NADP-SPECIFIC MALATE DEHYDROGENASE AND GLYCERATE KINASE IN LEAVES AND EVIDENCE FOR THEIR LOCATION IN CHLOROPLASTS *

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The operation of both malate dehydrogenase and glycerate kinase in certain phases of photosynthesis has been implied from previous studies. Malate has been recognized as a quantitatively minor photosynthetic product in algae and some plants in which the Calvin cycle is operative (Calvin and Bassham, 1962), and as an intermediate in the C_4-dicarboxylic acid pathway of photosynthesis (Hatch and Slack, 1966; Hatch, Slack and Johnson, 1967; Johnson and Hatch, 1968). Although NADP appears to be the predominant pyridine nucleotide in chloroplast oxidation-reduction reactions, an NADP-malate dehydrogenase has been found neither in leaves nor indeed from any other source. A glycerate kinase appears not to have been reported previously from plant tissues although it has been proposed to operate in the glycollate pathway, the photosynthetic bypass cycle in which glycollate derived from sugar phosphates is apparently converted to 3-phosphoglycerate (Tolbert, 1963). Glycerate kinase has been detected in liver and yeast (Black and Wright, 1956; Ichihara and Greenberg, 1957).

This communication reports the presence of an NADP-specific malate dehydrogenase and a glycerate kinase in leaves of both Calvin cycle plants and plants in which the C_4-dicarboxylic acid pathway is operative. Some properties of these enzymes are described and evidence for their location in the chloroplasts of maize leaves is presented.

METHODS

For the survey of enzyme activity in different plants, extracts were prepared and treated on Sephadex G-25 as previously described (Slack, 1968). NADP-glyceraldehyde 3-phosphate dehydrogenase, acid phosphatase, chlorophyll, protein (Slack and Hatch, 1967) and pyruvate,P_i dikinase (Hatch and Slack, 1968) were determined as previously described. Reagent enzymes were obtained from Sigma Chemical Co., St. Louis, Mo.

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Malate dehydrogenase activities were measured by the change in absorbance at 340 mμ in reaction mixtures containing enzyme, 25mM tris-HCl buffer, pH 7.9, 1mM EDTA, 0.5mM oxaloacetate and 0.2mM $NADH_2$ or $NADPH_2$. EDTA was added to bind traces of divalent metal ions thus preventing the operation of the "malic enzyme" present in some of the extracts.

For the assay of glycerate kinase in Sephadex G-25 extracts reactions contained enzyme, 30mM tris-HCl, pH 8.3, 7mM dithiothreitol, 7mM ATP, 10mM $MgCl_2$, 5mM [1-^{14}C] glycerate, 2mM fluoride and 2mM K_2HPO_4. Reactions were stopped by adding formic acid to give a final concentration of 5% (^{w}/v) and samples were chromatographed on No. 1 Whatman paper using pentanol saturated with 5M formic acid as the developing solvent to separate and determine the proportions of radioactivity in the substrate and product. The partially-purified enzyme was also assayed spectrophotometrically by measuring ADP formation in reactions containing in addition to the above reactants phosphopyruvate, $NADH_2$, pyruvate kinase and lactate dehydrogenase or by phosphoglycerate formation in reactions supplemented with 3-phosphoglycerate mutase, enolase, pyruvate kinase, lactate dehydrogenase, and ADP. For the latter assay phosphate and fluoride, added in the other assays to inhibit enolase, were omitted.

RESULTS AND DISCUSSION

NADP-malate dehydrogenase. With all leaf extracts tested $NADPH_2$ was oxidised following the addition of oxaloacetate (Table 1). Activities in plants with the C_4-dicarboxylic acid pathway were comparable to or greater than the maximum photosynthesis rates (Slack and Hatch, 1967; Hatch and Johnson, 1968) and were about five times the activity in Calvin cycle plants. To confirm that the observed activities were not mediated by NAD-malate dehydrogenase, following the action of either transhydrogenase or phosphatase on $NADPH_2$, samples of extracts were incubated with $NADPH_2$ in a reaction mixture containing lactate dehydrogenase and pyruvate. In all cases changes in optical density were negligible although added $NADH_2$ was rapidly oxidised.

In extracts of maize leaf the malate dehydrogenase activity with $NADPH_2$ was about 25% of that with $NADH_2$. However, with the protein fraction precipitating between 51% and 56% saturation of $(NH_4)_2SO_4$ the activity with $NADPH_2$ was about 2.5 times that with $NADH_2$. When this fraction was treated on a Sephadex G-100 column the NADP-malate dehydrogenase emerged well after the void volume but before the NAD-specific enzyme. In the earlier emerging fractions the activity with $NADPH_2$ was about 30 times that with $NADH_2$.

Table 1. NADP-malate dehydrogenase and glycerate kinase activities in leaves.

Plant	Pathway of photosynthesis*	Enzyme Activity (μmoles/mg chlorophyll/min.) NADP-malate dehydrogenase	Glycerate kinase
Sugarcane (Saccharum hybrid)	C_4-dicarboxylic acid pathway	4.6	1.4
Maize (Zea mays)		6.5	2.5
Sorghum (Sorghum hybrid)		3.7	3.2
Amaranthus (A. palmeri)		2.4	2.6
Spinach (Spinacea oleracea)	Calvin pathway	0.9	9.6
Silver-beet (Beta vulgaris)		0.2	6.0
Carrot (Daucus carota)		0.2	8.7
Pea (Pisum sativum)		0.8	-

* See Hatch, Slack and Johnson (1967) and Johnson and Hatch (1968).

Table 2. Distribution of malate dehydrogenase and glycerate kinase compared with chlorophyll and other enzymes in non-aqueously isolated maize-leaf fractions. *

Density of fraction	% of total in each fraction: Chloro-phyll	NADP-malate dehydrogenase	Glycerate kinase	Pyruvate, P_i dikinase	NADP-G3P dehydrogenase	NAD-malate dehydrogenase	Acid phosphatase
<1.30	61	62	56	56	51	15	4
1.30-1.33	31	30	29	28	35	21	8
1.33-1.36	4	4	6	5	6	13	3
1.36-1.40	2	3	4	5	5	19	8
>1.40	2	1	5	6	3	32	77

* Fractions were prepared from destarched leaves as previously described (Slack, 1969). Plants were briefly illuminated prior to collecting leaves to activate pyruvate,P_i dikinase (Hatch and Slack, 1969).

With this partially-purified NADP-malate dehydrogenase maximum activity was observed between pH 7.5 and 8.3. The Km for oxaloacetate was less than 0.05mM and above 2.5mM oxaloacetate was inhibitory. At pH 8.9 the rate in the direction of $NADPH_2$ oxidation was about 30 times that for the reverse direction.

Our previous studies implicate dicarboxylic acids, including malate, as intermediates in photosynthesis by plants with the C_4-dicarboxylic acid pathway (Hatch and Slack, 1966; Slack and Hatch, 1967; Johnson and Hatch, 1968; Hatch and Slack, 1968). The results of Table 2 provide evidence that the NADP-malate dehydrogenase is associated with chloroplasts, having the same distribution as chlorophyll, NADP-glyceraldehyde 3-phosphate dehydrogenase and pyruvate,P_i dikinase. The latter enzyme, previously named phosphopyruvate synthase, is present only in plants with the C_4-dicarboxylic

acid pathway (Hatch and Slack, 1968). The results for NAD-malate dehydrogenase and acid phosphatase, two enzymes not characteristically photosynthetic in function, are included for comparison. We suggest that the NADP-malate dehydrogenase contributes to malate formation during the operation of the C_4-dicarboxylic acid pathway of photosynthesis, and that it possibly also has a photosynthetic function in Calvin cycle plants. It is not possible to say whether the small part of the total NAD-malate dehydrogenase which appears in the fractions containing chlorophyll is associated with the chloroplasts. Leaves certainly contain large quantities of this enzyme (Slack and Hatch, 1967) and there is evidence that in spinach at least a part of this activity is located in chloroplasts (Rocha, Mukerji and Ting, 1968).

Glycerate kinase. Evidence for glycerate kinase in C_4-dicarboxylic acid pathway plants was sought because of its possible involvement in the transfer of the C-4 of dicarboxylic acids to the C-1 of 3-phosphoglycerate. Activity was detected in the leaves of these plants and was comparable to the photosynthesis rates, but even higher activities were present in leaf extracts of the Calvin cycle plants (Table 1). These activities were determined using the procedure with radioactive glycerate as substrate (see Methods section). Activity was dependent upon the addition of ATP and was linear until at least 80% of the glycerate was consumed. The product formed with maize and spinach Sephadex G-25 extracts co-chromatographed with 3-phosphoglycerate in butanol-propionic acid water (10:5:7 by vol.) and chromatographed with glycerate in the above solvent and in pentanol saturated with 5M formic acid after treatment with alkaline phosphatase. When similar reactions were provided with unlabelled glycerate, 3-phosphoglycerate mutase, enolase, phosphopyruvate carboxylase, aspartate aminotransferase, glutamate and $NaH^{14}CO_3$, radioactive aspartate was formed. The formation of aspartate was dependent upon the addition of ATP.

When maize and spinach extracts were fractionated with $(NH_4)_2SO_4$ most of the activity precipitated between 50% and 65% saturation. Further purification of the spinach enzyme was achieved by filtration on Sephadex G-100, the enzyme apparently having a fairly low molecular weight since it was considerably retarded. Spinach enzyme prepared in this way could be assayed spectrophotometrically by measuring either production of ADP or phosphoglycerate by coupling to lactate dehydrogenase (see Methods section). Since preparations were not completely freed of 3-phosphoglycerate mutase, present in large quantities in the original extracts, it was not possible to prove whether 3-phosphoglycerate or 2-phosphoglycerate was the initial product.

The pH optimum for the spinach enzyme was approximately 8.5 and the Km for glycerate was less than 1.0mM. Mg^{2+} was optimal at 1mM in the presence of

5mM ATP. There was apparently no metal ion requirement other than to provide Mg^{++}-ATP complex. The partially-purified enzyme was stable when frozen.

Glycerate kinase, like NADP-malate dehydrogenase, was apparently associated with chloroplasts prepared in non-aqueous media (Table 2). The significance of its pattern of distribution in relation to the distribution of other enzyme activities has already been discussed. It remains to be determined whether chloroplast glycerate kinase has any function in the glycollate bypass as has been proposed (Tolbert, 1963), or in the C_4-dicarboxylic acid pathway.

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