$ for 3 h and ribosomes were isolated. A number of ribosomal phosphoproteins were detected after SDS-PAGE and autoradiography (Fig. 1B, lane T). These included a major phosphoprotein of 31 kD that is the size of ribosomal protein S6 of maize (Pérez-Méndez et al., 1993), a group of proteins between 20 and 25 kD, a minor phosphoprotein of 36 kD that is the expected size of P0, and a 15-kD protein that is the approximate size of P1 and P2. The 36-kD phosphoprotein corresponds to the acidic protein of 36 kD that was eluted from ribosomes at low levels with 1.0 M NH_4Cl/50% ethanol (Fig. 1A). Extraction of proteins from in vivo-labeled ribosomes by use of 0.4 M NH_4Cl/50% ethanol confirmed that the 15-kD phosphoprotein(s) eluted as 15.5-, 15-, and approximately 13-kD proteins (Fig. 1B, compare lanes T and 0.4). This confirms that acidic ribosomal protein(s) with an apparent molecular mass of 15 kD is phosphorylated in vivo and resolved as a group of 13- to 15.5-kD proteins after the extraction procedure.

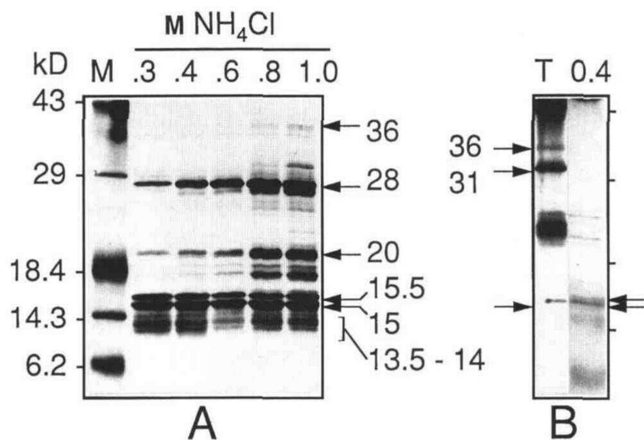

Figure 1. Ribosomal proteins extracted with NH_4Cl/50% ethanol. A, Ribosomes from aerobic roots were pelleted through a Suc cushion and resuspended in a buffer containing 0.3 to 1.0 M NH_4Cl/50% ethanol. Ribosome cores were removed by centrifugation, and proteins in the eluate were precipitated with acetone, separated by 14% (w/v) SDS-PAGE, and visualized by silver-staining. Molecular mass standards were electrophoresed in the lane designated M. B, Maize roots were phosphorylated in vivo by incubation in $[^{32}P]Pi$ for 3 h. Total ribosomes were isolated by pelleting through a Suc cushion, and proteins were eluted by 0.4 M NH_4Cl/50% ethanol, separated by 14% (w/v) SDS-PAGE, and visualized by autoradiography. Lane T, Total ribosomal proteins; lane 0.4, 0.4 M NH_4Cl/50% ethanol-extracted proteins.

Identification and Characterization of Four Distinct Forms of P1/P2-Type Proteins in Maize

In parallel to the biochemical analysis of the acidic ribosomal proteins, maize cDNAs that encode P1- and P2-type proteins were isolated and characterized. Yeast P1 and P2 protein sequences were used to search the GenBank database for plant cDNAs encoding putative P1- and P2-type proteins using the BLAST algorithm (Altschul et al., 1990). We identified a maize EST cDNA that encodes a putative P2-type protein, and rice EST cDNAs that encode putative P1- or P2-type proteins. The maize and rice cDNAs were used as gene probes to isolate cDNAs from a library made from immature ear mRNA of maize. cDNA sequence analysis revealed at least one P1-type protein (P1), two P2-type proteins (P2a, P2b), and a fourth, P1/P2-like protein (P3). These proteins of maize possess features in common with P1 and P2 of other eukaryotes; hence, we collectively refer to them as P1/P2-type proteins. All of these proteins have a unique N-terminal region of 70 to 76 residues, followed by a stretch of 16 to 20 Ala, Pro, and Gly residues, a block of 9 to 10 acidic residues (Arg and Glu), and a highly conserved 12-residue C terminus (Fig. 2).

The presence of four distinct acidic proteins in the ribosomes of roots of maize that correspond to P1, P2a, P2b, and P3 was confirmed by microsequencing of the 15- and 15.5-kD ribosomal proteins. C-18 HPLC fractionation and protein microsequencing were carried out to identify the small, acidic ribosomal proteins. Proteins released from ribosomes with 0.4 M NH_4Cl/50% ethanol were resuspended in 3 M guanidine-HCl, 100 mM β-mercaptoethanol before fractionation over a C-18 HPLC column (Fig. 3). Only a small amount of the 13.5- to 15.5-kD proteins eluted in fractions with a single protein species (Fig. 3, fractions B–D, F, and L). Most of the 15.5- and 15-kD proteins eluted together (Fig. 3, fractions O and P), and a small amount eluted with a 20-kD protein (Fig. 3, fractions H–J). The 13.5- to 14-kD proteins eluted as a group or with a 29-kD protein (Fig. 3, fractions M and N). The elution pattern indicates that most of these proteins were released as complexes from the ribosome. In addition, the elution profile indicates that the apparent molecular mass and charge of these proteins is more heterogeneous than predicted from the one-dimensional SDS-PAGE profile (Fig. 1A). When proteins released from the ribosome with 0.4 M NH_4Cl/50% ethanol were resuspended in 2% SDS sample buffer, precipitated with acetone, and digested with alkaline phosphatase, the protein complexes were dissociated into individual proteins, but only a small fraction of the proteins were recovered (data not shown). Fractions containing single polypeptides of 13.5 to 15.5 kD, obtained by the various solubilization techniques described in "Materials and Methods," were microsequenced by Edman degradation.

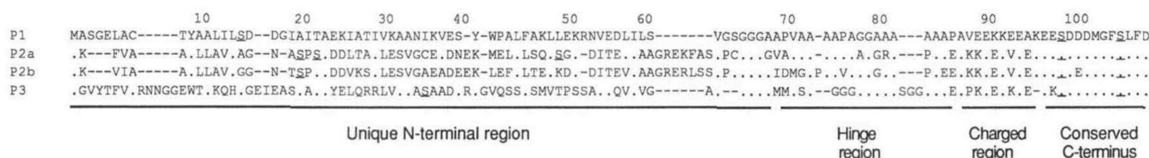

Figure 2. Comparison of deduced amino acid sequences of maize cDNAs encoding P1/P2-type proteins. Alignment of peptide sequences of four forms of small acidic ribosomal proteins of maize were deduced from cDNA sequences. The CLUSTAL W alignment program was used to align the four sequences. Gaps represented by dashes were introduced to ensure maximum homology. Dots represent identity with the P1. Underlined Ser residues are CK II phosphorylation target sites. Features in common with P1 and P2 of other eukaryotes are labeled.

Peptide sequences were obtained by Edman degradation for two proteins, with an apparent molecular mass of 15 kD, that were homologous to the sequences deduced from cDNAs encoding two very similar P2-type proteins of maize. A 12-residue sequence was obtained for a 15-kD protein released from the complex by SDS solubilization and alkaline phosphatase digestion. This sequence matched 9 out of 11 residues of the N terminus of a P2-type ribosomal protein, designated P2a, deduced from a maize cDNA (Table I). P2a is encoded by a small gene family (K. Szick, M. Springer, and J. Bailey-Serres, unpublished data); hence, differences between the peptide and cDNA sequence could reflect inaccuracies in the peptide sequence or variations among P2a gene family members. This cDNA encodes a protein with a calculated molecular mass of 11.5 kD and pI of 4.1. P2a has 75 and 79% sequence similarity to yeast P2α and P2β, respectively (Table II). A 15-kD polypeptide released from the complex by solubilization in 2% SDS sample buffer yielded a 14-residue sequence of a similar protein. The peptide sequence matched the deduced N terminus of a cDNA encoding a P2-type ribosomal protein, designated P2b (Table I). This cDNA encodes a 113-residue protein with a calculated molecular mass of 11.8 kD and pI of 4.1. P2b has 71 and 72% sequence similarity to yeast P2α and P2β, respectively (Table II). The deduced peptide sequences of P2a and P2b are the most similar of the P1/P2-type proteins that were identified (Fig. 3; Table II).

The peptide sequence of a 15.5-kD protein was identical to an internal portion of a P1-type protein. Many fractions that contained a single polypeptide of 15.5 kD (such as that shown in Fig. 3, fraction F) provided either a low or no yield of peptide sequence by Edman degradation, suggesting that many 15.5-kD acidic ribosomal proteins have blocked N termini. Microsequencing of a 15.5-kD protein yielded the sequence DGIA at low levels, most likely from a degradation product of the 15.5-kD protein. This sequence is identical to residues 17 to 20 of a protein encoded by a maize P1-type cDNA (Table I). The maize P1 cDNA encodes a 109-residue protein with a calculated molecular mass of 11.0 kD and pI of 4.1. P1 has 70% sequence similarity to the two P1 isoforms and approximately 60% similarity to the two P2 isoforms of yeast (Table II). Failure to obtain an N-terminal sequence beginning at the initiator Met for P1 may indicate that P1 is N-terminally acetylated in maize, as is P1 in yeast and L7 in *E. coli* (Santos et al., 1993).

In two independent experiments a 15.5-kD protein yielded a sequence that was identical to the N terminus of a novel P1/P2-type protein. The 15.5-kD protein was obtained by guanidine-HCl solubilization of the 0.4 M NH_4Cl/50% ethanol-extracted proteins and HPLC fractionation (Fig. 3, fraction D). Protein microsequencing yielded a peptide sequence identical to residues 2 to 17 of the deduced N terminus encoded by a maize cDNA that was designated P3 (Table I). This cDNA encodes a 120-residue protein with a calculated molecular mass of 12.2 kD and pI of 4.3. By comparison of the deduced peptide sequences shown in Figure 2, it is apparent that the N-terminal region of P3 is unlike that of P1, P2a, or P2b. Indeed, this protein cannot be unambiguously classified as a P1- or P2-type protein by comparison with P1 and P2 of maize or yeast (Table II). Because this novel P1/P2-type protein has an N-terminal region that is clearly distinct from that of P1 and P2, we designated it P3.

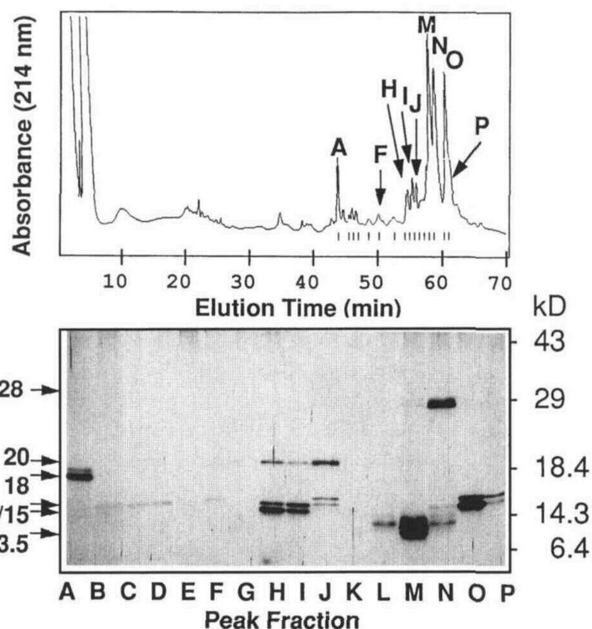

Figure 3. C-18 HPLC fractionation of acidic ribosomal proteins. Proteins were eluted from ribosomes with 0.4 M NH_4Cl/50% ethanol, concentrated by acetone precipitation, resuspended in 3 M guanidine-HCl and 100 mM β-mercaptoethanol, heated at 100°C for 5 min, and fractionated on a C-18 column by HPLC. Peak fractions were solubilized with 2% SDS sample buffer, fractionated by 14% (w/v) SDS-PAGE, and visualized by silver-staining. Apparent molecular mass (kD) of proteins is indicated.

Table I. *N-terminal amino acid sequences of proteins eluted from maize ribosomes with 0.4 or 1.0 M NH_4Cl/50% ethanol and sequenced by Edman degradation*

Protein	Source of Peptide Sequence	Sequence	GenBank Accession No.
P2a N terminus			
Maize 15 kD	Protein	KYVAAYLVAVL	
Maize P2-type	cDNA	MKFVAAYLLAVL	U29383
P2b N terminus			
Maize 15 kD	Protein	MKVIAAYLLAVLGG	
Maize P2-type	cDNA	MKVIAAYLLAVLGG	U62753
P1 internal			
Maize 15.5 kD	Protein	DGIA	
Maize P1-type	cDNA	. . .DGIA. . .	U62752
P3 N terminus			
Maize 15.5 kD	Protein	GVYTFVCRNNGGEXTA	
Maize P3-type	cDNA	MGVYTFVCRNNGGEWTA	U62751
P0 N terminus			
Maize 36 kD	Protein	AIKRTKAEKLI	
Rice P0	cDNA	MAIKRTKAEKKV	D21130
L15 N terminus			
Maize 20 kD[a]	Protein	MFKRRKGGREIKYLFT	No match
L35 N terminus			
Maize 17 kD	Protein	ARIKVNELRGTSK	
Rice L35	cDNA	MARIKVHELRGKNK	D23391

[a] Identified as ribosomal protein L15 by immunological cross-reaction to antiserum against yeast L15 (data not presented).

Identification of the 36- and 36.5-kD Acidic Ribosomal Proteins as P0

The molecular mass, charge, and in vivo phosphorylation of 36- to 36.5-kD acidic ribosomal proteins of maize (Bailey-Serres and Freeling, 1990) is characteristic of P0. P0 is present in phosphorylated and nonphosphorylated forms in ribosomes of yeast and mammals (Elkon et al., 1986; Mitsui et al., 1988). Despite the efficient release of P1/P2-type proteins, acidic proteins of 36 and 36.5 kD were poorly released from ribosomes washed with 1.0 M NH_4Cl/50% ethanol (Fig. 1A). Inefficient elution of the 36- and 36.5-kD acidic proteins was not unexpected, since P0 is a core ribosomal protein (Towbin et al., 1982). The acidic proteins of 36 and 36.5 kD that eluted from maize ribosomes with 1.0 M NH_4Cl/50% ethanol were fractionated by 12% SDS-PAGE, transferred to a PVDF membrane, and subjected to N-terminal peptide sequence analysis by Edman degradation. Both polypeptides yielded a sequence that was nearly identical to the N terminus of P0 that was deduced from a rice cDNA (Table I). Our analyses confirm that the 36- to 36.5-kD acidic phosphoproteins of maize ribosomes are P0 (Fig. 1B; Table I).

Table II. *Percent sequence identity[a] comparison of deduced peptide sequences of P1/P2-type proteins from maize and yeast*

Z. mays Gene	*Z. mays* Protein	*Z. mays* Protein and GenBank Accession No.				*S. cerevisiae* Protein and GenBank Accession No.			
		P1 (U62752)	P2a (U29383)	P2b (U62753)	P3 (U62751)	P1α (M26504)	P1β (M26507)	P2α (M26503)	P2β (M26505)
Rpp1	P1	100				70	70	57	63
Rpp2a	P2a	41	100			62	61	75	79
Rpp2b	P2b	39	71	100		61	62	71	72
Rpp3	P3	36	30	29	100	59	60	59	60

[a] Calculated using BESTFIT from the Genetics Computer Group software.

We also attempted to identify the basic proteins that eluted along with P1, P2, and P3 when maize ribosomes were washed with 0.4 M NH_4Cl/50% ethanol. Only the 20- and 17-kD proteins yielded a peptide sequence after Edman degradation (Table I). The microsequence obtained for the 20-kD protein did not match the N terminus of a ribosomal protein deduced from any plant cDNA in the public databases. However, a polyclonal antiserum against yeast ribosomal protein L12 (previously known as yeast L15) specifically cross-reacted with the maize 18-kD ribosomal protein (data not shown). The microsequence of the 17-kD protein matched 10 of 13 residues of the deduced N terminus of rice ribosomal protein L35.

Oxygen Deprivation of Maize Roots Alters the in Vitro Phosphorylation of P0- and P1/P2-Type Ribosomal Proteins

A comparison of ribosomal proteins from oxygen-deprived and aerobic roots of 5-d-old seedlings revealed a number of changes in ribosomal proteins, including a reduction in the abundance of a class of acidic proteins in response to flooding (Bailey-Serres and Freeling, 1990). Our previous report showed that 36- and 10-kD ribosomal proteins, which we now know correspond to P0- and P1/P2-type proteins, were labeled in vivo with [^{32}P]Pi and [γ-^{32}P]ATP by ribosome-associated kinases (Bailey-Serres and Freeling, 1990). We examined whether in vitro phosphorylation of these proteins with ribosome-associated kinases would reveal changes in the P-proteins in response to oxygen deprivation. We presume that the P-proteins of maize form a lateral stalk complex, as in other eukaryotes, since the maize P1/P2-type proteins eluted as a complex and the wheat P-protein stalk was visualized by cryoelectron microscopy (Verschoor et al., 1996).

The effect of short-term, long-term, and recovery from oxygen deprivation on the in vitro phosphorylation potential of the acidic ribosomal proteins was examined. Ribosomal proteins were phosphorylated in vitro with [γ-^{32}P]ATP by ribosome-associated kinases, fractionated by NEpHGE/SDS-PAGE, and visualized by autoradiography (Fig. 4A-D). Protein phosphorylation and mobility in this assay reflects existing phosphorylation status, ribosome three-dimensional structure, and presence of ribosome-associated kinases and/or phosphatases. Acidic proteins with apparent molecular masses of 56, 36.5, 36, 15.5, 15, and 13 kD were labeled in the in vitro assay. The various treatments reproducibly affected the phosphorylation and electrophoretic mobilities of proteins of approximately 36.5, 36, 15.5, 15, and 13 kD. On the basis of the purification and N-terminal sequence analysis of acidic protein from maize ribosomes, the 36.5- and 36-kD proteins are forms of P0 and the 15.5- to 13-kD proteins are the P1/P2-type proteins.

In vitro phosphorylation of intact ribosomes by ribosome-associated kinases revealed changes associated with P0 in response to flooding (Fig. 4). Phosphorylation of the 36.5-kD form of P0 (white arrow) was higher relative to that of the 36-kD form (black arrow) in aerobic ribosomes (Fig. 4A). In ribosomes of roots deprived of air for 4 or 24 h

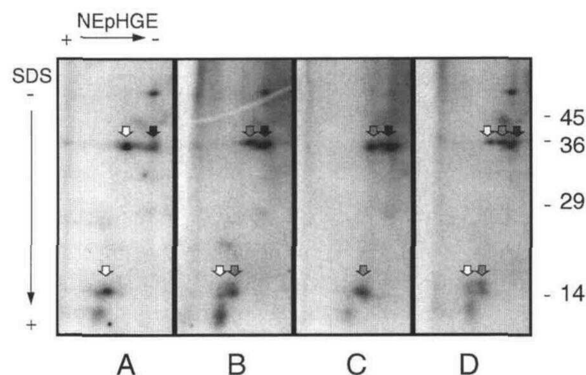

Figure 4. Two-dimensional gel analysis of ribosomal proteins of maize phosphorylated in vitro by ribosome-associated kinases. Proteins were fractionated in pH 3.5 to 10 NEpHGE gels in the first dimension, and 14% (w/v) SDS-polyacrylamide gels in the second dimension, and visualized by autoradiography. The region of the gel containing acidic ribosomal proteins of roots grown in air (A), oxygen-deprived for 4 h (B), oxygen-deprived for 24 h (C), or oxygen-deprived for 4 h and allowed to recover in air for 2 h (D) is shown. The position of molecular mass standards (kD) are indicated. White arrows indicate proteins detected at higher levels in aerobic roots. Gray and black arrows indicate proteins detected at higher levels in oxygen-deprived roots.

(Fig. 4, B and C) the 36.5-kD form of P0 (gray arrows) had a higher pI than in the aerobic ribosomes. In addition, phosphorylation of the 36-kD form of P0 was higher relative to that of the 36.5-kD form in oxygen-deprived ribosomes. In ribosomes from roots deprived of oxygen for 4 h and allowed to recover in air for 2 h (Fig. 4D), P0 was resolved as two 36.5-kD proteins (white and gray arrows) and a 36-kD protein (black arrow). Thus, recovery resulted in the detection of a mixture of the electrophoretically distinct forms of P0 that were present in aerobic and 4-h-stressed roots.

In vitro phosphorylation of intact ribosomes by ribosome-associated kinases revealed that oxygen deprivation also stimulates changes in the P1/P2-type proteins (Fig. 4). The P1/P2-type proteins were resolved as a single 15-kD protein of in vivo-phosphorylated ribosomes (Fig. 1B, lane T), and a single 15-kD protein (white arrow) was detected after in vitro phosphorylation in ribosomes from aerobic roots (Fig. 4A). Although no change in the in vivo phosphorylation pattern of these proteins was detected in response to oxygen deprivation (Bailey-Serres and Freeling, 1990; data not shown), in vitro phosphorylation of ribosomes of roots deprived of oxygen for 4 h resulted in labeling of four proteins of approximately 15 kD (Fig. 4B). In this sample, the most abundant 15-kD protein (gray arrow) was more basic in charge than in aerobic ribosomes; in addition, small amounts of two 15.5-kD proteins were detected. In ribosomes of roots deprived of oxygen for 24 h, a single protein of 15 kD (gray arrow) was detected (Fig. 4C). This protein has electrophoretic mobility similar to that of the major 15-kD phosphoprotein detected in the 4-h oxygen-deprived sample. The pattern of phosphoproteins detected in ribosomes from roots deprived of oxygen for 4 h and allowed to recover in air for 2 h demonstrated that

oxygen deprivation induces reversible changes in ribosomes that influence the in vitro phosphorylation potential of the P1/P2-type proteins (Fig. 4D). Similar amounts of two phosphoproteins of 15 kD (white arrow) and two of 15.5 kD (gray arrow) were resolved in samples allowed to recover from oxygen deprivation. The pattern of these phosphoproteins is a mixture of that observed in the aerobic and 4-h oxygen-deprived samples. The 13-kD phosphoprotein(s), which are perhaps either degradation products or distinct structural forms of the P1/P2-type proteins (discussed below), varied slightly in quantity and apparent molecular mass in these samples.

These results clearly demonstrate that the in vitro phosphorylation potential of P0 and the P1/P2-type acidic ribosomal proteins of intact maize ribosomes is responsive to oxygen deprivation and reoxygenation. However, the in vivo phosphorylation of the P1/P2-type proteins was not affected by oxygen deprivation (Bailey-Serres and Freeling, 1990; data not shown). When proteins were released from ribosomes of aerobic and 12-h oxygen-deprived roots by use of 0.4 M NH_4Cl/50% ethanol, the stoichiometric ratio of the 15.5- and 15-kD acidic proteins was very similar (Fig. 5A). In addition, we could not detect any clear differences in the proteins released from ribosomes of aerobic and oxygen-deprived roots by NEpHGE/SDS-PAGE, IEF/SDS-PAGE, or C-18 HPLC fractionation (data not shown). Therefore, it is unlikely that the variations in the in vitro phosphorylation of the 15.5- to 13-kD proteins are due to differences in the in vivo phosphorylation state; instead these changes may reflect variations in ribosome-associated kinases/phosphatases or the presence and accessibility of phosphoryl acceptor sites that are the targets of these enzymes.

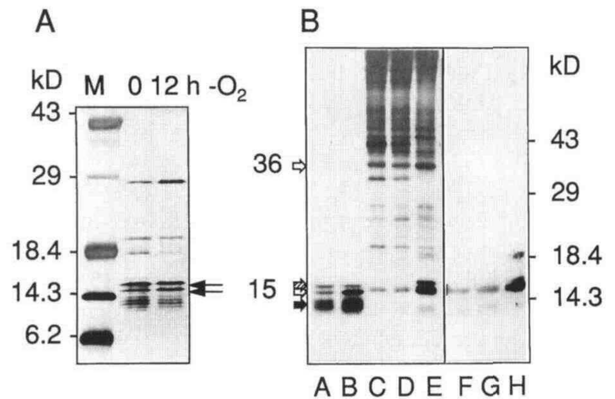

Figure 5. Phosphorylation of proteins from ribosomes of aerobic and oxygen-deprived roots with rabbit CK II. A, Proteins extracted from ribosomes with 0.4 m NH_4Cl/50% ethanol as in Figure 1. 0, Aerobic roots; 12 h, 12-h hypoxic roots. Molecular mass standards were electrophoresed in the lane marked M. Apparent molecular mass (kD) of proteins is indicated. B, Ribosomal proteins were phosphorylated in vitro with rabbit CK II in the presence of [γ-^{32}P]ATP and fractionated by 14% (w/v) SDS-PAGE. Proteins (6 mg) extracted from ribosomes with 0.4 m NH_4Cl/50% ethanol from aerobic (A) or 12-h oxygen-deprived (B) roots. Total ribosomes (0.5 OD) were phosphorylated with CK II from aerobic (C), 3-h oxygen-deprived (D), or 24-h oxygen-deprived (E) roots. Proteins extracted from ribosomes (5 OD) phosphorylated in vitro with CK II (10 units) with 0.4 m NH_4Cl/50% ethanol from aerobic (F), 3-h oxygen-deprived (G), or 24-h oxygen-deprived (H) roots.

In Vitro Phosphorylation of Acidic Ribosomal Proteins by CK II Demonstrates Differences in the Structure of Ribosomes of Aerobic and Oxygen-Deprived Roots

P-proteins of yeast and mammals can be phosphorylated in vitro with CK II (Hasler et al., 1991) and in vivo and in vitro by a CK II-type kinase associated with the 60S subunit, most likely at a Ser within the conserved C terminus (Pilecki et al., 1992; Grankowski et al., 1993). On the basis of the sequence of a cDNA that putatively encodes P0 of rice, this protein contains two target sites for phosphorylation by CK II (Hihara et al., 1994). cDNA sequence analysis revealed that deduced P1, P2, and P3 proteins of maize contain multiple target sequences for phosphorylation by CK II (Fig. 2). We took advantage of the presence of CK II target sites to examine whether phosphorylation in vitro with this enzyme reveals differences between these proteins from ribosomes of aerobic and oxygen-deprived roots.

Proteins extracted from ribosomes of aerobic and 12-h oxygen-deprived roots with 0.4 M NH_4Cl/50% ethanol (Fig. 5A) were phosphorylated in vitro with rabbit reticulocyte CK II in the presence of [γ-^{32}P]ATP (Fig. 5B, lanes A and B). Autoradiography detected phosphoproteins with apparent molecular masses of 15.5 kD (striped arrow) and 15 kD (white arrow), which correspond to the profile of purified P1/P2-type proteins (Fig. 5A). The 13.5- to 14-kD phosphoproteins (black arrow) are also forms of P1/P2-type proteins on the basis of their phosphorylation and purification characteristics. A greater amount of the 15-kD and 13.5- to 14-kD phosphoproteins was detected in the oxygen-deprived sample. The 0.4 M NH_4Cl/ethanol-extraction procedure results in the release of a protein complex, as demonstrated by the C-18 HPLC analysis; thus, the variation in phosphorylation potential observed reflects a difference in the presence or accessibility of phosphorylation sites in the ribosomal protein complex.

CK II was used to phosphorylate proteins of intact ribosomes isolated from 0-, 3-, and 24-h oxygen-deprived roots, and protein phosphorylation patterns were detected by autoradiography (Fig. 5B, lanes C–E). Phosphorylation of intact ribosomes resulted in a more complicated pattern than that observed for partially purified P1/P2-type proteins. Similar results were obtained when ribosomes were heated to 70°C for 30 min to inactivate endogenous kinases before phosphorylation by CK II (data not shown). We observed a number of differences in the pattern of protein phosphorylation from ribosomes of aerobic and oxygen-deprived roots. These differences support our previous conclusion that oxygen deprivation stimulates changes in ribosomes (Bailey-Serres and Freeling, 1990). Of particular interest, a 36-kD form of P0 was labeled in all of the samples, whereas the 36.5-kD form of P0 was detected only in aerobic ribosomes (see also Fig. 6, Aerobic). The pattern of P1/P2-type protein phosphorylation by CK II was distinct for aerobic and oxygen-deprived ribosomes. CK II phosphorylated a 15-kD protein of aerobic and 3-h oxygen-deprived ribosomes, and 15.5- and 15-kD proteins of 24-h

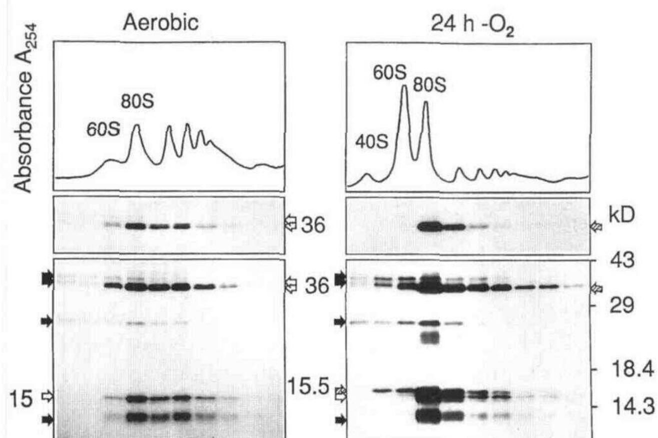

Figure 6. Suc gradient fractionation and phosphorylation of ribosomes with rabbit CK II. Polysomes (7.5 OD) from aerobic and 24-h oxygen-deprived roots were pelleted through a Suc cushion for 18 h, resuspended, and separated by centrifugation through continuous 20 to 65% (w/v) Suc gradients. Gradients were fractionated with an ISCO fractionator and UV A_{254} monitor, ribosomal proteins were labeled in vitro with [γ-^{32}P]ATP by CK II, separated by 14% (w/v) SDS-PAGE, and detected by exposure to x-ray film. The top gel panel show a short exposure of the region of the gel with 36-kD proteins. The bottom gel panel show a longer exposure of the gel. The apparent molecular mass (kD) of P-proteins is indicated. Autophosphorylated subunits of CK II of 43, 39, and 26 kD are indicated by black arrows. Other arrows indicate differences in the labeling pattern in response to the two treatments.

oxygen-deprived ribosomes. The phosphorylation of these proteins was reproducibly higher in the 24-h oxygen-deprived ribosomes than in the other two samples. Proteins of 13.5 to 14 kD (black arrow) were labeled in all of the samples, with labeling slightly more pronounced in the 24-h oxygen-deprived ribosomes. Extraction of the in vitro-labeled phosphoproteins with 0.4 NH_4Cl/50% ethanol confirmed that the 13- to 15.5-kD ribosomal proteins correspond to the P1/P2-type proteins (Fig. 5B, lanes F–H). Thus, oxygen deprivation results in changes in ribosomes that are revealed by quantitative and qualitative differences in the in vitro phosphorylation of proteins by CK II.

Suc Density Gradient Fractionation of Ribosomes followed by Phosphorylation in Vitro by CK II

The pattern of ribosomal protein phosphorylation by CK II may be distinct for ribosomal subunits, monoribosomes, and polyribosomes. This is particularly relevant, since separation of total ribosomes over Suc density gradients demonstrates that oxygen deprivation stimulates an increase in the amount of ribosomal subunits (40S and 60S) and monoribosomes (80S) relative to the amount of polyribosomes (>80S) (Bailey-Serres and Freeling, 1990) (Fig. 6). Suc gradient fractions were collected and ribosomal proteins were in vitro phosphorylated with rabbit CK II. As observed in Figure 5 (lanes C and E), the pattern of protein phosphorylation by CK II was distinct for aerobic and 24-h oxygen-deprived ribosomes. For example, the 36.5- and 36-kD forms of P0 (Fig. 6, middle panels) were detected in the large ribosomal subunit, monoribosome, and polyribosome fractions of the aerobic root sample, whereas only a 36-kD form of P0 was detected in 24-h oxygen-deprived roots. A longer exposure of the gel revealed that the qualitative pattern of labeling of P0 was the same in all fractions of the aerobic or oxygen-deprived root samples (Fig. 6, bottom panels). The longer exposure time also resulted in visualization of the autophosphorylated subunits of CK II (α [43 kD], α' [39 kD], and β [26 kD]); autophosphorylation of these proteins parallels that of ribosomes due to stimulation by basic proteins (Palen and Traugh, 1991) (Fig. 6, upper three black arrows). In vitro phosphorylation of Suc-gradient-fractionated ribosomes revealed that oxygen deprivation induced clear changes in the 15.5- and 15-kD proteins that correspond to P1/P2-type proteins. A 15-kD phosphoprotein (white arrow) was detected in aerobic root ribosomes, whereas 15.5- (striped arrow) and 15-kD (white arrow) phosphoproteins were observed in 24-h oxygen-deprived ribosomes. Only the 15.5-kD protein was detected in 60S subunits of the oxygen-deprived sample, indicating a difference in these proteins or the acidic protein complex in 80S ribosomes and 60S ribosomal subunits. The 13.5- to 14-kD (black arrow) forms of P1/P2-type proteins were observed in both aerobic and oxygen-deprived root samples. In summary, in vitro phosphorylation by CK II revealed changes in the accessibility of phosphorylation sites on P0- and P1/P2-type proteins of monoribosomes and polyribosomes that occur in response to oxygen deprivation.

DISCUSSION

Maize Ribosomes Contain Homologs of P0, P1, and P2 and a Novel P1/P2-Type Protein

We demonstrated that maize seedling root ribosomes contain acidic phosphoproteins that correspond to P0, P1, and P2, the proteins that form the body and stalk of a protuberance that is a universal feature of the large ribosomal subunit. In addition, a P1/P2-type protein that is unique to plant ribosomes was identified and designated P3.

A complex of acidic and basic ribosomal proteins was released from maize ribosomes with 0.4 M NH_4Cl/50% ethanol. Edman degradation of the proteins revealed that the released proteins included one form of P1, two forms of P2, and a unique, P1/P2-type protein. Analysis of maize cDNAs encoding P1/P2-type proteins corroborated the protein purification and microsequencing data. We characterized cDNAs encoding one P1, two very similar forms of P2 (P2a and P2b), and a novel P1/P2-type protein that was designated P3. Comparison of the deduced amino acid sequence of the maize P1, P2, and P3 proteins (Fig. 2) indicates that these proteins have highly homologous C termini and unique N termini. To our knowledge, P3, a P1/P2-type protein that is distinct from P1 and P2 (Fig. 2; Table II), is the first plant ribosomal protein identified to date that does not have an animal or yeast homolog. GenBank contains EST accessions from rice (D15754) and Arabidopsis (Z18207) that are homologous to maize P3, indicating that this protein is present in ribosomes of monocotyledonous and dicotyledonous plants.

The deduced P1, P2, and P3 proteins of maize have the characteristic molecular mass (approximately 11 kD), pI (approximately 4), and structural features of the small, acidic ribosomal proteins of other eukaryotes. The conserved features include a flexible hinge region rich in Ala, Gly, and Pro, followed by an acidic region, and the highly conserved, 12-residue C terminus. The apparent molecular mass of in vivo phosphorylated P1/P2-type proteins in intact ribosomes was 15 kD, as determined by SDS-PAGE. The apparent molecular mass of P1, P2, and P3 proteins after extraction from ribosomes was 15.5 (P1 and P3) and 15 kD (P2a and P2b). These estimates are higher than that predicted from the cDNA sequences, as expected for proteins such as these that have high levels of α-helical secondary structure. A group of proteins ranging in size from 13.5 to 14 kD co-purified and were phosphorylated in vivo and in vitro along with P1, P2, and P3. Detection of the 13.5- to 14-kD proteins by in vitro phosphorylation of intact ribosomes by CK II suggests that these are forms of P1/P2-type proteins that are present in maize ribosomes. The 13.5- to 14-kD ribosomal proteins are most likely P1, P2, and/or P3 that are N- or C-terminally truncated or have altered α-helical structure.

The number of forms of P1 and P2 varies among eukaryotes. Ribosomes of animals contain multiple molecules of a single form of P1 and P2 (for review, see Wool et al., 1991). Ribosomes of fission and baker's yeast contain two different forms of P1 and P2 (Beltrame and Bianchi, 1990; Newton et al., 1990); gene sequence data indicate that multiple forms of P2 may be present in ribosomes of *Trypanosoma cruzi* (Schijman et al., 1995). Our protein purification and microsequencing data indicate that a single form of the P1 and P3 proteins and multiple forms of P2 proteins are present in ribosomes from maize roots. Together, these observations provide convincing evidence that the lateral protuberance of the 60S subunit has evolved into a distinct complex in animals, yeast, protozoa, and plants. A critical question is whether there are functional differences between the individual types (i.e. P1, P2, or P3) or forms (i.e. P2a and P2b) of these proteins.

P1/P2-Type Proteins Are Released in a Complex from Ribosomes with Other Proteins

Extraction of the P1/P2-type proteins from maize ribosomes yielded additional information about interactions between plant ribosomal proteins. Four basic proteins (28, 20, 18, and 17 kD) eluted along with P1, P2, and P3 when maize ribosomes were washed with 0.4 M NH_4Cl/50% ethanol. Our initial expectation was that these basic proteins could be separated from P1/P2-type proteins by HPLC fractionation. Nonetheless, the 28- and 20-kD proteins co-fractionated with the 15.5- to 13-kD proteins, suggesting that complexes of basic and acidic proteins were eluted from the ribosome. Co-elution of P1, P2, and basic ribosomal proteins L7a (previously known as yeast L10a) and L12 was observed after extraction of rat and yeast ribosomes with 0.5 M KCl/50% ethanol (Lavergne et al., 1988). An attempt to microsequence the 28-kD protein that co-fractionated with the 13.5- to 14-kD forms of P1, P2, and/or P3 was unsuccessful, most likely because of an acetylated N terminus. We speculate that this protein may be L7a, since the rice homolog has a deduced molecular mass of 29.3 kD (GenBank accession no. D12631). The 20-kD protein that co-fractionated with forms of the acidic ribosomal proteins that ranged in size from 16 to 15 kD was microsequenced (Table I), but the sequence did not match the N terminus of a ribosomal protein deduced from any plant cDNA in the public databases. However, an anti-yeast L12 antiserum (previously known as yeast L15) specifically cross-reacted with the maize 20-kD ribosomal protein (data not shown). L12 is functionally analogous to bacterial ribosomal protein L11, which is located at the base of the acidic protein stalk of the large subunit and interacts with the $(L7/L12)_2$-L10 complex (Saenz-Robles et al., 1988). After extraction with 1.5 M NH_4Cl/50% ethanol, yeast L12 immunoprecipitates in a complex with P1 and P2 (Saenz-Robles et al., 1988). Hence, the co-purification of L12 with P1/P2-type proteins from maize ribosomes further supports the notion that interactions between these proteins has been conserved among prokaryotes and eukaryotes (Thompson et al., 1993). A 17-kD protein that eluted from ribosomes with 0.4 M NH_4Cl did not co-fractionate with P1/P2 proteins. This indicates that the 17-kD moiety, identified as ribosomal protein L35 by Edman degradation (Table I), is not complexed with P1, P2, and P3 but is loosely associated with maize ribosomes. L35 also eluted at low levels from rat ribosomes treated with 0.5 M KCl/50% ethanol (Lavergne et al., 1988). Finally, the observation that alkaline phosphatase digestion was necessary to separate the 15.5- and 15-kD proteins by HPLC indicates that hydrophobic interactions and phosphorylation are required for the structural integrity of P-protein complexes eluted with 0.4 M NH_4Cl/50% ethanol.

Oxygen Deprivation Affects the In Vitro but Not the in Vivo Phosphorylation of the P-Proteins

In vivo labeling of maize roots with [^{32}P]Pi, followed by isolation of ribosomes and extraction of proteins with 0.4 M NH_4Cl/50% ethanol, confirmed that the 15.5- and 15-kD proteins that correspond to P1/P2-type proteins are phosphorylated in maize (Fig. 1B) as in other eukaryotes (Tsurugi et al., 1978; Hasler et al., 1991). No change in the in vivo phosphorylation of these was detected in oxygen-deprived maize seedling roots (Bailey-Serres and Freeling, 1990) or in the 12.7- to 13.8-kD acidic proteins of the 60S ribosomal subunit of heat-shocked tomato suspension-culture cells (Scharf and Nover, 1987). It is not surprising that these ribosomal proteins are phosphorylated in vivo under normal and stress conditions, since their phosphorylation is correlated with assembly into ribosomes (Sanchez-Madrid et al., 1981; Naranda and Ballesta, 1991). To date little is known about the kinases that phosphorylate or phosphatases that dephosphorylate P-proteins in vivo. A 38-kD, ribosome-associated, CK II-type kinase from maize embryos was shown to in vitro phosphorylate acidic ribosomal proteins released with NH_4Cl/50% ethanol (Sepúlveda et al., 1995). A 70-kD kinase from Arabidopsis in vitro phosphorylated 14- and 16-kD proteins of intact

ribosomes at high specificity (Zhang et al., 1994), presumably the P1/P2-type proteins. The 38-kD kinase is an integral component of the ribosome, whereas the 70-kD kinase is cytosolic and its level is developmentally regulated.

In light of the finding that unique phosphorylation sites on the P1 and P2 isoforms may play a role in regulation of translation in yeast (Naranda et al., 1993), it would be worthwhile to examine the role of these kinases in the phosphorylation of the different P1/P2-type proteins during plant development and in response to environmental cues. A comparative analysis of in vivo and in vitro phosphorylation sites is necessary to determine whether the in vitro phosphorylation by ribosome-associated kinases or CK II provide information on phosphorylation sites that are of biological significance. Nevertheless, the in vitro phosphorylation by CK II is dependent on the accessibility of phosphorylation sites and thereby provides information on ribosome structure.

The acidic phosphoprotein P0 is located at the base of the lateral protuberance of the 60S subunit of eukaryotes. We determined that P0 of maize ribosomes is phosphorylated, has an apparent molecular mass of 36 to 36.5 kD, and is poorly eluted from ribosomes with 1.0 M NH_4Cl/50% ethanol, as expected for an integral ribosomal protein. We previously demonstrated variations in the steady-state levels and electrophoretic mobility of forms of a 36- to 36.5-kD protein in ribosomes of seedling roots in response to oxygen deprivation (Bailey-Serres and Freeling, 1990). We now know that these proteins correspond to P0. Electrophoretic variants of P0 could be forms that have undergone different posttranslational modifications such as phosphorylation or acetylation, or may be the products of distinct loci.

The in vitro phosphorylation of P0 by ribosome-associated kinases also confirmed changes in this protein in response to oxygen deprivation that are reversible upon return to aerobic conditions. In vitro phosphorylation of intact ribosomes with CK II was performed to elucidate further the changes in P0 that occur in response to oxygen deprivation. In mammals all of the P-proteins can be phosphorylated by CK II at a Ser within the conserved C terminus (Hasler et al., 1991). Two CK II target sites are present at the C terminus of the deduced peptide sequence of P0 from rice (Hihara et al., 1994). Phosphorylation of P0 in vitro with CK II resulted in the detection of 36- and 36.5-kD forms of P0 in aerobic roots and only a 36-kD form in oxygen-deprived roots. Since all of the accessible phosphorylation target sites should be labeled in vitro, the observed variations in P0 phosphorylation by ribosome-associated kinases reflect differences in the activity of kinases or the presence or accessibility of target sites, whereas phosphorylation by CK II indicates changes in the presence or accessibility of target sites. These analyses predict that oxygen deprivation alters the phosphorylation state or phosphorylation site(s) accessibility on P0. These alterations in P0 are readily reversible; additional analyses are necessary to determine if the transient nature of these changes correlates with ability of roots to recover from oxygen deprivation.

Further analysis of Suc density gradient-fractionated ribosomes that were in vitro phosphorylated by CK II demonstrated that oxygen deprivation stimulates uniform changes in the P-proteins of 80S ribosomes (monoribosomes and polyribosomes). Distinctions between the 60S ribosomal subunits of aerobic and oxygen-deprived roots were also seen. As mentioned, oxygen deprivation resulted in slight changes in the steady-state levels of electrophoretic variants of P0 resolved by NEpHGE/SDS-PAGE (Bailey-Serres and Freeling, 1990). It is important to emphasize that the changes observed by in vitro phosphorylation of the P-proteins are in contrast to the absence of detectable changes in the in vivo phosphorylation of these proteins in response to oxygen deprivation (Bailey-Serres and Freeling, 1990) or heat shock (Scharf and Nover, 1987). Furthermore, oxygen deprivation had no clear affect on the relative levels of the 13- to 15.5-kD proteins analyzed by SDS-PAGE (Fig. 5A), or electrophoretic mobility of P1/P2-type proteins of intact ribosomes analyzed by NEpHGE/SDS-PAGE (Bailey-Serres and Freeling, 1990). We conclude that oxygen deprivation stimulates a reversible change in the P-protein complex of the 60S ribosomal subunit.

Oxygen Deprivation Stimulates Dynamic Alterations in the 60S Ribosomal Subunit P-Protein Complex

There are a number of ways in which the P-protein complex may be altered in response to oxygen deprivation. A change in the conformation of the stalk formed by the P-proteins relative to the body of the 60S subunit could have ramifications on the accessibility of phosphorylation sites. A conformational change could result from altered posttranslational modification of P0 or another ribosomal protein. Alternatively, a structural modification in the ribosome, such as a change in the quantity of P1/P2-type proteins in the ribosome, could affect the accessibility of kinases to phosphorylation sites. This seems likely, since NEpHGE/SDS-PAGE analysis revealed a reduction in the quantity of acidic ribosomal proteins in response to oxygen deprivation (Bailey-Serres and Freeling, 1990). Similarly, in yeast the stoichiometry of P1 and P2 per ribosome is higher in rapidly dividing than in stationary-phase cultures (Saenz-Robles et al., 1990). Finally, the discovery of one P1, two forms of P2, and a novel P3 in plant ribosomes raises the possibility that the quantity of these individual proteins may be modulated in response to stress. We plan to use antisera to quantitate the levels of the distinct P1/P2-type proteins in ribosomes from aerobic and oxygen-deprived roots. Changes in the structure or position of the P-protein stalk of ribosomes might be resolved by immunoelectron microscopy or three-dimensional electron microscopy.

It is not known whether the changes in the 60S ribosomal subunit P-protein stalk relate to the translational control observed in response to oxygen deprivation of maize roots. We have demonstrated that oxygen deprivation results in the competitive differences between mRNAs for initiation of translation (Fennoy and Bailey-Serres, 1995; Bailey-Serres and Dawe, 1996). Regulation also occurs after initiation, since certain mRNAs loaded onto polyribosomes are not efficiently translated (Webster et al., 1991; Fennoy and Bailey-Serres, 1995). In yeast, ribosomes lacking P1 and P2 selectively translate a subset of cellular mRNAs, and the

amount of P-proteins associated with the ribosome can be modulated by growth conditions (Remacha et al., 1995). We speculate that modification of the P-protein stalk in response to oxygen deprivation could play a role in or be reflective of changes involved in the mechanism of selective mRNA translation. The oxygen-deprivation response of maize seedling roots could be used to elucidate further the significance of dynamic changes in the structure and components of the 60S ribosomal subunit P-protein complex in translational regulation.

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