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Three Enzymes of Carbon Metabolism or their Antigenic Analogs in Pea Leaf Nuclei[1]

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Antigens closely resembling or identical to the three glycolytic enzyme proteins phosphate-glycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and aldolase are found in situ in the nucleus of the leaf mesophyll cells of pea (*Pisum sativum* L.). These proteins have already been identified in vertebrate nuclei. Apparently, these enzymes are nuclear proteins with "secondary" roles not directly related to their enzymatic function in carbon metabolism in both animals and plants.

Glycolytic enzymes have been recruited repeatedly for alternative uses and function during evolution. The lens proteins of the mammalian eye, for example, consist of a mixture that includes glyceraldehyde-3-P dehydrogenase (Lenstra et al., 1982). In vertebrates, proteins similar or identical to the glycolytic enzymes P-glycerate kinase (Viswanatha et al., 1992), glyceraldehyde-3-P dehydrogenase (Singh and Green, 1993), and aldolase (Ronai et al., 1992) are found in the nucleus. Here we present in situ immunological evidence for the occurrence of these or antigenically similar proteins in the nucleus of leaf mesophyll cells of the higher plant *Pisum sativum*.

MATERIALS AND METHODS

Plants

Pea (*Pisum sativum* L. var Little Marvel) plants were grown from seed as described previously (Anderson et al., 1995) but in a growth room under sodium vapor and fluorescent lamps, which provided a PPFD of 450 to 650 μmol photons m^{-2} s^{-1} during the 14-h photoperiod.

Fixation

Pea leaf tissue from 12-d-old plants was fixed with 1% (v/v) acrolein, 0.1% (v/v) glutaraldehyde in 100 mM sodium phosphate buffer, pH 7.2, for 2 h at 4°C, was washed three times for 10 min per wash in the phosphate buffer, and then was dehydrated in a graded (25, 50, 75, 100% [v/v]) series of ethylene glycol (10 min at each concentration, 4°C). Infiltration was continued with one part ethylene glycol to two parts LR White resin for 2 h, and the mixture was replaced with pure LR White resin three times over 24 h, at 4°C. Then the samples were polymerized in Eppendorf tubes with 1 mL of pure LR White resin in a vacuum oven at 50°C for 3 d. Cured blocks were trimmed and thin-sectioned with a diamond knife on a Riechert Ultra Cut E ultra microtome (Leica, Inc., Deerfield, IL) and the sections were picked up on 200-hex-mesh paralodion carbon-coated Ni grids.

Antibodies

The anti-aldolase used in these experiments was the antiserum that was used by Razdan et al. (1992) for immunoscreening a cDNA library for aldolase. It was raised in mice against the pea plastid isozymes. Anti-phosphoglycerate kinase was raised in mice against a band of the purified pea plastid isozyme (Macioszek et al., 1990) excised from an SDS gel. The gel piece was frozen, pulverized with a mortar and pestle, and mixed 1:1 with Freund's complete adjuvant for the first injection and with incomplete adjuvant for the succeeding injections. The protocol that was described by Razdan et al. (1992) for the production of antibody in mice was followed, except that there was a third injection before the first bleeding and less protein (approximately 10 μg) was used per injection. Likewise, anti-NADP-linked glyceraldehyde-3-P dehydrogenase was raised in mice against both bands of the purified pea leaf enzyme (Anderson et al., 1995) excised from an SDS gel essentially as described for anti-phosphoglycerate kinase except that there was a series of four injections of 25 μg of protein and adjuvant. This antisera cross-reacted with a band on a gel that corresponded to pea chloroplast subunit A expressed in *Escherichia coli* and was therefore assumed to be directed against glyceraldehyde-3-P dehydrogenase. Anti-Rubisco was a monoclonal antibody against the large subunit of spinach Rubisco and was the gift of Archie Portis (University of Illinois, Champaign-Urbana). Rabbit anti-spinach ADP-glucose pyrophosphorylase was the gift of Jack Preiss (Michigan State University, East Lansing). Rabbit anti-spinach Fru-bisphosphatase and anti-maize sedoheptulosebisphosphatase were the gifts of Bob Buchanan (University of California, Berkeley). Rabbit anti-yeast Glc-6-P dehydrogenase was purchased from Sigma. This antiserum inhibits pea chloroplast Glc-6-P dehydrogenase (data not shown).

Antisera were used without purification except for the anti-aldolase, which had been passed through a column of *E. coli* protein prior to use in screening an expression

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library (see Razdan et al., 1992). Each of these antisera reacted with a single band of protein (two in the case of glyceraldehyde-3-P dehydrogenase and ADP-Glc pyrophosphorylase) from SDS gels of pea chloroplast stromal extracts (not shown). We also tested these antisera against blots of total leaf proteins. Extracts were prepared from pea leaves that had been frozen in liquid nitrogen, ground in 0.3 M Tris-H_2SO_4 (pH 8.3), 5 mM DTT, 1 mM PMSF, 1 mM benzamidine-HCl in a mortar. The brei was diluted 1:1 with distilled water and 1:1 with 0.125 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (w/w) glycerol, 0.016% (w/v) bromphenol blue, and 10% (v/v) 2-mercaptoethanol, was heated 5 min in a boiling water bath, and was centrifuged (microfuge) for 1 min. Proteins (96 μg/lane) were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred electrophoretically (Hoefer, San Francisco, CA, TE70 SemiPhor Semi-Dry Transfer Unit) to a Millipore Immobilon-P membrane according to Gershoni and Palade (1982) and the manufacturer's instructions.

The membrane was soaked in 10 mM Tris-HCl, pH 7, 150 mM NaCl, 0.05% (w/w) Tween 20 (TBST) overnight at 7°C, washed for 1 h with two changes of buffer at room temperature, incubated with gentle shaking for 1 h in 5% (w/v) teleostean gelatin, 75 mM potassium phosphate (pH 7.4), 70 mM NaCl, 0.02% (w/v) NaN_3, and incubated with anti-phosphoglycerate kinase (1:8,000 dilution), anti-glyceraldehyde-3-P dehydrogenase (1:2,000 dilution), anti-aldolase (1:4,000 dilution), or anti-ADP-Glc pyrophosphorylase (1:20,000 dilution) in TBST buffer for 30 min. The membrane was then incubated for 30 min with goat anti-mouse polyclonal immunoglobulin alkaline phosphatase conjugate (Sigma) (1:1,000 in TBST) for anti-phosphoglycerate kinase, anti-glyceraldehyde-3-P dehydrogenase, and anti-aldolase, or goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma) (1:1,000 in TBST) for anti-ADP-Glc pyrophosphorylase, blotted dry with filter paper, and incubated in 0.033% (w/v) nitroblue tetrazolium, 0.017% (w/v) 5-bromo-4-chloro-3-indolyl phosphate, 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$ until color developed. Then the membrane was rinsed in distilled water and stored in the dark. Only one band was seen with the antisera directed against P-glycerate kinase, aldolase, or ADP-Glc pyrophosphorylase (Fig. 1), which probably indicates that the chloroplastic and cytosolic isozymes were not well separated and/or that electrophoretic mobility was affected by other proteins. Morell et al. (1987) found only a single band when blots of crude spinach leaf extracts were probed with anti-ADP-Glc pyrophosphorylase. A broad band corresponding to the α and β subunits is seen with the antisera directed against glyceraldehyde-3-P dehydrogenase (Fig. 1, lane 3).

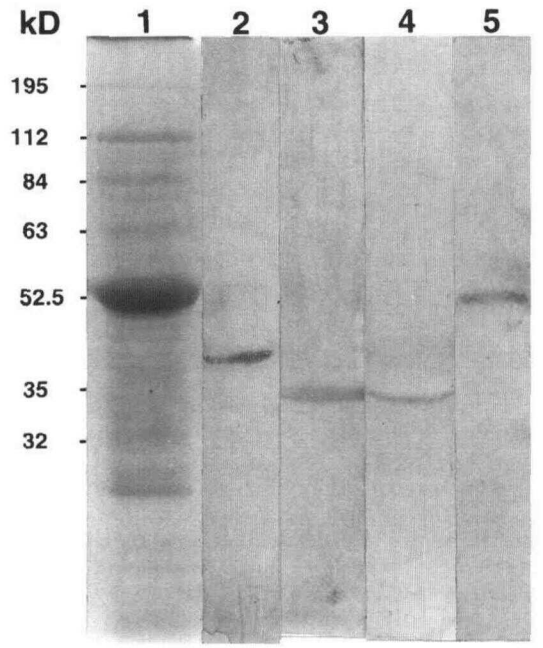

Figure 1. Cross-reaction between antisera used in these experiments and proteins in whole pea leaf extracts. Total leaf proteins were resolved by SDS-PAGE and blotted onto Immobilon. Numbers at left indicate positions of the molecular mass markers (in kD). Markers were prestained SDS Molecular Weight Markers from Sigma. Lane 1, Total pea leaf proteins stained with Coomassie blue. Lane 2, Reaction with anti-phosphoglycerate kinase. Lane 3, Reaction with anti-glyceraldehyde-3-P dehydrogenase. Lane 4, Reaction with anti-aldolase. Lane 5, Reaction with anti-ADP-Glc pyrophosphorylase. The molecular mass estimates for the pea leaf proteins are P-glycerate kinase, 44 kD; glyceraldehyde-3-P dehydrogenase, 36 kD, 37 kD; aldolase, 36 kD; and ADP-Glc pyrophosphorylase, 54 kD, in good agreement with published values.

Immunolabeling

The thin sections on the coated Ni grids were incubated at room temperature for 10 min in 0.5 M NH_4Cl, were washed twice in distilled water, and then were incubated in blocking solution (0.8% [w/v] BSA, 0.1% [w/v] immunogold-silver-stain-quality gelatin, 5% [v/v] normal goat serum in PBS, pH 7.4) for 45 min, washed twice in 0.8% (w/v) BSA, 0.1% (w/v) gelatin, 0.025% (v/v) Tween 20 in pH 7.4 PBS (washing solution), and incubated overnight with primary antibody diluted 1:500 (1:250 with anti-glyceraldehyde-3-P dehydrogenase) in 0.8% (w/v) BSA, 0.1% (w/v) gelatin, 1% (v/v) normal goat serum in PBS, washed twice in washing solution, and incubated with immunogold-labeled secondary antibody (goat anti-mouse IgM or goat anti-rabbit IgG heavy and light chain, as indicated; Amersham) diluted 1:25 for 4 h. Grids were washed six times with the washing solution for 5 min per wash, then twice with PBS, and the samples were fixed with 2% (v/v) glutaraldehyde in PBS for 10 min. Grids were washed twice in PBS and three times in distilled water, dried, stained with 2% (w/v) uranyl acetate (aqueous) for 3 min, dried, and photographed with a JEOL 1200 EX transmission electron microscope.

When preimmune mouse sera were used in place of the primary antibody, only a few gold particles were found on the micrographs (Fig. 2A). More gold particles were seen when preimmune rabbit sera were used, but the number of gold particles was still far lower than the number found with antisera directed against the specific proteins included in this study (Fig. 2B).

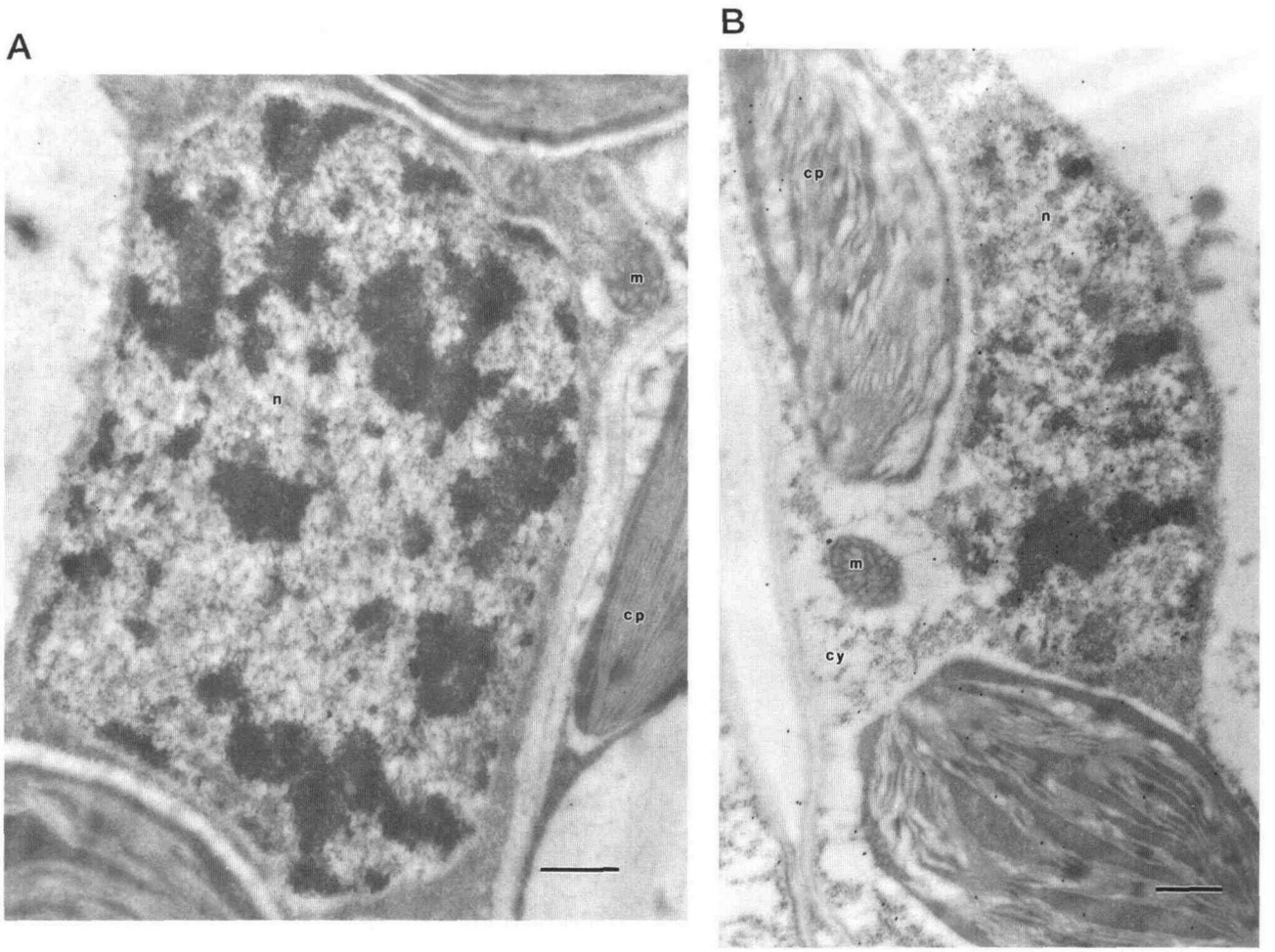

Figure 2. A, Gold particles in nucleus of pea leaf mesophyll cell when preimmune mouse antisera were substituted for primary antibody. cp, Chloroplast; m, mitochondrion; n, nucleus. Gold particles were 10 nm in diameter. Bar = 0.5 μm. B, Gold particles in nucleus of pea leaf mesophyll cell when preimmune rabbit antisera were substituted for primary antibody. cy, Cytosol. Other symbols as in A. Gold particles were 15 nm in diameter. Bar = 0.5 μm.

Measurements

We used the public domain program Image on a Macintosh IICX to measure areas occupied by chloroplasts and nuclei on the electron micrographs.

RESULTS AND DISCUSSION

Aldolase

There is cross-reactivity between anti-aldolase and material immediately outside the pea leaf nuclear envelope and, to a lesser extent, with material inside the nucleus (Fig. 3). Aldolase was initially identified as a DNA-binding protein when it was purified from SEWA sarcoma cells by DNA-affinity chromatography (Ronai et al., 1992). It is present in the perinuclear space in cells of vertebrate species (Minaschek et al., 1992; Ronai et al., 1992) and there is specificity in the DNA binding with respect to both the sequence of the DNA and to the aldolase species (Ronai et al., 1992). Apparently, aldolase is also located in the perinuclear space (Fig. 3, arrow) and functions as a nuclear protein in plants.

In our hands there was almost complete cross-reactivity between the chloroplast and cytosolic aldolases and antibody against either isozyme (see Razdan et al., 1992). These experiments, thus, do not distinguish between the chloroplast and cytosolic isozyme proteins. The nuclear aldolase protein could be the chloroplast isozyme, or the cytosolic isozyme, or a third isozyme specific to the nucleus. It might also be an antigenic analog of aldolase.

Phosphoglycerate Kinase

Our antibody apparently reacts with both the chloroplastic and cytosolic P-glycerate kinase isozymes; gold particles are distributed both inside and outside of the chloroplast when pea leaf sections are incubated with anti-P-glycerate kinase and gold-labeled secondary antibody (Fig. 4). Gold particles are also found throughout the nucleus. P-glycerate kinase has been localized in the nucleus of HeLa cells (Viswanatha et al., 1992), where it is the 41-kD subunit of

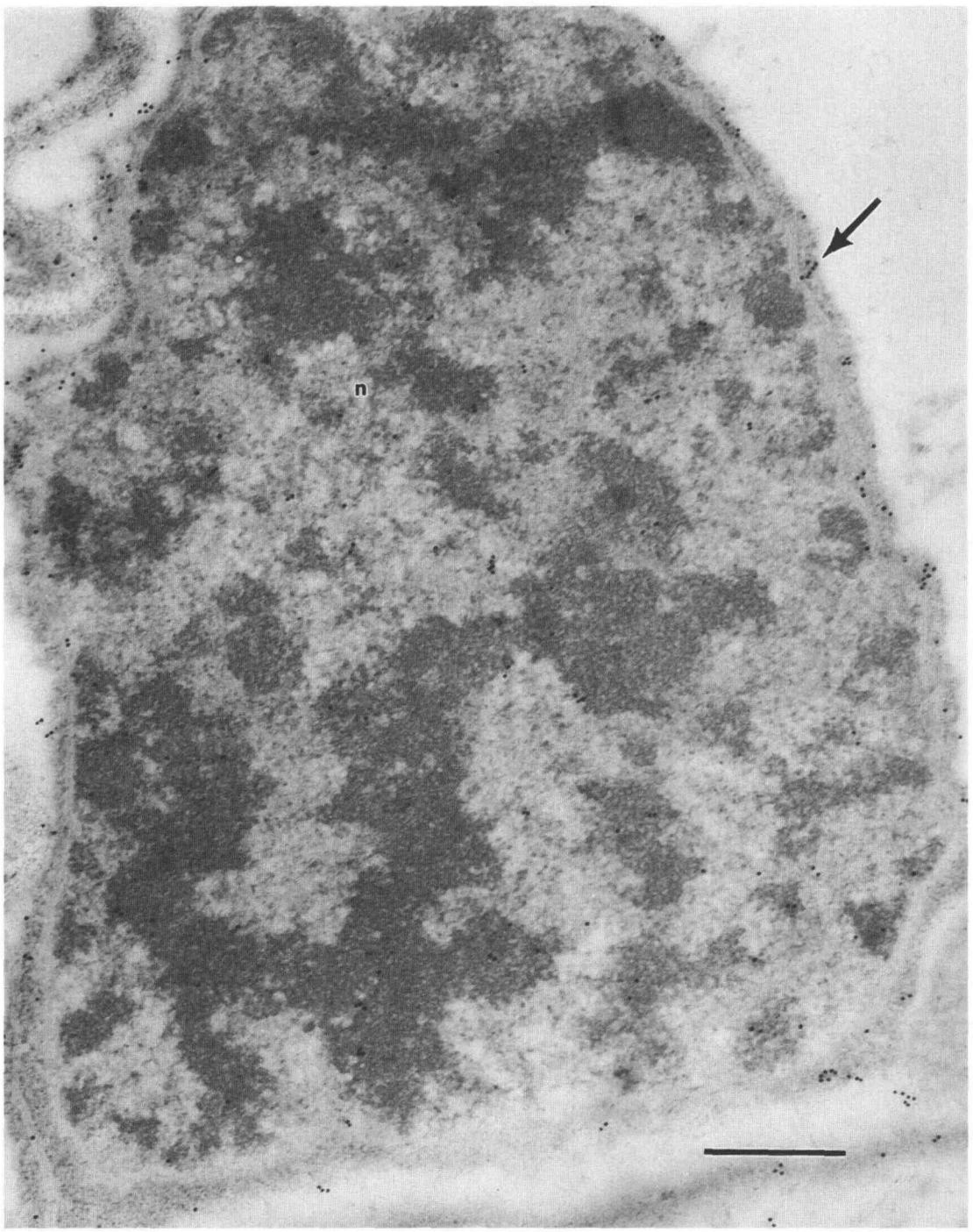

Figure 3. Localization of aldolase in and around the nucleus in a pea leaf mesophyll cell. Arrow points to a cluster of gold particles in the nuclear periplasm. There were 1.6 times as many particles per unit area in the nucleus as in the chloroplast (1339 particles counted in nuclei on four micrographs, 122 particles counted in chloroplasts on two micrographs) when the primary antibody was anti-chloroplastic pea aldolase. n, Nucleus. Gold particles were 10 nm in diameter. Bar = 0.5 μm.

the primer recognition protein involved in DNA synthesis on the lagging strand (Jindal and Viswanatha, 1990a, 1990b). Notably, the 41-kD circadian clock protein in *Chlorella fusca* is P-glycerate kinase (Walla et al., 1994). To our knowledge, this is the first observation of P-glycerate kinase (or of an antigen that is recognized by antibody against P-glycerate kinase) in the nucleus in higher plants.

The N termini of the plant P-glycerate kinases are distinct from those of the microbial and animal enzymes (not shown). Dingwall and Laskey (1991) have defined a bipartite nuclear localization sequence that occurs almost exclusively in nuclear proteins and is found in the N termini of the wheat cytosolic kinase and of the spinach chloroplastic isozyme (Fig. 5). It is not present in the N terminus of the

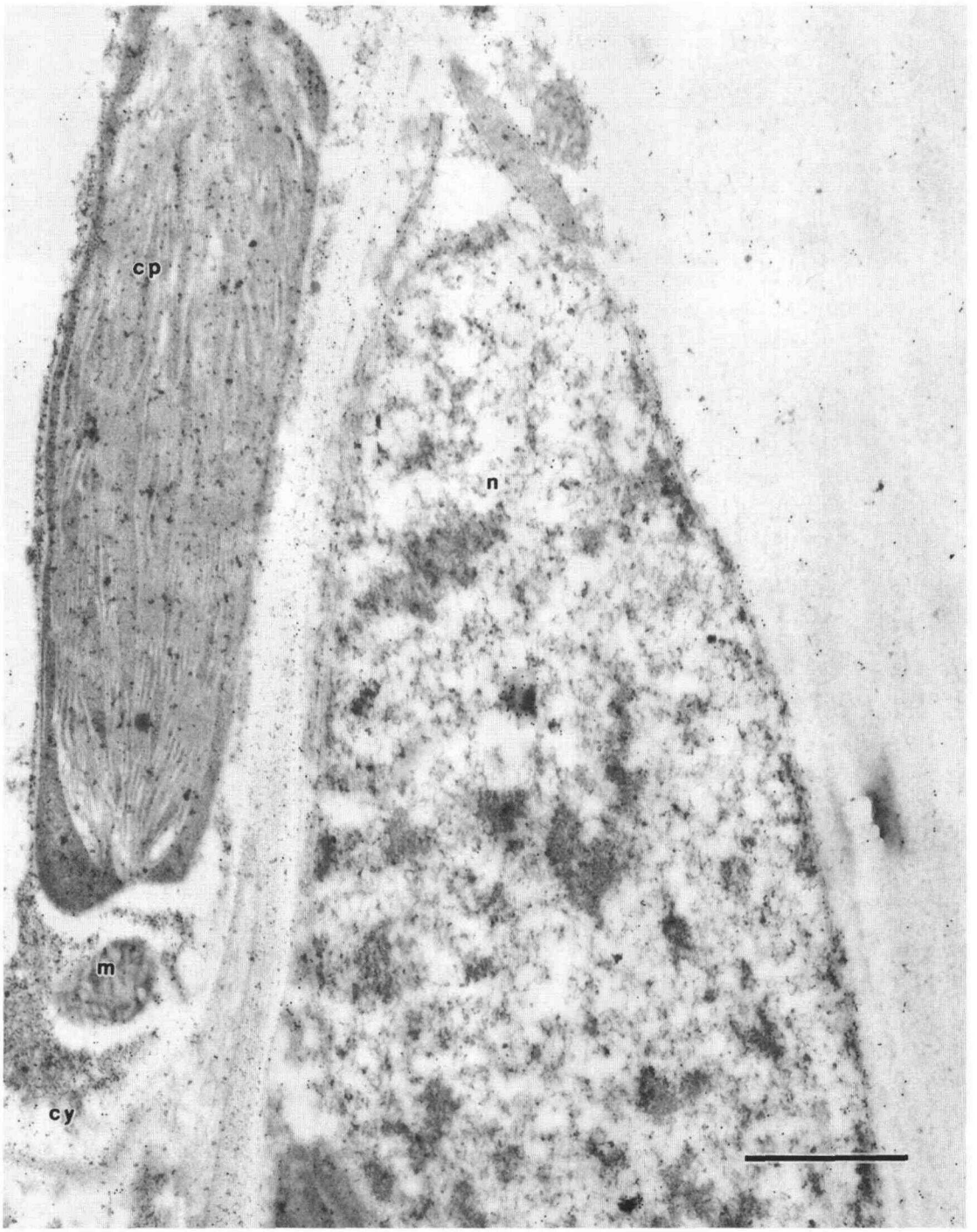

Figure 4. Localization of phosphoglycerate kinase in the nucleus of a pea mesophyll cell. There were as many particles per unit area in the nucleus as in the chloroplast (469 particles counted in nuclei on four micrographs, 279 particles counted in chloroplasts on five micrographs) when the primary antibody was anti-chloroplastic pea P-glycerate kinase. n, Nucleus. Other symbols as in Figure 2. Gold particles were 10 nm in diameter. Bar = 1 μm.

nonplant P-glycerate kinases. This stretch of residues is exposed on the surface of the enzyme in the three kinases for which crystal structures are available (yeast, horse, and *Bacillus stearothermophilus*, entries 3PGK, 2PGK, and 1PHP, respectively, in the Brookhaven Protein Data Base). It is reasonable to assume that it will be in approximately the same location in the plant P-glycerate kinases. A nuclear localization sequence in this position should be easily recognized by its receptor.

There is significant cross-reactivity between antibody prepared against one isozyme and the isozyme from the other cell compartment in barley (McMorrow and Brad-

Wheat Cytosolic	MATKRSVGTLGEADLKGKKV
Spinach Chloroplast	AKKSVGDLTSADLKGKKV
Pea Chloroplast	AKKSVGDLSAADLEGKKV
Wheat Chloroplast	AKKSVGDLSTADLEGKRV
Chlorella fusca	AKKSVGDLTKADLEGKRV

Figure 5. N-terminal sequence of plant P-glycerate kinases. The bipartite nuclear localization sequence (Dingwall and Laskey, 1991), which consists of two basic residues followed by a 10-residue spacer and then three basic residues in the next five positions, is in uppercase bold. The SwissPro data base numbers are spinach chloroplastic, P29409; wheat chloroplastic, P12782; *Chlorella fusca*, P36232; and wheat cytosolic, P12783. Pea chloroplast P-glycerate kinase, X. Wang and L. Anderson, unpublished data.

beer, 1990). Our antibody probably reacts with both isozymes. Therefore, these experiments do not allow us to identify the specific isozyme protein in the nucleus.

Glyceraldehyde-3-P Dehydrogenase

Anti-NADP-linked glyceraldehyde-3-P dehydrogenase reacts with protein both inside and outside of the chloroplast and with material in the nucleus (Fig. 6). There are several reports already indicating that this enzyme might have a second function unrelated to carbon metabolism in yeast and mammals. Singh and Green (1993) identified glyceraldehyde-3-P dehydrogenase as a tRNA-binding protein in HeLa cell nuclear extracts and demonstrated the presence of the enzyme in the nucleus by immunofluorescence. They suggested that the enzyme might be involved in tRNA export from the nucleus. Nagy and Rigby (1995) found that mammalian glyceraldehyde-3-P dehydrogenase binds adenine-uracil-rich RNA. NAD, NADH, and ATP inhibit binding, which indicates involvement of the pyridine nucleotide binding site in RNA binding.

Earlier, Meyer-Siegler et al. (1991) cloned and sequenced the nuclear enzyme uracil DNA glycosylase of humans and found that it was identical to, and could be replaced by, monomeric glyceraldehyde-3-P dehydrogenase. Hamster fibroblast glyceraldehyde-3-P dehydrogenase has been shown to bind single-stranded but not double-stranded DNA (Perucho et al., 1977; Grosse et al., 1986). A nucleic acid helicase in yeast is identical with glyceraldehyde-3-P dehydrogenase (Karpel and Burchard, 1981). Because there is significant homology in the sequences of the chloroplast and cytosolic forms of glyceraldehyde-3-P dehydrogenase, it is likely that the antisera react with the NAD-linked cytosolic form as well as with the chloroplastic enzyme and might interact with a nuclear-specific form as well. Therefore, we cannot identify the isozyme protein present in the nucleus. To our knowledge, this is the first observation of glyceraldehyde-3-P dehydrogenase in the nucleus of higher plants.

Other Proteins

Gold particles decorate the chloroplast and appear with far less frequency in the nucleus when the primary antibody is directed against several other stromal enzymes. The ratio of particles per unit area in nucleus versus chloroplast was 0.11 with anti-ADP-Glc pyrophosphorylase (Fig. 7), 0.25 with anti-sedoheptulosebisphosphatase, 0.4 with anti-Rubisco, 0.44 with anti-Glc-6-P dehydrogenase, and 0.65 with anti-Fru-bisphosphatase (not shown).

CONCLUSIONS

To our knowledge, this is the first report of the three glycolytic (and reductive pentose phosphate pathway) enzymes, P-glycerate kinase, glyceraldehyde-3-P dehydrogenase, and aldolase, in the plant nucleus. The association seems to be fairly specific because neither sedoheptulosebisphosphatase, Fru-bisphosphatase, Glc-6-P dehydrogenase, Rubisco, or ADP-Glc pyrophosphorylase appears to be nuclear-localized. The appearance of these enzymes or closely related antigens in the nuclei of both plants and animals suggests that these proteins are characteristic nuclear proteins in most or all eukaryotes. They may then have important secondary roles not directly related to carbon metabolism. The secondary roles of these three glycolytic enzymes may be related to the fact that each, including aldolase (Kochman and Mas, 1981; Sytnik et al., 1991), contains a nucleotide binding site and may therefore bind to DNA or RNA in the nucleus.

These enzyme proteins are now known to occur in three compartments in the green leaf. Whether the nuclear forms are specific to the nucleus or are the cytosolic and/or the chloroplastic form with a second function remains to be determined. In at least one case, a single enzyme protein is targeted to both mitochondrion and nucleus (Li et al., 1989), and it therefore seems possible that a bifunctional protein might also be targeted to both chloroplast and nucleus. The presence of a putative nuclear localization sequence in the spinach chloroplast isozyme (Fig. 5) suggests that P-glycerate kinase may be such an enzyme.

Interestingly, levels of glyceraldehyde-3-P dehydrogenase protein in the nucleus of bovine endothelial cells increase in response to hypoxia (Graven et al., 1994). NADP-linked glyceraldehyde-3-P dehydrogenase is reductively activated in the chloroplast (Anderson, 1986), and thioredoxin and NADP-thioredoxin reductase have been shown to be present in the nucleus of rat hepatocytes (Rozell et al., 1988). The possibility of redox control of this dehydrogenase in its secondary function in the plant nucleus is intriguing.

Figure 6. (On opposite page.) Localization of glyceraldehyde-3-P dehydrogenase in the nucleus of a pea leaf mesophyll cell. There were 1.9 times as many gold particles per unit area in the nucleus as in the chloroplast (943 particles counted in nuclei on 4 micrographs, 349 particles counted in chloroplasts on 10 micrographs) when the primary antibody was anti-pea NADP-linked glyceraldehyde-3-P dehydrogenase. nu, Nucleolus. Other symbols as in Figure 2. Gold particles were 10 nm in diameter. Bar = 0.5 μm.

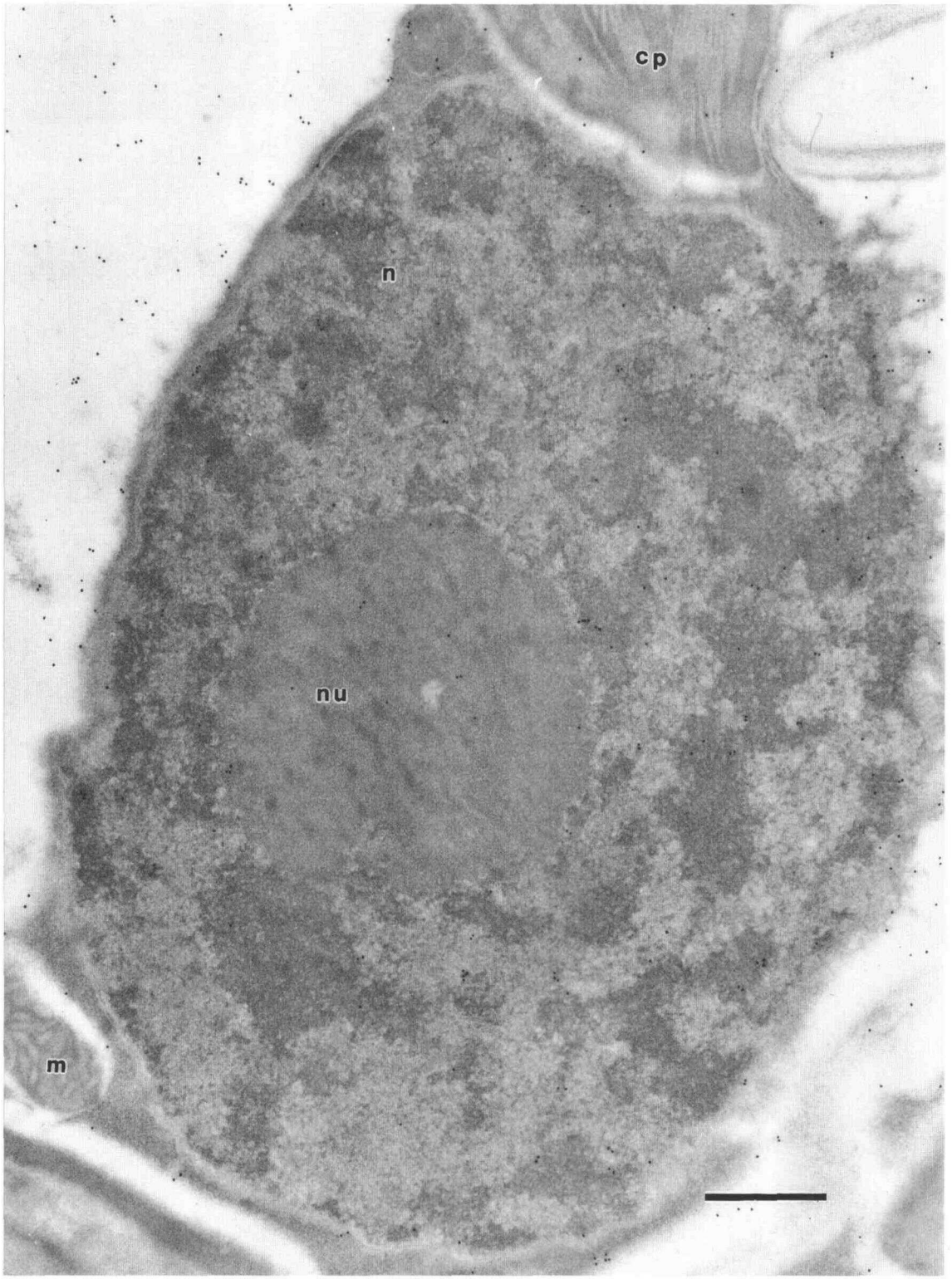

Figure 6. (Legend appears on opposite page.)

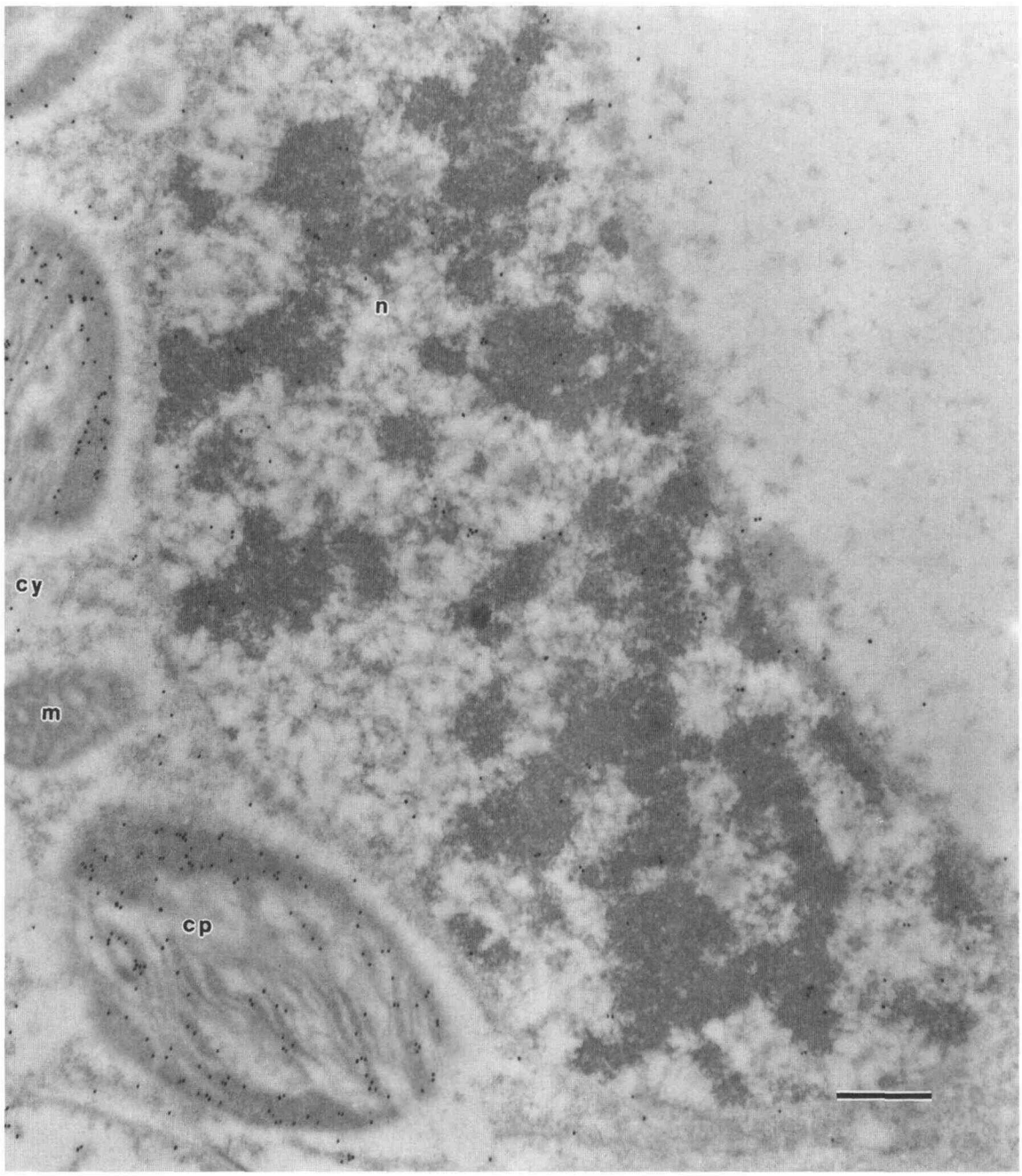

Figure 7. Localization of ADP-Glc pyrophosphorylase in a pea leaf mesophyll cell. There were 9 times as many gold particles per unit area in the chloroplast as in the nucleus (702 particles counted in chloroplasts on eight micrographs, 1524 particles counted in nuclei on five micrographs) when the primary antibody was anti-spinach ADP-Glc pyrophosphorylase. Symbols as in Figure 2. Gold particles were 15 nm in diameter. Bar = 0.5 μm.

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