Extrachloroplastic Site of Synthesis of Three Chloroplast Proteins in Maize *(Zea mays)*

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Acetyl-CoA Synthetase, Adenylate Kinase, Glycerate Kinase, *iojap* Maize

Activities of acetyl-CoA synthetase (EC 6.2.1.1), adenylate kinase (EC 2.7.4.3) and glycerate kinase (EC 2.7.1.31) were found in leaf extracts of *iojap* mutant maize *(Zea mays)* which lacks the protein-synthesizing apparatus in plastids. These activities represented at least one-half of the rates observed for normal maize plants. Since the proteins studied are localized either preferentially (adenylate kinase) or exclusively (acetyl-CoA synthetase, glycerate kinase) in chloroplasts, the high activities seen for *iojap* mutant can not be attributed to some outerchloroplastic isoforms of these enzymes. The data indicate that in maize the three proteins are synthesized by an outerchloroplastic translation system.

Introduction

The recessive nuclear gene *iojap* conditions a permanent heritable deficiency in the ability of maize plastids to differentiate [1, 2]. Affected plastids contain a normal genome but have no detectable ribosomes nor high molecular weight RNA and do not incorporate exogeneous amino acids into proteins [2]. The ribosome-deficient plastids are chlorotic in the light and thus are unable to perform photosynthesis. *Iojap* plants may be regarded as a convenient and simple system for identification of proteins which are synthesized on 70 ribosomes of chloroplasts. The ribosome-deficient plastids should not contain proteins formed by normal chloroplasts, and thus presence of a given protein in *iojap*-affected plants would certainly rule out chloroplasts as its site of biosynthesis.

In the present study, *iojap* maize seedlings were investigated for the activity of acetyl-CoA synthetase (ACS), adenylate kinase (AK) and glycerate kinase (GK). These proteins are involved in three widely divergent aspects of metabolism in leaf cells such as fatty acid biosynthesis, ACS [3–5], energy metabolism, AK [6, 7], and photorespiration, GK [8–10]. However, they have also one common characteristic in that they are localized either predominantly (AK) or exclusively (ACS and GK) in chloroplasts [3, 8, 11]. The question put forth in the present study was whether these proteins are synthesized *in situ* in chloroplasts or whether they need to be imported into the chloroplasts from the cytosol.

Abbreviations: ACS, acetyl-CoA synthetase; AK, adenylate kinase; GK, glycerate kinase; LSU, large subunit; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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Materials and Methods

Plant material

Seedlings of *iojap* mutant and normal maize (*Zea mays* L.) plants were grown in greenhouse as previously described [9].

Preparation of leaf extracts

Samples of leaves from 8–9 day old seedlings were frozen with liquid nitrogen and then extracted in a grinding medium using mortar and pestle (0–4 °C). The grinding buffer contained 40 mM Tricine, pH 7.8, 2 mM $MgCl_2$ and 1 mM EDTA. The extract was squeezed through four layers of cheesecloth and one layer of Miracloth and centrifuged at 10,000 *g* for 10 min. Aliquots of supernatants were desalted on a small Sephadex G-25 column which had been pre-equilibrated with the grinding buffer.

Purification of LSU of Rubisco and immunological studies

Rubisco was purified from fescue (*Festuca arundinacea* Schreb.) leaves as described in [12]. The

small and large subunits of the enzyme were separated by preparative SDS-polyacrylamide gel electrophoresis. The band corresponding to the LSU was cut from the gel and the protein eluted using 5 mM phosphate buffer (pH 7.5).

Antibodies against LSU of Rubisco were obtained by injecting a New Zealand white male rabbit with a single dose of the purified fescue protein. The dose contained 300 μg of protein mixed with Freund's complete adjuvant (Cappel Laboratories) in a 1:1 (v/v) ratio. Serum samples were collected six weeks after the immunization. The IgG fraction (1.2 mg protein/ml) containing antibodies against the LSU was obtained by chromatography of crude serum on DEAE-Affi Gel Blue (Bio-Rad) column followed by dialysis against 5 mM phosphate buffer. Antibodies were routinelly aliquoted into 1–2 ml samples and kept at −20 °C.

Western immunoblotting was performed according to a slightly modified procedure of Towbin *et al.* [13], as described in [14]. An alkaline phosphatase-labelled goat anti-rabbit IgG (Sigma) was used as a linking antibody. Specific immunoreactions on nitrocellulose were detected using Fast Red (Sigma).

Enzyme assays

Both AK and GK were assayed spectrophotometrically at 340 nm (25 °C). Activity of AK was monitored in the direction of ADP formation as previously described [7], with the exception that $MgCl_2$ was maintained at 3 mM. Conditions of the assay and thiol activation of GK were also previously published [10]. ACS activity was measured by the radioassay described in [15]. The reaction mixture contained, in 0.35 ml, 50 mM Hepes-KOH, pH 8.0, 5 mM $MgCl_2$, 2 mM ATP, 0.5 mM CoA, 2 mM dithiothreitol, and 1 mM [1-^{14}C]acetate (1 μCi/nmol). The reaction was initiated by the addition of [^{14}C]acetate and assayed for 30 min at 30 °C. One unit of either AK or GK activity was defined as the amount of the enzyme required to oxidize 1 μmol NADH/min under conditions of assays. One unit of ACS was taken as 1 μmol acetyl-CoA produced/min.

Other methods

Determination of protein was done according to Bradford [16]. Slab gel electrophoresis was done according to Laemmli [17]. Protein standards for SDS-electrophoresis were from Bio-Rad.

Results and Discussion

Studies on the site of synthesis of ACS, AK and GK were carried out using *iojap* and normal maize seedlings. Leaves of *iojap* plants contained considerable activities of each of the three enzymes under study (Table I). Activity of GK, which in maize is light-activated *in vivo* and thiol-activated *in vitro* [9, 10], was almost identical in both *iojap* and normal plants, regardless of whether the assay was carried out in the presence or absence of 2-mercaptoethanol (activated and inactive form of GK, respectively). Both ACS and AK had lower activities in *iojap* plants, however, their magnitude still accounted for at least one-half of rates observed for normal maize seedlings.

Table I. Activities of acetyl-CoA synthetase, adenylate kinase and glycerate kinase in leaf extracts of *iojap* and normal maize.

Enzyme	Activity [μmol/min/mg protein] Normal	Iojap
Acetyl-CoA synthetase	0.004	0.002
Adenylate kinase	5.6	3.7
Glycerate kinase (−SH)	0.011	0.009
(+SH)*	0.069	0.065

* GK was pre-incubated with 100 mM 2-mercaptoethanol as previously described [10].

To ascertain that plastids of the mutant plants used in the present study had no protein-synthesizing ability, we investigated crude leaf extracts of both *iojap* and normal plants for the presence of LSU of Rubisco which is known to be synthesized on 70S ribosomes of chloroplasts [18]. The LSU was assayed qualitatively by Western blot technique [13] using crude leaf extracts of *iojap* and normal plants probed with rabbit antibodies against fescue LSU (Fig. 1). Since the LSU is produced in chloroplasts, probing for this protein could be used as an indirect test of the translational ability of plastids. The lack of immunoreaction for *iojap* proteins and the appearance of a single band corresponding to the LSU for normal maize (Fig. 1) as well as spinach (data not shown) was consistent with ribosome deficiency in plastids of the mutant plants.

The use of *iojap* maize allowed us to rule out the plastid 70S ribosome translation apparatus as the site of synthesis of leaf ACS, AK and GK. The occur-

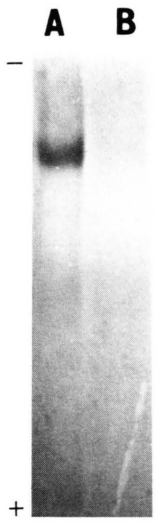

Fig. 1. Immunodetection of LSU of Rubisco in extracts of normal and *iojap* maize leaves after SDS-polyacrylamide gel electrophoresis. Proteins from leaf extracts were loaded on 10%-polyacrylamide gel and electrophoresed in the SDS buffer. After electrophoresis, resolved proteins were transferred on the nitrocellulose paper and incubated with rabbit anti-fescue LSU IgG (1:50 dilution). Formation of the specific antigen-antibody complex(es) was determined as described in the Materials and Methods section. Lane A – normal maize; lane B – *iojap* maize.

rence of these chloroplastic proteins in an *iojap* tissue provided unequivocal evidence that they are formed outside the plastids, irrespectively of some quantitative changes. The relatively lower activities observed in *iojap* plants for either ACS, AK and GK (Table I) could perhaps readily be explained by the fact that *iojap* plastids are unable to perform photosynthesis, while activities of each of these three enzymes had been found to be positively correlated with the development of photosynthetic apparatus in leaves [4, 10, 19]. Therefore, one would rather expect the activities under study to be lower in the chlorotic tissue *(iojap)* when compared to the green one (normal maize). The similar conclusions have recently been reached for chloroplast enzymes of nitrogen metabolism found in heat-treated 70S ribosome-deficient rye seedlings [20].

To our knowledge, this is the first report on the site of synthesis of leaf ACS and GK. Concerning AK, our data are analogous to those obtained by Höinghaus and Feierabend [21] who investigated AK activity in heat-bleached 70S ribosome-less rye seedlings. This indicates that AK is synthesized outside the chloroplasts in both C_3 (rye) and C_4 (maize) species.

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