Photoactive ATP Dependent Glutamine Synthetase from Chloroplasts of *Setaria italica* Beauv.

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Glutamine Synthetase, Photophosphorylation, Mesophyll and Bundle Sheath Chloroplasts

Light and ATP dependent glutamine synthetase (E. C. 6.3.1.2) activity was predominantly located in the mesophyll chloroplasts of *Setaria italica* Beauv., a C_4 plant. ATP served the kinetic requirement while ADP exerted inhibitory effects on the enzyme activity. Sucrose stimulated the enzyme activity both in the light and in the dark. The inhibitors of both the cyclic and noncyclic photophosphorylation have suppressed the enzyme activity which suggested the specific requirement for ATP.

Introduction

Incorporation of nitrogen into the ∞-amino groups of amino acids through the reductive amination of ∞-ketoglutarate is now considered to be a minor or insignificant pathway. The recently established GS/GOGOT (glutamine synthetase/glutamate synthase) pathway is regarded to be the major alternate route for nitrogen assimilation in higher plants [1, 2].

Glutamine synthetase mediates the ATP driven addition of ammonia to glutamate to produce glutamine [3, 4] which is of considerable importance as a readily metabolised intermediate in plants. Glutamine synthetase activity has been reported to be associated with chloroplasts, [1, 5 – 7] as well as proplastids [8]. Although is has been purified to study the properties [3] the precise relationship between glutamine synthetase, photosynthetic electron transport and photophosphorylation is not clearly elucidated. Hence the present study is oriented towards an understanding the requirement of light and ATP and to study the process by which chloroplast glutamine synthetase is coupled to photosynthetic photo-phosphorylation, by using specific inhibitors.

Materials and Methods

Setaria italica Beauv. var. H – 1 was field grown using farm yard manure as fertilizer. Young and fully expanded leaves of about three weeks old were chosen as the experimental material.

Isolation of chloroplasts: The chloroplasts were isolated in the medium consisting of 0.2 M Tris-HCl buffer pH 7.4, 0.33 M sorbitol, 2 mM EDTA, 2mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM sodium ascorbate, 0.5 mM cyteine, 2 mM polyvinylpyrrolidone, 5 mM dithiothreitol and 0.1% bovine serum albumin.

5 g leaves were chopped, blended for 10 sec and the slurry was filtered through four layers of muslin. The filtrate was centrifuged at 1000 x *g* for 10 min and the pellet contained exclusively mesophyll chloroplasts.

The residue on the muslin was blended for 30 sec at full line voltage and the homogenate was filtered through two layers of muslin. The remaining residue on the muslin was vigorously ground with 8 ml of isolation medium in a prechilled mortar. The bundle sheath chloroplast pellet was recovered after centrifugation at 1000 x *g* for 10 min.

To isolate the whole leaf chloroplasts, the macerate after initial homogenation was filtered through two layers of muslin cloth, cell debris was removed at 600 x *g* for 1 min. The supernatant was centrifuged at 1500 x *g* for 10 min and the pellet consisted of whole leaf chloroplast fraction. Routine microscopic examination was made to assess the purity of the chloroplast preparations.

The three chloroplasts types were separately suspended in either 15 mM Tris-HCl pH 7.4, 0.33 M sorbitol, 1 mM $MgCl_2$, 0.5% bovine serum albumin for assaying photophosphorylation or 50 mM Tris-HCl pH 7.9, 4 mM $MgSo_4$, 7 mM β-mercaptoethanol and Triton X-100 to a (final concentration of 0.1%) for assaying glutamine synthetase activity.

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Abbreviations: GS, Glutamine synthetase; Tris, tris (hydroxymethyl) aminomethane; ATP, adenosine triphosphate; ADP, adenosine diphosphate; DCMU, 3-(3,4-dichlorophenyl) 1, 1-dimethylurea; 2,4-DNP, 2,4-dinitrophenol; Chl, Chlorophyll; PMS, N-methylphenazonium methosulphate.

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Photophosphorylation rates were determined by studying the light dependent ^{32}P incorporation [9]. Biosynthetic assay based on glutamyl hydroxamate synthesis [3] was adopted to determine the enzyme activity. The reactions were carried out either in the dark or in the light (25 Klux). Various inhibitors at the specified concentrations were included in the reaction mixtures.

Chlorophyll concentration was determined according to the method of Arnon [10].

Results and Discussion

The conversion of glutamate to glutamine mostly by mesophyll chloroplasts suggest the operation of GS/GOGOT pathway which is in agreement with the view that the nitrate assimilating enzymes are predominantly localized in the mesophyll cells of C_4 plants [11, 12]. 70% of the total activity on chlorophyll basis was observed in the mesophyll chloroplast preparation (Table I) and higher activities are recorded in the light than in the dark.

Table I. Glutamine synthetase activity by whole leaf, mesophyll and bundle sheath chloroplasts from *S. italica*. Reaction mixture in 3 ml consisted of 0.05 M tris HCl pH 7.8, 20 mM $MgSO_4$, 1 mM EDTA, 80 mM L-glutamate, 6 mM NH_2OH, 8 mM ATP and β-mercaptoethanol, 5 mM and chloroplast suspension (35–50 μg Chl). GS activity expressed as μmol γ-gluamyl hydroxamate formed/mg Chl×h.

Chloroplast type	GS Activity	
	Light	Dark
whole leaf	27.91	13.84
mesophyll	23.62	8.57
bundle sheath	9.67	2.09

Chloroplast glutamine synthetase has an absolute requirement for ATP. Increasing concentrations of ATP enhanced (Table II) the *L*-glutamate + NH_3 + ATP $\xrightarrow[Mg^{2+}]{GS}$ L-glutamine + ADP + P_i enzyme activity while ADP has an inhibitory effect since it acts as a competitive inhibitor with respect to ATP [13]. It is virtually certain that glutamine synthesis by isolated chloroplasts depends on ATP generated by photophosphorylation [14]. The photophosphorylation data suggest that *S. italica* chloroplasts are capable of synthesizing considerably high amount of ATP through cyclic as well as noncyclic photophosphorylation. The PMS catalysed cyclic photophosphorylation was inhibited by salicylaldoxime

Table II. Influence of ATP or ADP on chloroplast glutamine synthetase activity from *S. italica* Beauv. Reaction mixture as in Table I. ATP was added at the specified concentrations. ADP at said concentrations was included besides 8×10^{-3} M ATP already present in the reaction mixture. GS activity is expressed as μmol γ-glutamyl hydroxamate formed/mg Chl×h.

Additives [M]	GS Activity	% Relative Activity
ATP –	3.02	10.8
ATP 2×10^{-3}	3.89	13.9
4×10^{-3}	11.45	41.0
6×10^{-3}	20.37	72.9
8×10^{-3}	27.91	100.0
1×10^{-2}	26.23	93.7
ADP –	27.91	100.0
2×10^{-3}	27.34	98.7
4×10^{-3}	27.08	97.0
6×10^{-3}	22.89	81.8
8×10^{-3}	14.75	52.6
1×10^{-2}	13.10	46.9

and 2, 4-DNP while ferricyanide supported noncyclic photophosphorylation was suppressed by DCMU (Table III). The ATP utilized in glutamine synthesis is apparently not derived from the conventional noncyclic photophosphorylation [14] evidently from a different source *i. e.* the endogenous phosphorylation which might be either cyclic or possibly pseudocyclic. Mitchell and Stocking [4] suggested that ATP supporting glutamine synthesis may be derived from photosystem I mediated cyclic process. DCMU besides being an inhibitor of photosystem II me-

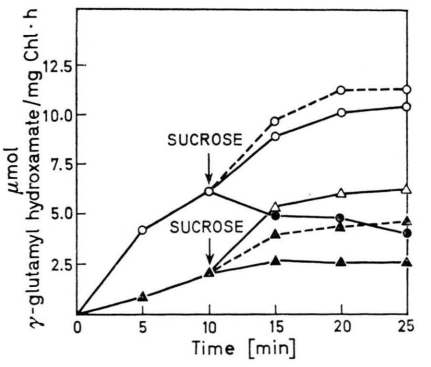

Fig. 1. Influence of sucrose (0.1 M) on glutamine synthetase activity by total leaf chloroplasts either in the light (25 Klux) or in the dark. Reaction mixture as in Table I. Sucrose was added after 10 min. ○—○, light; ○ - - - ○, light + sucrose; ○—●, light to dark; ▲—▲, dark; ▲ - - - ▲, dark + sucrose; ▲—△, dark to light.

diated reactions [15] involving noncyclic photophosphorylation, high concentrations (2×10^{-5} M) might knock out the possibility of electron transport as well as endogenous and pseudocyclic phosphorylations.

Glutamine synthetase activity was suppressed by inhibitors of photophosphorylation like salicylaldoxime (an inhibitor of photosystem I [16]) and 2,4-DNP [17] (Table IV) since they have suppressed the ATP generation through photosystem I mediated cyclic photophosphorylation. The stimulation of the enzyme activity by sucrose (Fig. 1) is possibly due to the provision of ATP generated by glycolytic breakdown.

The localization of leaf glutamine synthetase hence thought to be an advantage to the plant from compartmentalization of nitrogen metabolism, particularly in the chloroplasts where it allows the process to be closely linked to the provision of energy and to carbon fixation.

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Table III. Photophosphorylation capabilities of *S. italica* chloroplasts in the presence of cofactors and inhibitors. Reaction mixture consisted in 3.0 ml 33 mM Tris-HCl pH 8.0, 2 mM $MgCl_2$, 10 mM NaCl, 0.5% bovine serum albumin, 1 mM ADP, 2 mM KH_2PO_4 containing ^{32}P with $10^{-5}-10^{-6}$ cpm, 0.05 mM PMS (cyclic) or 5 mM ferricyanide (noncyclic) and chloroplasts equivalent to 15—20 μg of chlorophyll. Inhibitors at specified concentrations were included in the reaction mixtures.

	μmol ATP formed/mg Chl×h		
	Whole leaf chloroplasts	Mesophyll chloroplasts	Bundle sheath chloroplasts
Control	27	18	12
ferricyanide catalysed noncyclic photophosphorylation	226	105	31
in presence of DCMU 2×10^{-6} M	114	43	11
2×10^{-5} M	41	11	8
PMS catalyzed cyclic photophosphorylation	604	316	187
in presence of Salicylaldoxime 2×10^{-4} M	231	204	67
2×10^{-3} M	104	69	37
in presence of 2,4-DNP 1×10^{-4} M	192	161	136

Table IV. Glutamine synthetase activity of mesophyll and bundle sheath chloroplasts from *S. italica* as influenced by photosynthetic inhibitors in light. Reaction mixture as in Table I: various additives were added to the reaction mixture at the specified concentrations

Additives	Mesophyll chloroplasts		Bundle sheaths chloroplasts	
[M]	GS Activity *	% Inhibition	GS Activity	% Inhibition
Control	23.62	—	9.67	—
DCMU 2×10^{-6}	18.61	21.2	7.93	17.1
2×10^{-5}	16.57	30.2	7.67	18.8
salicylaldoxime 2×10^{-4}	19.28	18.4	8.53	9.7
2×10^{-3}	17.10	25.9	8.39	13.2
2,4-DNP 1×10^{-4}	6.31	73.2	3.42	64.6

* GS activity is expressed as μmol γ-glutamyl hydroxamate formed/mg Chl×h.

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