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LOCALIZATION OF CERTAIN PHOTOSYNTHETIC ENZYMES IN MESOPHYLL AND PARENCHYMA SHEATH CHLOROPLASTS OF MAIZE AND *AMARANTHUS PALMERI*

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Abstract—An attempt has been made to separate by non-aqueous density fractionation the mesophyll and parenchyma sheath chloroplasts present in leaves of maize and *Amaranthus palmeri*. The results suggest that ribulose diphosphate carboxylase and phosphoribulokinase are localized mainly in parenchyma sheath chloroplasts while enzymes operative in the C_4 dicarboxylic acid pathway, pyruvate-P_i dikinase and adenylate kinase are restricted mainly to the mesophyll chloroplasts. In addition, glyceraldehyde phosphate dehydrogenase (NADP) appears to be localized mainly in the mesophyll chloroplasts.

INTRODUCTION

RECENT studies[1, 2] have indicated that photosynthetic CO_2 fixation in tropical grasses proceeds via a pathway which differs from the reductive pentose phosphate cycle[3] in the steps leading to 3-phosphoglycerate formation. This pathway has been referred to as the C_4 dicarboxylic acid pathway of photosynthesis[4] since CO_2 is incorporated first into oxaloacetate by the carboxylation of phosphopyruvate[5] then transferred to an acceptor forming 3-phosphoglycerate. The observation that ribulose diphosphate (RuDP) carboxylase is present in leaves of tropical grasses, although in relatively small amounts,[5] has raised the question as to the function of the enzyme in these species. Leaves of tropical grasses contain two morphologically distinct types of chloroplast. Those in the sheath parenchyma, are large, contain starch grains, but no grana, whereas the chloroplasts in the surrounding mesophyll are smaller, contain grana and are essentially starch-free.[6] It was of interest, therefore, to determine whether RuDP-carboxylase and enzymes of C_4 dicarboxylic acid pathway are equally distributed between two types of chloroplast, or whether certain enzymes are localized in mesophyll chloroplasts and others in chloroplasts of the parenchyma sheath.

Stocking[7] has shown that destarched chloroplasts isolated from lyophylized tobacco leaf in non-aqueous media are lighter than most other cell constituents and Thalaker and Behrens[8] demonstrated that starch increases the chloroplast density. Consequently, it seemed possible that the starch-free mesophyll chloroplasts might separate from the starch-containing parenchyma sheath chloroplasts during a density fractionation of lyophylized

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[6] SISTER M. C. JOHNSON, Ph.D. Thesis, University of Texas, Austin, Texas, U.S.A. (1964).
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leaf. In the present paper the distribution of enzymes in fractions obtained by density fractionation of illuminated and destarched leaf are compared.

RESULTS AND DISCUSSION

Enzymes investigated were glyceraldehyde phosphate dehydrogenase (NADP) which is operative in the reductive pentose phosphate cycle and probably also in the path of photosynthetic CO_2 fixation in tropical grasses,[1,5] phosphoribulokinase and RuDP-carboxylase, which are generally considered to be exclusively associated with the reductive pentose phosphate cycle, and adenylate kinase and pyruvate-P_i dikinase which are thought to have a specific role in the C_4 dicarboxylic acid pathway.[4] The latter enzyme catalyses the conversion of pyruvate to phosphopyruvate by the overall reaction

$$\text{pyruvate} + \text{ATP} + P_i \rightleftharpoons \text{phosphopyruvate} + \text{AMP} + PP_i$$

it was purified recently from sugar cane leaves and identified in leaves of a number of other tropical grasses[4] and *Amaranthus* species[9] but not in several temperate species. The enzyme was originally referred to as phosphopyruvate synthase but has been renamed pyruvate-P_i dikinase, on the suggestion of Reeves,[10] to distinguish it from the enzyme described by Cooper and Kornberg.[11] We have suggested[4] that adenylate kinase, which is present in large amounts in leaves of tropical grasses, operates in association with purivate-P_i dikinase converting AMP to ADP.

In previous studies the distribution of RuDP-carboxylase[5,12] and glyceraldehyde phosphate dehydrogenase[5] paralleled that of chlorophyll when destarched maize leaf was fractionated using a density range of 1·35–1·40. For the present studies it was desirable to use a range of densities which afforded the best chance of separating starch-free mesophyll chloroplasts from the starch-containing chloroplasts of the parenchyma sheath. Hence, preliminary experiments were performed using destarched leaf to ascertain the density at which most of the chlorophyll-containing material remained suspended after centrifugation. It was hoped that with preparations of illuminated leaf the mesophyll chloroplasts would still remain suspended at this density while the starch-containing parenchyma sheath chloroplasts would be pelleted. Suitable densities for maize and *Amaranthus palmeri* were found to be 1·30 and 1·28 respectively.

When destarched maize leaf was fractionated, very little chlorophyll was associated with material of density greater than 1·33 (Table 1). This suggested that both the mesophyll and destarched parenchyma sheath chloroplasts were isolated together in the light fractions (density $< 1{\cdot}33$). Most of the activity of the enzymes shown in Table 1 were present in these light fractions which, however, contained only 40 per cent of the soluble protein and 20 and 60 per cent of total acid phosphatase and malate dehydrogenase activity respectively. Smillie[13] suggested that the ratio of enzyme activity to chlorophyll content for each fraction could be used to distinguish plastid enzymes from cytoplasmic enzymes; the ratio for plastid enzymes being theoretically the same for all fractions. However, the inaccuracy inherent in measuring the small amount of chlorophyll in the heavy fractions and in assaying low enzyme activity, using techniques of varying sensitivity, could undoubtedly lead to variations in the

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ratio for plastid enzymes. The variations in the ratios obtained for RuDP-carboxylase, phosphoribulokinase and pyruvate-P_i dikinase using destarched leaf are very similar and it is assumed that these enzymes, together with glyceraldehyde phosphate dehydrogenase, are localized in plastids. The activity of adenylate kinase in the heaviest fraction, when expressed on a chlorophyll basis, was 10-fold that in the lightest fraction, this suggests, as might be expected, that a fraction of this enzyme is not associated with the chloroplasts.

When illuminated leaf was fractionated a considerable amount of chlorophyll was present in fractions of density greater than 1·33 (Table 1). A microscopic examination of iodine-stained suspensions of the various fractions indicated that the two types of chloroplast had partially separated. The light fractions (density $< 1{\cdot}33$) contained starch-free chloroplasts whereas starch-containing chloroplasts were present in the heavier fractions. Thus, the light

TABLE 1. DISTRIBUTION OF CHLOROPHYLL AND ENZYMES IN FRACTIONS OF DESTARCHED AND ILLUMINATED MAIZE LEAF PREPARED IN NON-AQUEOUS MEDIA

				Enzyme activity (mμmole/min/fraction)			
	Fraction density	Chlorophyll (μg/fraction)	Pyruvate-P_i dikinase	Adenylate kinase	Glyceraldehyde phosphate dehydrogenase (NADP)	Ribulose diphosphate carboxylase	Phospho-ribulokinase
Destarched leaf	$< 1{\cdot}30$	56·4	80·0	5050	1110	84·0	1290
	1·30–1·33	10·2	13·0	1400	51	18·0	350
	1·33–1·36	1·8	5·4	630	0	3·5	68
	1·36–1·40	1·5	5·4	570	0	5·3	68
	$> 1{\cdot}40$	1·5	3·2	1400	0	3·5	70
Illuminated leaf	$< 1{\cdot}30$	49·5	95·0	2600	570	16·7	230
	1·30–1·33	28·6	40·5	2000	320	10·7	210
	1·33–1·36	7·5	13·7	630	130	16·7	230
	1·36–1·40	10·3	23·8	630	150	34·3	500
	$> 1{\cdot}40$	8·1	16·3	580	83	21·4	390

Illuminated leaves were harvested at 10.00 a.m. (sunrise 5.30 a.m.). Destarched leaves were obtained from plants held in darkness for 24 hr then illuminated for 10 min in full sunlight immediately before harvest.

fractions presumably contained mesophyll chloroplasts and any parenchyma sheath chloroplasts from which starch grains had been lost during isolation and the heavy fractions intact parenchyma sheath chloroplasts. With illuminated leaf the distribution of RuDP-carboxylase and phosphoribulokinase did not parallel that of chlorophyll and only about 27 per cent of the total activity of enzymes were isolated in the light fractions (density $< 1{\cdot}33$) compared with approximately 88 per cent with destarched leaf. In contrast, most of pyruvate-P_i dikinase and glyceraldehyde phosphate dehydrogenase activity was isolated in the light fractions from illuminated leaf.

In an attempt to test the validity of this observation a similar experiment was performed using leaves of *A. palmeri*. Evidence for the operation of the C_4 dicarboxylic acid pathway in this species was presented recently.[9] The leaf anatomy of *A. palmeri* resembles that of tropical grasses in that the chloroplast containing cells are localized around vascular bundles,[9] and the mesophyll chloroplasts are essentially free of starch whereas the larger parenchyma

sheath chloroplasts, which in this species contain grana, accumulate starch.[14] The results obtained (Table 2) were essentially the same as those for maize. The distribution of pyruvate-P_i dikinase amongst the various fractions was similar to that of chlorophyll in preparations from both illuminated and destarched leaf. The low activity of the enzyme in the destarched leaf no doubt reflects the rapid inactivation of the enzyme that occurs when leaves are darkened.[15] In contrast, there was a marked difference between illuminated and destarched leaf in the distribution patterns of RuDP-carboxylase and phosphoribulokinase. The bulk of these enzymes was isolated in the light fractions (density $< 1{\cdot}32$) when destarched leaf was used and their distribution paralleled that of chlorophyll, but with illuminated leaf a large proportion of the total activity of both enzymes was associated with material isolated

TABLE 2. DISTRIBUTION OF CHLOROPHYLL AND ENZYMES IN FRACTIONS OF DESTARCHED AND ILLUMINATED *Amaranthus* LEAF PREPARED IN NON-AQUEOUS MEDIA

				Enzyme activity (mμmole/min/fraction)		
	Fraction density	Chlorophyll (μg/fraction)	Pyruvate-P dikinase	Adenylate kinase	Ribulose diphosphate carboxylase	Phospho-ribulokinase
Destarched leaf	$< 1{\cdot}28$	107·0	9·8	5250	224·0	1500
	1·28–1·30	4·5	0·1	520	4·5	58
	1·30–1·32	18·3	2·8	2100	28·5	240
	1·32–1·34	9·1	1·3	1890	11·6	140
	1·34–1·36	5·1	0·5	830	4·5	58
	$> 1{\cdot}36$	14·3	0·8	2650	18·9	100
Illuminated leaf	$< 1{\cdot}28$	50·0	74·0	6100	72·5	480
	1·28–1·30	3·8	10·9	550	5·4	71
	1·30–1·32	23·0	54·0	3150	67·5	430
	1·32–1·34	15·5	27·0	2000	82·0	535
	1·34–1·36	10·8	20·4	1830	112·0	625
	$> 1{\cdot}36$	28·0	8·1	3500	107·0	550

Starch-containing leaves were harvested at 10.00 a.m. Sunrise 5.30 a.m. Destarched leaves were obtained from plants held in darkness for 24 hr prior to harvest.

in heavier fractions. The data for adenylate kinase are consistent with the view that part of the leaf's complement of this enzyme is not associated with chloroplasts.

The most plausible explanation for the finding that RuDP-carboxylase and phosphoribulokinase are iolated with heavier material from illuminated leaf than from destarched leaf is that these enzymes are wholly or mainly localized in the starch-accumulating chloroplasts of the parenchyma sheath. It has been suggested that these chloroplasts are specialized for starch accumulation and that they play only a minor role in photosynthetic CO_2 fixation.[6, 16, 17] In this regard, it is possible that the supply of external CO_2 to RuDP-carboxylase

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may be limited since CO_2 entering the leaf must pass a compact layer of chloroplast-containing mesophyll cells before reaching the parenchyma sheath. It may be, as Kursanov[18] has suggested, that the supply of CO_2 to the parenchyma sheath is augmented by respiratory CO_2 moving from the vascular tissue. The fact that the bulk of the pyruvate-P_i dikinase, glyceraldehyde phosphate dehydrogenase and adenylate kinase activity was associated with the light material isolated from illuminated leaf suggests that these enzymes are mainly localized in the mesophyll chloroplasts.

Information about the localization of other enzymes involved in the C_4 dicarboxylic acid pathway and reductive pentose phosphate cycle, and about the localization of intermediates of photosynthetic CO_2-fixation is required before the precise roles of the two types of chloroplast in the overall process of CO_2 fixation can be defined.

EXPERIMENTAL

Plant Material

Plants were grown in vermiculite culture at 28° under natural illumination. Young, fully expanded leaves were used. Illuminated leaf was harvested from plants at 10.00 a.m. after about 4·5 hr illumination. Leaf was destarched by holding plants in darkness for 24 hr at 28°. The maize plants were illuminated subsequently in full sunlight for 10 min immediately prior to harvest to allow the photoactivation of pyruvate, P_i dikinase.[15]

Fractionation of Leaf Material

Immediately after harvest the leaf was frozen and lyophilized by the procedure of Stocking[7] and the dried leaf stored over P_2O_5 at −15° until required. The leaf was homogenized using a glass tissue grinder in hexane–CCl_4 and fractionated by density as described by Smillie.[13] All procedures were carried out at 3°. Fractions were suspended in hexane, subsampled, dried *in vacuo* and stored at −15° over silica gel. For enzyme assays the material was suspended in Tris-HCl buffer (pH 8·3) containing 0·005 M $MgCl_2$ and 0·005 M dithiothreitol (DTT). Suspensions were centrifuged at 20,000 × *g* when spectrophotometric assays were employed.

Pyruvate-P_i-Dikinase Assay

The enzyme was assayed as described previously[4] using the system linked to pyruvate carboxylase and aspartate aminotransferase; β-mercaptoethanol was replaced by DTT.

Ribulose Diphosphate Carboxylase and Phosphoribulokinase Assays

Ribulose diphosphate carboxylase was assayed as previously described.[5] Phosphoribulokinase was assayed in a similar system containing 0·005 M ATP, spinach ribulose diphosphate carboxylase and phosphoriboisomerase and 0·01 M ribose-5-phosphate in place of ribulose diphosphate. The latter enzymes were prepared by the procedure of Racker[19] and Hurwitz *et al.*[20] respectively.

Adenylate Kinase Assay

The enzyme was assayed by measuring the absorbance change at 340 nm in a system containing 0·02 M tris-HCl buffer (pH 7·6), 0·0025 M ATP, 0·0012 M phosphopyruvate, 0·005 M $MgCl_2$, 0·02 M KCl, 0·0002 M $NADH_2$, 0·0025 M AMP and pyruvate kinase and lactic dehydrogenase (obtained from Sigma Chemical Co.) at 22°.

Other Assays

Glyceraldehyde phosphate dehydrogenase (NADP) was assayed by the method of Gibbs.[21] Acid phosphatase and malate dehydrogenase were assayed as described previously.[5] Protein and chlorophyll were measured by the procedures of Lowry *et al.*[22] and Arnon[23] respectively.

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