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A HIGH-ACTIVITY ATP TRANSLOCATOR IN MESOPHYLL CHLOROPLASTS OF *DIGITARIA SANGUINALIS*, A PLANT HAVING THE C-4 DICARBOXYLIC ACID PATHWAY OF PHOTOSYNTHESIS

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SUMMARY

The effect of exogenous adenine nucleotides on CO_2 fixation and oxygen evolution was studied with mesophyll protoplast extracts of the C_4 plant *Digitaria sanguinalis*. Exogenous ATP was found to stimulate the rate of pyruvate and pyruvate+oxalacetate induced CO_2 fixation, as well as reverse the inhibition of CO_2 fixation by carbonyl cyanide *m*-chlorophenyl hydrazone and several electron transport inhibitors. The ATP-dependent stimulation of CO_2 fixation varied from 40 to 70 μmol CO_2 fixed/mg chlorophyll per h, suggesting that ATP was crossing the chloroplast membranes at rates of 80-140 μmol/mg chlorophyll per h, since 2 ATP are required for each CO_2 fixed. Fixation of CO_2 could also be induced in the dark by exogenous ATP, in which case ADP accumulated outside the chloroplasts. This suggests that external ATP is exchanging for internal ADP. In contrast, ADP and AMP were found not to traverse chloroplast membranes, on the basis that neither nucleotide inhibited CO_2 fixation or stimulated oxygen evolution that was limited by available ADP for phosphorylation. Further evidence that ATP can enter the chloroplasts was obtained by direct measurements of the increase in ATP in the chloroplasts due to addition of exogenous ATP in the dark. These studies yielded minimal rates of ATP uptake on the order of 30–40 μmol/mg chlorophyll per h. It is suggested that a membrane translocator exists that specifically transports ATP into the chloroplasts in exchange for ADP. The significance of these findings are considered with respect to the C_4 pathway of photosynthesis.

INTRODUCTION

Transport across chloroplast membranes is thought to occur via specific translocator systems that exchange exogenous for endogenous molecules. In C_3 mesophyll

Abbreviations: C_3 plant: plant having only the Calvin-Benson pathway of photosynthesis; C_4 plant: plant having the C-4 dicarboxylic acid pathway of photosynthesis; DBMIB: 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; CCCP: carbonyl cyanide *m*-chlorophenyl hydrazone; DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

chloroplasts, three translocators have been identified: the dicarboxylic acid [1], the phosphate [1, 2], and the ATP [3] translocator. The first two function at rates of the order of 100 μmol/mg chlorophyll per h, whereas the ATP translocator has a maximum rate of 5 μmol/mg chlorophyll per h [4]. As defined by Heldt [3], the adenine nucleotide translocator is specific for exogenous ATP and is thought to transport cytoplasmic ATP into the chloroplasts in the dark to maintain various biosynthetic functions. To further characterize mesophyll chloroplasts of C_4 plants, we have indirectly studied the transport of adenine nucleotides by monitoring their effects on CO_2 fixation and O_2 evolution and directly by measuring the uptake of ADP and ATP.

With mesophyll protoplast extracts of C_4 plants, CO_2 fixation requires pyruvate, as a precursor for the carboxylation reaction, and ATP, which can come from either cyclic, noncyclic, or pseudocyclic phosphorylation [5]. Two ATP are required for the conversion of pyruvate to phosphoenolpyruvate, which is then carboxylated to oxalacetate. Four partial reactions are required for CO_2 fixation.

(a) $\text{pyruvate} + P_i + \text{ATP} \xrightarrow{\text{pyruvate, } P_i \text{ dikinase}} \text{phosphoenolpyruvate} + \text{AMP} + PP_i$

(b) $\text{AMP} + \text{ATP} \xrightarrow{\text{adenylate kinase}} 2\ \text{ADP}$

(c) $PP_i \xrightarrow{\text{pyrophosphatase}} 2\ P_i$

(d) $\underline{\text{phosphoenolpyruvate} + CO_2 \xrightarrow{\text{phosphoenolpyruvate carboxylase}} \text{oxalacetate} + P_i}$

Net: $\text{pyruvate} + 2\ \text{ATP} + CO_2 \rightarrow \text{oxalacetate} + 2\ \text{ADP} + 2\ P_i$

There is evidence that enzymes catalyzing reactions a, b and c are localized in chloroplasts of C_4 mesophyll cells [6] while phosphoenolpyruvate carboxylase is cytoplasmic [7].

If the concentration of oxalacetate becomes sufficiently high, or if exogenous oxalacetate is added during in vitro studies with C_4 mesophyll chloroplasts, O_2 evolution will proceed rapidly as the oxalacetate is reduced to malate by NADP-malate dehydrogenase. This reaction is not required for CO_2 fixation. If adenine nucleotides are able to enter the chloroplast, predictable effects should be observed either on CO_2 fixation or O_2 evolution. The finding that added ATP can stimulate a reaction catalyzed by a chloroplast enzyme is taken as evidence that ATP can enter the chloroplast. This is supported by direct measurements of ATP uptake into chloroplasts.

MATERIALS AND METHODS

For most experiments, fully matured (2–3 weeks old) leaves of *Digitaria sanguinalis* were collected from plants grown outdoors during the summer months in Madison, Wisc. Mesophyll protoplasts were enzymatically isolated according to the procedure of Kanai and Edwards [8, 9] using the optimum conditions described by Huber and Edwards [10]. Mesophyll protoplast extracts were prepared by mechanically rupturing mesophyll protoplasts as previously described [11]. "Mesophyll protoplast extract" refers to a mixture of chloroplasts and extrachloroplastic material. When prepared in this way, chloroplasts were 75–90 % intact, based on the rate of ferricyanide reduction before and after osmotic shock (Ku and Edwards, unpub-

lished), and retention of chloroplastic enzymes [11]. Mesophyll protoplast extracts were used for CO_2 fixation studies because extrachloroplastic phosphoenolpyruvate carboxylase is required for CO_2 fixation [11].

CO_2 fixation assays

Reaction mixtures (0.25 ml) for CO_2 fixation contained: 0.3 M sorbitol, 2 mM $MgCl_2$, 1 mM KH_2PO_4, 50 mM Tricine-KOH (7.8), 4 mM $NaH^{14}CO_3$ (spec. act., 1–2 Ci/mol), 2.5 mM pyruvate (potassium salt) and 5–10 μg chlorophyll. Where used, oxalacetate was added to give a final concentration of 0.5 mM. All solutions of adenine nucleotides were prepared equimolar with $MgCl_2$, to prevent chelation of Mg^{2+} in the reaction mixtures by the adenylates. Except when indicated, assays were performed at 35 °C and with 80 nEinstein/cm^2 per s, between 400 and 700 nm by a General Electric Lucalox sodium discharge lamp. At various intervals, 40-μl aliquots were removed and the incorporation of $^{14}CO_2$ into acid-stable products was determined by liquid scintillation counting in a cocktail composed of 70 % toluene and 30 % ethanol containing 6 g/l 2,5-diphenyloxazole (PPO) and 0.2 g/l 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene (POPOP). Samples were counted immediately. Rates, calculated from the linear phase of Co_2 fixation, are expressed as μmol CO_2 fixed/mg chlorophyll per h.

Oxygen evolution

Reaction mixtures for oxygen evolution were the same as for CO_2 fixation, except that the final volume was 1.5 ml and $NaH^{12}CO_3$ was substituted for the radioactive bicarbonate. Oxygen evolution was measured polarographically in a water-jacketed Plexiglass chamber maintained at 35 °C. Light was provided by two General Electric projector flood lamps giving a quantum flux density of 120 nEinstein/cm^2 per s between 400 and 700 nm at the cuvette surface.

Exchange measurement

The measurement of the exchange was carried out by a back exchange technique, similar to that of Pfaff and Klingenberg [12]. First, washed chloroplasts were loaded with [^{14}C]ATP (100 μM, 10–20 Ci/mol) in a medium containing 0.3 M sorbitol, 2 mM $MgCl_2$, 1 mM KH_2PO_4 and 50 mM Tricine-KOH (pH 7.8) with 25–50 μg chlorophyll in 0.25 ml. The chloroplasts were incubated for 15–30 min at 4 °C, and were then washed twice afterwards. Loaded chloroplasts were observed to have incorporated 10–20 μmol [^{14}C]ATP/g chlorophyll by this method. Total adenylates in the mesophyll chloroplasts of *D. sanguinalis* averaged 20–30 nmol/mg chlorophyll, as measured with the luciferin-luciferase assay (Huber and Edwards, unpublished), which is slightly less than previously reported for spinach chloroplasts [3]. To measure the back exchange, the loaded chloroplasts (10–15 μg) were resuspended in 0.15 ml of medium similar to that used for loading. The reaction was started by the addition of unlabeled nucleotides. Reactions were usually run in the light at 20 °C. After 4 min of exchange, the entire reaction mixture was centrifuged ($960 \times g$ for 30 s) and the supernatant and pellet were checked for radioactivity. For correction of unspecific leakage, a control sample without addition of nucleotides was run simultaneously. The percent exchange was calculated according to Kleineke et al. [13].

Silicon oil centrifugal filtration

For the experiment in Fig. 7, chloroplasts were separated from the incubation mixture by spinning them through a layer of silicon oil, essentially according to the procedure outlined by Heldt et al. [1] but with the following modifications. All work was done with a Beckman Microfuge B, capable of reaching $10\,000 \times g$ well within 5 s. Centrifugations were performed in 0.5 ml polystyrene tubes containing three layers: a bottom layer of 10 μl of 7 % $HClO_4$; a middle layer of 70 μl of General Electric SF-50 silicon oil, and a top layer of 50 μl containing chloroplasts. After a 10 s centrifugation, more than 85 % of the chlorophyll originally applied was found in the bottom layer. Chloroplasts pass intact through the silicon oil layer, as assessed by the nearly complete recovery of the chloroplast enzyme NADP triose phosphate dehydrogenase into a bottom layer of 0.8 M sorbitol (Huber and Edwards, unpublished). After recovering the chloroplasts in the bottom acid layer, samples were neutralized with NaOH and 0.8 M HEPES, pH 7.4, and kept on ice until analyzed for adenylates.

Analysis of adenine nucleotides

The adenylates were analyzed by the luciferin-luciferase method, using an Aminco Chem-Glow photometer, model J4-7441, coupled to a recorder with a 100-mV input. Samples were either recovered from the bottom acid phase if the silicon oil centrifugal filtration technique was used, or alternatively, 50 μl aliquots of the reaction mixture were taken and acidified with 10 μl $HClO_4$ 10 %, followed by neutralization as before. Samples for ATP determination were injected into 0.4 ml of a mixture containing 63 mM Tris · SO_4 (pH 8.0), 3 mM $MgSO_4$ and reconstituted firefly lantern extract (Sigma). After each sample addition, 10 nmol of ATP were added to correct for quenching (10–30 %) due to the perchloric acid and chlorophyll. ADP and AMP were calculated in separate aliquots by difference after conversion to ATP using pyruvate kinase and adenylate kinase, essentially as described by Chapman et al. [14].

RESULTS AND DISCUSSION

Effect of adenylates on CO_2 fixation

Mesophyll protoplast extracts of *D. sanguinalis* fix CO_2 at high rates in the light, without exogenous ATP, when supplied with pyruvate, and the rate is stimulated by the addition of catalytic amounts of oxalacetate (Fig. 1). The dependence of CO_2 fixation on pyruvate is consistent with the role of mesophyll cells in vivo in C_4 plants and has been discussed at length elsewhere [11, 15]. The stimulation of CO_2 fixation by low levels (0.5 mM) of oxalacetate is thought to be due to the extra ATP formed as a result of the induction of noncyclic electron flow as oxalacetate is reduced to malate via NADP-malate dehydrogenase. As shown, CO_2 fixation in the light is increased by the addition of ATP, with the total increase in rate roughly the same for both induction systems. To determine whether ATP was stimulating CO_2 fixation by broken chloroplasts, pyruvate-dependent CO_2 fixation by mesophyll protoplast extracts containing intact or osmotically lyzed chloroplasts was measured. As shown in Table I, intact chloroplasts fix CO_2 in the light in the absence of ATP, and the rate is roughly doubled by the addition of ATP, whereas broken chloroplasts do not fix

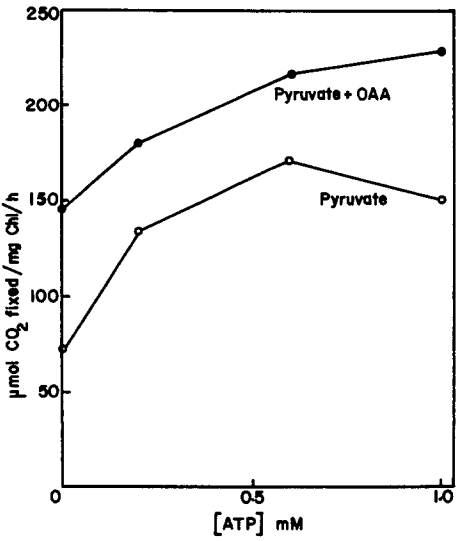

Fig. 1. Effect of ATP on $^{14}CO_2$ fixation by mesophyll protoplast extracts of *D. sanguinalis* induced by pyruvate and pyruvate+oxalacetate (OAA). See Materials and Methods section for details.

appreciable amounts of CO_2 in the absence or presence of ATP. Broken chloroplasts would not be expected to contribute to CO_2 fixation since pyruvate P_i dikinase is irreversibly inactivated when released from the chloroplasts in the absence of a reducing agent [16].

While ATP stimulated CO_2 fixation, ADP and AMP did not significantly affect the rate of CO_2 fixation (Fig. 2). If AMP could penetrate the chloroplast membrane, an inhibition of CO_2 fixation might be expected for several reasons: (1) AMP is a potent inhibitor of pyruvate, P_i dikinase [16], which is required for CO_2 fixation, and (2) if the concentration of AMP were to rise in the chloroplast, adenylate kinase would act to reduce the concentration of ATP in the chloroplast.

TABLE I

EFFECT OF ADDED ATP ON $^{14}CO_2$ FIXATION BY MESOPHYLL PROTOPLAST EXTRACTS CONTAINING INTACT OR BROKEN CHLOROPLASTS

The chloroplast preparations were prepared by separating the chloroplasts from the extrachloroplastic material by centrifugation (600 × *g*, 90 s). One part of the chloroplast pellet was resuspended with osmoticum ("intact") and one part without osmoticum ("broken"). The resuspension medium contained 2 mM $MgCl_2$, 1 mM KH_2PO_4, 50 mM Tricine-KOH (pH 7.8), and for intact chloroplasts, 0.3 M sorbitol as osmoticum. To each preparation, an equal volume of supernatant from the initial centrifugation was added. Other conditions are as described in Materials and Methods section.

Chloroplast	µmol/mg chlorophyll per h	
	Control	2 mM ATP
Intact	32	72
Broken	6	5

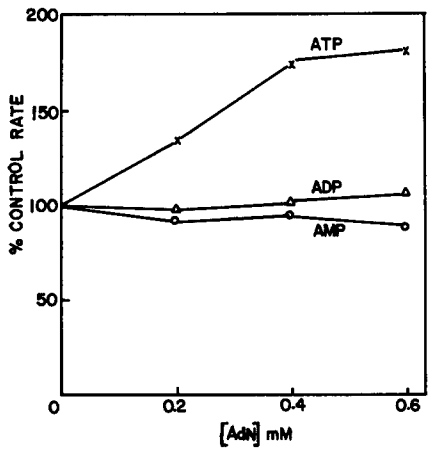

Fig. 2. Effect of adenine nucleotides (AdN) on pyruvate induced $^{14}CO_2$ fixation by mesophyll protoplast extracts of *D. sanguinalis*. The control rate was 65 μmol/mg chlorophyll per h under 21 % O_2. See text for discussion.

Since no effect is seen with either ADP or AMP, it suggests that neither nucleotide is able to penetrate the chloroplast membrane.

Pyruvate-dependent CO_2 fixation is strictly light-dependent. However, fixation at roughly 85 % of the light control rate, after an initial lag, can be induced in the dark by the addition of ATP (Fig. 3). The fact that ATP can induce CO_2 fixation in the dark suggests that the uptake of ATP is not a light-driven process, and that in the dark, there is sufficient activity of pyruvate P_i dikinase to catalyze the ATP dependent CO_2 fixation.

ATP reversal of effect of inhibitors of CO_2 fixation

Light-dependent CO_2 fixation by C_4 mesophyll preparations can be inhibited,

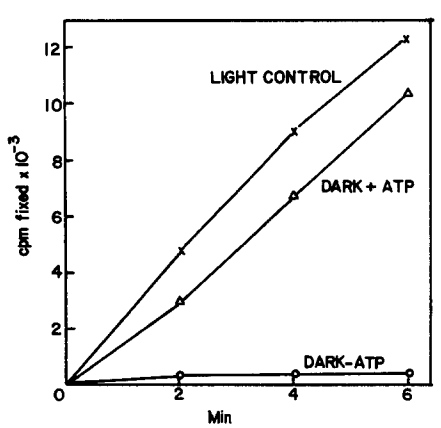

Fig. 3. The effect of ATP on pyruvate-induced $^{14}CO_2$ fixation in the light and dark by mesophyll protoplast extracts of *D. sanguinalis*. The rate of CO_2 fixation in the light control was 72 μmol/mg chlorophyll per h.

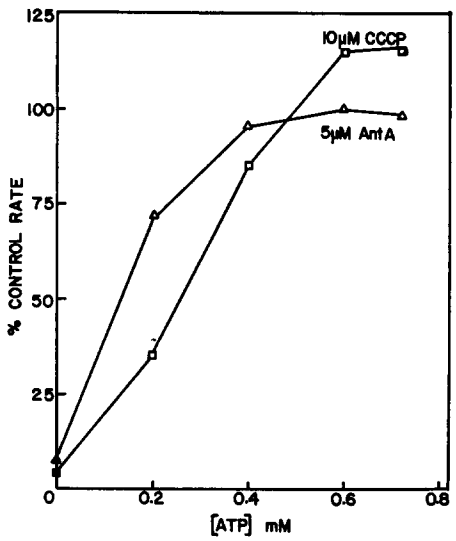

Fig. 4. Effect of added ATP on pyruvate induced $^{14}CO_2$ fixation by mesophyll protoplast extracts inhibited by 10 μM CCCP or 5 μM antimycin A. All assays were performed in sealed ampules under 2 % O_2. The control rate was 54 μmol/mg chlorophyll per h.

to varying degrees, by inhibitors and uncouplers of electron flow. Antimycin A, which blocks cyclic electron flow [17], inhibits pyruvate induced CO_2 fixation under 2 % oxygen and the inhibition can be completely reversed by exogenous ATP (Fig. 4). In similar fashion, the inhibitory effect of the uncoupler CCCP can be reversed by ATP (Fig. 4). Also, the effect of two other electron flow inhibitors, DCMU which blocks noncyclic electron flow [18] and DBMIB which blocks cyclic and noncyclic electron flow [19], can be partially reversed by 0.6 mM ATP (Table II). The reversal by ATP of the inhibition caused by a number of electron flow inhibitors and CCCP

TABLE II

EFFECT OF ADDED ATP ON $^{14}CO_2$ FIXATION IN THE PRESENCE OF THE INHIBITORS DCMU AND DBMIB

CO_2 fixation with DBMIB was induced by pyruvate, and with DCMU, CO_2 fixation was induced by pyruvate+oxalacetate. Rates of CO_2 fixation are given in parentheses as μmol CO_2/mg chlorophyll per h

Inhibitor	Concentration (μM)	Addition	
		None (% control)	+0.6 mM ATP (% control)
DBMIB	0	100 (58)	100 (86)
	1.5	36	68
	3.0	4	49
DCMU	0	100 (150)	100 (189)
	0.8	31	50
	1.2	21	42

indicates that ATP can enter the chloroplast, where it is used in CO_2 fixation. The reversal of the CCCP inhibition is particularly interesting, as Heldt [3] found that the adenine nucleotide translocator in C_3 chloroplasts is sensitive to this uncoupler.

Effect of atractyloside on CO_2 fixation

Atractyloside is a competitive inhibitor of the adenine nucleotide translocator in mammalian mitochondria [20], but not with Jerusalem artichoke mitochondria [21] or spinach chloroplasts [3]. Pyruvate-induced CO_2 fixation by mesophyll chloroplast preparations that is dependent on exogenous ATP (i.e., antimycin A poisoned under low O_2) is inhibited only at very high concentrations of atractyloside (Fig. 5); the inhibition was greater with the lower concentration of ATP. This fits with atractyloside acting as a competitive inhibitor with respect to the extrachloroplastic adenine nucleotide, but the concentration required was much higher than that required in rat liver mitochondria (1–3 μM). Atractyloside also inhibited, although to a less degree, light-dependent CO_2 fixation in the absence of added ATP, suggesting that at least some of the effect was nonspecific. However, this could also reflect an inhibition of phosphoenolpyruvate transport, as occurs in mitochondrial membranes [22].

Effect of adenylates on oxygen evolution

In the presence of pyruvate+oxalacetate, mesophyll protoplast extracts of *D. sanguinalis* evolve oxygen at high rates as the oxalacetate is reduced to malate in the chloroplasts via NADP malate dehydrogenase [5]. If pyruvate is not present, coupled noncyclic electron flow is limited by the turnover of ATP. Exogenous ATP is very inhibitory to oxygen evolution induced by pyruvate+oxalacetate (Fig. 6). At 1.0 mM ATP, the rate of oxygen evolution was reduced almost to the level obtained in the absence of pyruvate, i.e., the uncoupled rate. The inhibition observed

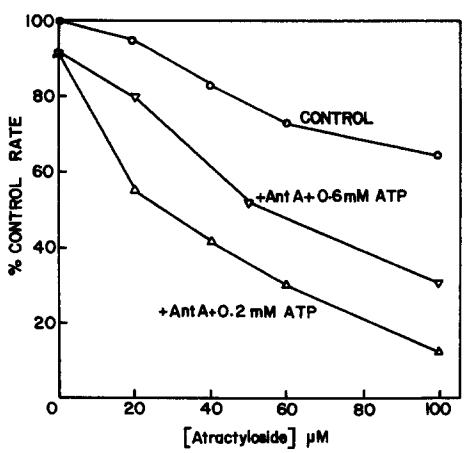

Fig. 5. Effect of atractyloside on $^{14}CO_2$ fixation by mesophyll protoplast extracts of *D. sanguinalis* in the presence of 5 μM antimycin A and either 0.2 mM or 0.6 mM ATP or without antimycin A and ATP. The percent control rate refers to the rate in the absence of antimycin A, ATP and atractyloside, and was equal to 78 μmol CO_2 fixed/mg chlorophyll per h. All assays were performed under 2 % O_2. See text for discussion.

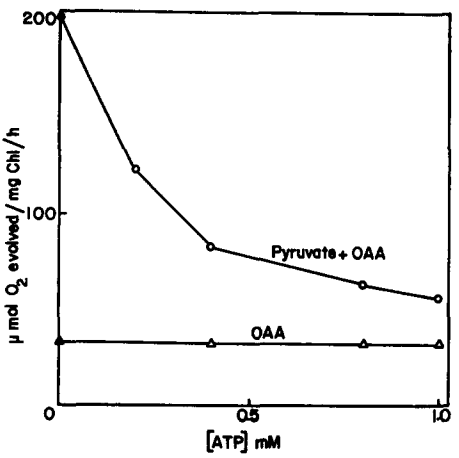

Fig. 6. The effect of ATP on O_2 evolution induced by pyruvate+oxalacetate (OAA) and oxalacetate (minus pyruvate). See text for discussion.

could result from an exchange of exogenous ATP for endogenous ADP, thus shifting the ATP/ADP ratio to one unfavorable for coupled electron flow. This finding further suggests that ATP can enter the chloroplast possibly in exchange for ADP.

Oxygen evolution with oxalacetate, in the absence of pyruvate, should be stimulated by AMP or ADP if they can traverse the chloroplast membrane; ADP would stimulate by directly allowing coupled electron flow to proceed and AMP would stimulate by the action of adenylate kinase, which would form ADP from ATP and AMP, thus allowing coupled electron flow to proceed. No stimulation was observed at up to 2 mM levels of either ADP or AMP (data not shown), which again suggests that neither ADP or AMP can enter the chloroplast.

Back exchange studies

To study another aspect of the adenine nucleotide translocator, chloroplasts were loaded with [^{14}C]ÀTP, and the back exchange of label was determined under various conditions. As shown in Table III, the exchange activity with unlabelled ATP

TABLE III

THE EFFECT OF ILLUMINATION AND VARIOUS INHIBITORS ON THE BACK EXCHANGE OF CHLOROPLASTS LOADED WITH [^{14}C]ATP

The loaded chloroplasts used in this experiment had incorporated 14.8 μmol of [^{14}C]ATP/g chlorophyll. Cold ATP was added to give a final concentration of 1 mM. For details, see Materials and Methods section.

Conditions	Exchange (%)
Light	78
Dark	47
Light+50 μM atractyloside	48
Light+10 μM CCCP	59
Light+ 5 μM antimycin A	75

TABLE IV

BACK EXCHANGE OF CHLOROPLASTS LOADED WITH [^{14}C]ATP WITH VARIOUS EXTRACHLOROPLASTIC ANIONS

The final concentration of all external anions was 1 mM. The figures given are average values of at least two separate experiments. The labeled chloroplasts in these experiments had incorporated 12–18 μmol of [^{14}C]ATP/g chlorophyll.

Anion	Exchange (%)
ATP	77
ADP	9
AMP	14

was considerably higher in the light than in the dark; hence, all exchange studies were performed with illumination. When ATP induces the back exchange, it is unknown whether the label is appearing in the supernatant as ATP or ADP, both of which are possible with mitochondria [12, 22] and chloroplasts, since adenylate kinase in the chloroplast could allow the labeling of all the adenine nucleotides. Antimycin A did not affect the degree of back exchange while CCCP caused a slight inhibition of the exchange. Atractyloside, which inhibits adenine nucleotide translocation in mitochondria, only partially inhibited the back exchange in our system. This is consistent with the work of Heldt [3] with the ATP translocator of C_3 chloroplasts, which suggested that the ATP translocator is not sensitive to 100 μM atractyloside.

The specificity of this translocator was studied by measuring the back exchange induced by different metabolites with chloroplasts loaded with [^{14}C]ATP (Table IV): unlabeled ATP was very effective while ADP and AMP were ineffective in the back exchange. The lack of back exchange by ADP and AMP further suggests that these nucleotides are not translocated into the chloroplast. The specificity observed agrees well with that reported by Heldt [3] for spinach chloroplasts.

The adenine nucleotide translocator of mitochondria also transports phosphoenolpyruvate [22]. Since C_4 photosynthesis requires the rapid transport of phosphoenolpyruvate across chloroplast membranes [11] the possibility that the ATP translocator we are observing serves also as a translocator for phosphoenolpyruvate cannot be dismissed.

Direct measurement of ATP uptake by chloroplasts

The results described above suggest that ATP can readily penetrate the membrane of C_4 mesophyll chloroplasts. The direct uptake of ATP by the chloroplasts was measured by incubating the chloroplasts in the dark with ATP and then separating the chloroplasts from the supernatant by centrifugation through a layer of silicon oil (see Methods). As shown in Fig. 7, a large burst of ATP uptake was observed during the first 5 s that was reduced in the presence of pyruvate. Following the burst, the concentration of ATP in the chloroplast gradually decreased to a lower level. The uptake kinetics at room temperature are too rapid to follow, but a minimal rate can be determined from the amount of ATP taken up after 5 s. On this basis, using incubation conditions similar to those used in CO_2 fixation experiments (1 mM free Mg^{2+}), the rate of ATP uptake was 33 μmol/mg chlorophyll per h (curve A, Fig. 7). When a

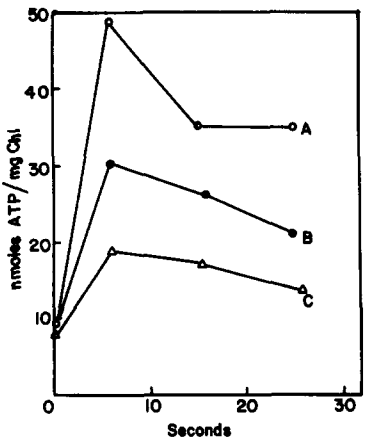

Fig. 7. Kinetics of [^{12}C]ATP uptake in the dark by mesophyll chloroplasts of *D. sanguinalis*. Chloroplasts were incubated with 0.6 mM Mg · ATP in the dark and, at times indicated, were separated from the incubation mixture by Si Oil centrifugal filtration. The chloroplast pellet was then assayed for ATP as described in the Materials and Methods section. Reaction mixtures contained 0.3 M sorbitol, 1 mM $MgCl_2$, 1 mM KH_2PO_4, 50 mM Tricine-KOH (pH 7.8). Curve A, C_4 mesophyll chloroplasts; Curve B, C_4 mesophyll chloroplast plus 2 mM pyruvate; Curve C, spinach mesophyll chloroplasts. Initial uptake rates were 33, 17 and 8.4 μmol ATP/mg chlorophyll per h for curves A, B and C, respectively.

similar experiment was performed in the presence of pyruvate (curve B, Fig. 7), the uptake was considerably lower. Whether pyruvate reduces the apparent uptake by forming ADP via the pyruvate, P_i dikinase reaction (see Introduction) or actually blocks the uptake of ATP needs to be determined. The rate of ATP uptake by spinach chloroplasts (curve C, Fig. 7) was several fold lower than that observed for the C_4 mesophyll chloroplasts, and agrees well with the maximum rates reported by Heldt [3].

The initial burst of uptake by C_4 mesophyll chloroplasts was highly temperature dependent; in one experiment, the rate of uptake at 20 °C was 38 μmol/mg chlorophyll per h and at 4 °C decreased to 6 μmol/mg chlorophyll per h (data not shown). After roughly 30 s of uptake, however, the final concentration of ATP in the chloroplast was the same at the two temperatures. Initial rates of ATP uptake in these experiments, which may be underestimations of the true rates due to saturation at 5 s, are of the same order of magnitude as the rates predicted from the ATP-dependent CO_2 fixation experiments.

When chloroplasts were incubated in the dark with 0.6 mM ADP, no increase in chloroplastic ADP was observed, which again suggests that ADP cannot traverse chloroplast membranes (data not shown). This finding also suggests that the observed uptake of ATP does in fact represent transport into the stroma. If the observed uptake of ATP was due to inclusion of label in the non-specific sucrose permeable space of the chloroplast, for example, then apparent uptake of ADP should also be observed.

Measurement of the ATP requirement in CO_2 fixation

In calculating the rate of ATP transport based on rates of ATP-dependent

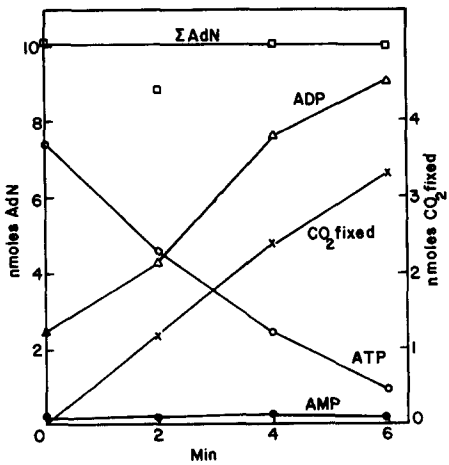

Fig. 8. Simultaneous measurement of $^{14}CO_2$ fixation and changes in adenine nucleotides. The reaction mixture contained 0.6 mM Mg · ATP and 2 mM pyruvate in the dark. The rate of CO_2 fixation was 23 μmol/mg chlorophyll per h. For details of CO_2 fixation and determination of adenylates, see Materials and Methods section.

CO_2 fixation, it has been assumed that 2 ATP are required for each CO_2 fixed (see Introduction). By simultaneously measuring CO_2 fixation and the consumption of exogenous ATP in the dark, it was possible to directly test this. As shown in Fig. 8, ATP was consumed and ADP was produced as CO_2 was fixed. With time, the total amount of adenylates remained constant while the increase in ADP paralleled the decrease in ATP. After 6 min, 6.38 nmol of ATP were consumed and 3.30 nmol of CO_2 were fixed. This yields a ratio of 1.92 ATP per CO_2 fixed. In other experiments, ratios of 2.08, 1.95 and 1.90 were obtained. Hence, the assumption that each CO_2 fixed requires 2 ATP is valid. In another experiment, the chloroplasts were separated from the reaction mixture by silicon oil centrifugal filtration prior to assay of adenylates. It was observed that ADP accumulates outside of the chloroplasts as ATP is utilized inside the chloroplast, suggesting that external ATP exchanged with internal ADP (data not shown). Without added pyruvate, no significant consumption of ATP was observed (data not shown).

Concluding remarks

C_4 mesophyll chloroplasts are capable of utilizing exogenous ATP for CO_2 fixation, both when endogenous ATP is supplied from photochemical electron transport (Figs. 1, 2) and when electron transport is blocked by various inhibitors (Fig. 3, Table II). ADP and AMP are apparently not translocated, as they have no effect on CO_2 fixation and do not stimulate oxygen evolution that is limited by the availability of ADP (oxygen evolution in the presence of oxalacetate without pyruvate). The ATP translocator of C_4 mesophyll chloroplasts is specific for ATP, and can transport ATP at rates ranging from 80–140 μmol/mg chlorophyll per h as suggested by the fact that ATP-dependent CO_2 fixation proceeds at rates ranging from 40–70 μmol/mg chlorophyll per h (Table I, Fig. 4) and 2 ATP are required for each CO_2 fixed (see Introduction and Fig. 8). Direct measurements of ATP uptake by the chloroplasts (Fig. 7)

showed saturation within 5 s at 20 °C and do not allow a determination of maximum uptake rates (minimum rate calculated at roughly 35 μmol ATP/mg chlorophyll per h by this method). In contrast to the ATP translocator defined by Heldt [3] for spinach chloroplasts, the translocator of C_4 mesophyll chloroplasts is relatively insensitive to CCCP and has a high transport velocity. Exogenous ATP is apparently exchanging for endogenous ADP, since ADP was observed to accumulate outside of the chloroplasts during ATP-dependent CO_2 fixation in the dark.

The physiological significance of this high activity ATP translocator in C_4 mesophyll chloroplasts is not known, although it could clearly operate to deliver ATP, synthesized by glycolysis or respiration, to the chloroplasts to drive some dark metabolism or biosynthesis. Alternatively, the observed transport of ATP may be a ramification of a system designed primarily to transport phosphoenolpyruvate across chloroplast membranes at high rates, which is apparently required for the operation of the C_4 pathway.

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