Biochimica et Biophysica Acta 810 (1985) 340–345
Elsevier

BBA 41893

Adenine nucleotide translocase-dependent anion transport in pea chloroplasts

Gebretateos Woldegiorgis [a], Steve Voss [a], Earl Shrago [a,b,*], Margaret Werner-Washburne [c] and Kenneth Keegstra [c]

Departments of [a] Medicine, [b] Nutritional Sciences and [c] Botany, University of Wisconsin, 1415 Linden Drive, Madison, WI 53706 (U.S.A.)

(Received July 31st, 1985)

Key words: Nucleotide transport; Adenine nucleotide translocase; Phospho*enol*pyruvate; Anion transport; (Pea chloroplast)

Pea chloroplasts were found to take up actively ATP and ADP and exchange the external nucleotides for internal ones. Using carrier-free [^{14}C]ATP, the rate of nucleotide transport in chloroplasts prepared from 12–14-day-old plants was calculated to be 330 μmol ATP/g chlorophyll/min, and the transport was not affected by light or temperature between 4 and 22°C. Adenine nucleotide uptake was inhibited only slightly by carboxyatractylate, whereas bongkrekic acid was nearly as effective an inhibitor of the translocator in pea chloroplasts as it was in mammalian mitochondria. There was no counter-transport of adenine nucleotides with substrates carried on the phosphate translocator including inorganic phosphate, 3-phosphoglycerate and dihydroxyacetone phosphate. However, internal or external phospho*enol*pyruvate, normally considered to be transported on the phosphate carrier in chloroplasts, was able to exchange readily with adenine nucleotides. Furthermore, inorganic pyrophosphate which is not transported by the phosphate carrier initiated efflux of phospho*enol*pyruvate as well as ATP from the chloroplast. These findings illustrate some interesting similarities as well as differences between the various plant phosphate and nucleotide transport systems which may relate to their role in photosynthesis.

Introduction

The inner membrane of the chloroplast envelope is impermeable to anions except by carrier-mediated transport, thus acting as a barrier between the chloroplast stroma and the cytoplasm of the plant cell [1,2]. Specific anion carriers in the chloroplast membrane have been identified. Of all the transport systems found so far in chloroplasts, the phosphate translocator has the highest activity, and it is apparent that it represents a major protein constituent of the inner envelope membrane [1,2]. An adenine nucleotide translocate in plants was first demonstrated in spinach chloroplasts [3], but its relatively low activity seemed to preclude any important physiological function. Furthermore, although the main purpose of the phosphate translocator is to export fixed carbon from the stroma to the cytoplasm, this shuttle could indirectly also account for the transfer of ATP and ADP. A more active adenine nucleotide exchange has been demonstrated in young pea leaf chloroplasts [4,5], and in mesophyll chloroplasts of *Digitaria sanguinales* the activity was reported to be almost equal to that of the phosphate translocator [6].

The observations indicating that pyrophosphate [5] and phospho*enol*pyruvate [7] can exchange with adenine nucleotides suggests some possible interesting ramifications of the ADP/ATP carrier, since

* To whom correspondence should be addressed.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P_i, inorganic phosphate.

both these metabolites in plants function in important photosynthetic reactions. Using the forward and back exchange reactions, we have, therefore, carried out an extensive study of the properties of the ADP/ATP carrier in pea chloroplasts. In addition to delineating anion specificity, we found differential effects of the inhibitors carboxyatractylate and bongkrekic acid on the translocator.

Experimental procedures

Pea seedlings (*Pisum sativum* var Laxton's Progress 9) were grown in vermiculite in a growth chamber at 18–20°C for 12–14 days. Maize (Illinois Xtra Sweet Hybrid, 85 day variety) was grown in vermiculite at ambient light in the greenhouse for 2–3 weeks.

Isolation of pea chloroplasts

Pea shoots were homogenized with a polytron blender at 4°C in an isolation buffer containing 330 mM sorbitol/50 mM Hepes (pH 7.5)/0.1% bovine serum albumin. The homogenate was then filtered through layers of cheese-cloth containing cotton according to the method of Cline et al. [8]. The chloroplasts were recovered by centrifugation at 2500 × *g* for 2 min, and the pellet resuspended in the isolation media. Intact chloroplasts were then obtained by centrifugation of the suspension on preformed percoll gradients for 30 min at 1000 × *g*. Chloroplasts forming a band near the botton of the gradient were recovered and centrifuged at 2000 × *g* for 7 min, and the pellet washed twice with 300 mM sorbitol/50 mM Hepes (pH 7.5). The final chloroplast pellet was suspended in the media at a concentration of 1 mg chlorophyll/ml.

Exchange assays

Forward exchange

[^{14}C]ADP or [^{14}C]ATP transport and binding studies in pea chloroplasts were carried out by the forward exchange assay of Pfaff and Klingenberg [9] as used in this laboratory [10]. Pea chloroplasts (20–30 μg chlorophyll) were suspended in 0.4 ml of the incubation media (0.33 M sorbitol/50 mM Hepes (pH 7.6)/1 mM $MgCl_2$/2 mM EDTA) and preincubated for 30 s under the conditions specified in the tables and figures. 10–15 μl of [^{14}C]ADP or [^{14}C]ATP was then added and the chloroplasts incubated for 20 s. Exchange was terminated by rapid centrifugation of a 300 μl aliquot of the chloroplast suspension through a layer of silicone oil into 20 μl of 1.0 M $HClO_4$ in a Beckman Model 152 microfuge. A 200 μl aliquot of the supernatant was transferred into vials for radioactive counting, and the bottom of the tube containing the denatured pellet was cut, transferred into a capped 1.5 ml microfuge tube and dissolved in 0.3 ml Soluene 100. The radioactivity in the pellet and supernate was then determined in a Tri-carb liquid scintillation spectrometer using Bray's solution [11].

Back exchange

Pea chloroplasts suspended in the incubation media (330 mM sorbitol/50 mM Hepes (pH 7.6)/1 mM $MgCl_2$/2 mM EDTA) at a concentration of 1 mg/ml chlorophyll were preloaded with [^{14}C]ATP (20 μCi; 54.2 mCi/nmol) or [^{14}C]phospho*enol*-pyruvate (19.6 mCi/nmol) at 0°C for 1 h. Labeled chloroplasts were then separated by centrifugation at 2000 × *g* for 7 min, washed twice and the pellet resuspended in the above incubation media.

[^{14}C]ATP or [^{14}C]phospho*enol*pyruvate loaded pea chloroplasts containing between 5000–10000 dpm were suspended in 0.4 ml of the incubation media under conditions specified in the tables. The back exchange was initiated by addition of the anions tested, and after 4 min the reaction was terminated by rapid centrifugation into a layer of silicone oil containing 20 μl of 1.0 M $HClO_4$ using a Beckman model 152 microfuge. The radioactivity in the pellet and supernatant was determined with a Tri-carb scintillation spectrometer using Bray's solution [11]. The percent exchange was calculated according to the method of Kelineke et al. [12].

Chlorophyll was assayed by the method of Arnon [13], chloroplast integrity determined by the ferricyanide exclusion test [14], and the internal space of the chloroplast measured as sorbitol impermeable space with [^{14}C]sorbitol and [^{3}H]H_2O [15]. Uptake of radioactive ATP, ADP or phospho*enol*pyruvate was corrected for the amount of label carried through the silicone layer and by nonspecific permeation of the intermembrane

space by adding 10 μl of labeled compounds to the chloroplast suspension as outlined by Heldt [15].

[^{14}C]ATP and [^{14}C]ADP were purchased from New England Nuclear and [^{14}C]phospho*enol*-pyruvate from Amersham. Bongkrekic acid was a gift from Professor H. Berends, Technical University of Delft, Delft, The Netherlands. All other reagents were of the highest grade commercially available.

Results

Chloroplasts prepared from peas grown for 12–14 days and incubated with [^{14}C]ATP took up the radioactive nucleotide at a rate of 330 μmol/min per g chlorophyll, whereas, chloroplasts prepared from 21-day-old peas showed approx. one-half the uptake of [^{14}C]ATP as those from 12–14-day-old peas. A lower activity of 34 μmol/min per g chlorophyll observed in spinach [3] and a higher activity of 600 μmol/min per g chlorophyll calculated for digitaria [6] chloroplasts are referred to for comparative purposes.

To obtain more definitive data of a carrier-mediated exchange process, the chloroplasts were preloaded with [^{14}C]ATP and allowed to exchange with cold nucleotides. It can be seen from the results in Table I that loss of radioactivity from the pellet occurred with addition of cold nucleotide. There was essentially no egress in the absence of external nucleotide (results not shown). Under the experimental conditions used, varying either the temperature or light-dark conditions did not seem to influence the exchange of internal for external nucleotides. However, the relatively long incubation time may have prevented any temperature effect. The relative specificity of the translocator is shown by the negligible transport of inorganic phosphate on this carrier. In contrast to the findings reported for spinach chloroplasts [3], however, ADP was noted to exchange quite actively on the pea chloroplast ATP/ADP carrier.

The poor inhibition of adenine nucleotide uptake by atractylate, generally observed in chloroplasts [3,4] could be interpreted to indicate that this inhibitor-insensitive transport was due to simple diffusion with a relatively minor amount of carrier-specific mediated transport. To obtain a more precise characterization of the adenine nucleotide translocase in pea chloroplasts, kinetic experiments were carried out to determine if the sites on the carrier could be saturated. Fig. 1 presents the results of such an experiment. The curve represents the difference between the uptake of carrier free [^{14}C]ADP and that of [^{14}C]ADP diluted with cold nucleotide. The results are consistent with the saturation process suggesting a high-affinity carrier-mediated transport of ADP.

Although there are reports documenting the generally poor response of the plant chloroplast and mitochondrial adenine nucleotide translocators to inhibition by carboxyatractylate [16,17], there have been no corresponding studies carried out on the sensitivity of the chloroplast ADP/ATP carrier to bongkrekic acid. The results shown in Fig. 2 indicate a much greater effect of bongkrekic acid than carboxyatractylate on carrier-mediated transport in pea chloroplasts, particularly in the concentration range found to be effective for mammalian mitochondria. These results with bongkrekic acid were, however, similar to those reported by Passam and Coleman [17] for Jerusalem-artichoke mitochondria.

Using a fixed time interval of 4 min we have observed that pea chloroplasts were able to counter transport ATP and ADP with phospho*enol*-pyruvate [7], an activity previously described in mammalian mitochondria [18]. In the present ex-

TABLE I

EFFECT OF LIGHT AND TEMPERATURE ON THE ADENINE NUCLEOTIDE TRANSLOCASE OF PEA CHLOROPLASTS

[^{14}C]ATP loaded pea chloroplasts (26.5 μg chlorophyll) were suspended in 0.4 ml of the incubation media and preincubated for 30 s under ambient light. Back exchange was initiated by addition of the anions (1.0 mM) tested and incubated for 4 min.

Additions	[^{14}C]ATP preloaded chloroplasts (% exchange)			
	dark		light	
	22°C	4°C	22°C	4°C
ATP	77.7	75.4	78.8	75.3
ADP	–	–	84.5	–
Inorganic phosphate	5.1	4.1	0	16.8

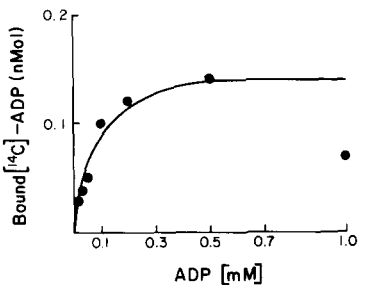

Fig. 1. Concentration-dependent uptake of [^{14}C]ADP by pea chloroplast adenine nucleotide translocase. Pea chloroplasts (30.2 μg chlorophyll) were suspended in 0.4 ml incubation media at room temperature under ambient light and preincubated for 30 s. Different concentrations of [^{14}C]ADP were then added and the chloroplasts were incubated for 20 s. Further exchange was stopped by rapid centrifugation and the radioactivity in the pellet determined.

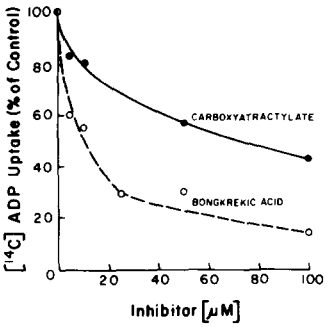

Fig. 2. Effect of carboxyatractylate and bongkrekic acid on pea chloroplasts adenine nucleotide translocase. Pea chloroplasts (40 μg chlorophyll) were suspended in 0.4 ml incubation media at room temperature under ambient light and preincubated for 2 min with or without carboxyatractylate and/or bongkrekic acid. Nucleotide exchange was initiated by addition of 10 μl [^{14}C]ADP (42.6 mCi/nmol) and the mixture was incubated for an additional 2 min. Exchange was terminated by rapid centrifugation of a 300 μl aliquot of the chloroplast suspension through silicone oil and the radioactivity in the pellet determined

periments, this counter transport system was further investigated by comparing the time-course of the efflux of preloaded [^{14}C]ATP from chloroplasts produced by external cold ATP and phospho*enol*pyruvate. The results in Fig. 3 indicate that both ATP and phospho*enol*pyruvate initiated a rapid exchange with adenine nucleotides in the chloroplast matrix. It also appears from the data that the [^{14}C]ATP-phospho*enol*pyruvate counter exchange in pea chloroplasts is almost equal to that of [^{14}C]ATP-ATP transport and therefore more active than that reported in liver mitochondria [18].

Anion transport dependent upon the ADP/ATP carrier was compared with that normally attributed to the phosphate translocator, which is considered primarily responsible for phospho*enol*-pyruvate transport in plant chloroplasts [1–3]. The results in Table II clearly show that external ATP and ADP readily exchange with internal phospho*enol*pyruvate as well as the internal nucleotides, and external phospho*enol*pyruvate exchanges with both internal phospho*enol*pyruvate and adenine nucleotides. The poor exchange of inorganic phosphate with adenine nucleotides is evidence for the specificity of the adenine nucleotide-phospho*enol*pyruvate counter transport system. This concept is further supported by the poor exchange of 3-phosphoglycerate and dihydroxyacetone phosphate, common substrates for the phosphate carrier, with adenine nucleotides. Externally added pyrophosphate, however, does exchange with internal ATP, ADP and phospho*enol*pyruvate. Robinson and Wiskich [5] previously reported that pyrophosphate caused the efflux of ATP from young pea chloroplasts, and they considered the possibility that this transport might play a role in regulating adenine nucleotide levels in the chloroplast and cytoplasm. Since py-

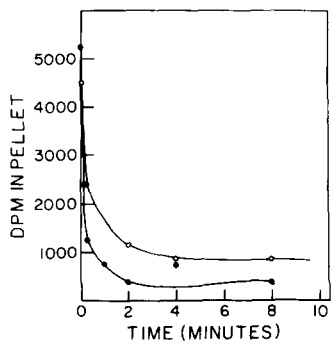

Fig. 3. Time-course of egress of [^{14}C]ATP from preloaded chloroplasts by external ATP and phospho*enol*pyruvate. [^{14}C]ATP preloaded chloroplasts (25.2 μg chlorophyll) were suspended in 0.2 ml of incubation media and preincubated for 30 s at ambient light and room temperature. Back exchange was initiated by addition of 1.0 mM ATP or phospho*enol*-pyruvate for the time indicated and further exchange terminated by centrifugation and radioactivity determined. ATP (●———●); phospho*enol*pyruvate (○———○).

TABLE II

INTERRELATIONSHIP OF ANION TRANSPORT DEPENDENT ON THE ADENINE NUCLEOTIDE AND PHOSPHATE CARRIERS

[^{14}C]ATP- and [^{14}C]phospho*enol*pypruvate-loaded chloroplasts (23–25 μg chlorophyll) were suspended in 0.2 ml of incubation media and preincubated for 30 s. Back exchange was initiated by addition of the anion (1.0 mM) tested and incubated for 4 min at room temperature under ambient light.

Additions	Preloaded chloroplasts (% exchange)	
	[^{14}C]ATP	[^{14}C]phospho*enol*-pyruvate
ATP	86	67
ADP	73	78
Phospho*enol*pyruvate	58	79
Inorganic pyrophosphate	67	71
Inorganic phosphate	17	90
3-Phosphoglycerate	26	71
Dihydroxyacetone phosphate	22	76

rophosphate was found to be poorly transported on the phosphate carrier [19], its exchange with phospho*enol*pyruvate observed in this study is likely to be via the adenine nucleotide translocase.

Discussion

There has been considerable speculation as to the role of the adenine nucleotide translocase in plant metabolism. It is apparent from the literature [3,5,6] and this study that the activity of the carrier varies with different plant species. In spinach chloroplasts where adenine nucleotide transport was found to be relatively low it was suggested [3] that the main function of the carrier was to deliver ATP synthesized by glycolysis or respiration in the cytoplasm to the chloroplast during the dark phase. Robinson and Wiskich [5] subsequently noted that the activity of the ATP/ADP carrier was greater in pea than in spinach chloroplasts, and they proposed that it played a role in ADP inhibition of CO_2 fixation [20]. They also observed that pyrophosphate induced ATP egress from the chloroplast and accounted for the potential role of pyrophosphate by either depleting chloroplasts of ATP or competing for entry of ADP [5]. Based on the present study, a functional exchange between pyrophosphate and phospho*enol*pyruvate should also be considered.

An adenine nucleotide-phospho*enol*pyruvate counter exchange has recently been demonstrated in chloroplasts of both C_3 and C_4 plants [7]. Furthermore, in the present study it was shown that the excahnge is fairly rapid, with phospho*enol*pyruvate able to deplete the chloroplast [^{14}C]ATP by 80% within at least two minutes. A role for the adenine nucleotide phospho*enol*pyruvate counter exchange could be envisaged in mesophyll chloroplasts of C_4 plants as an aid in the transport of phospho*enol*pyruvate from the chloroplast where it is synthesized by pyruvate P_i dikinase to the cytoplasm where it is carboxylated by phospho*enol*pyruvate carboxylase. However, the fact that phospho*enol*pyruvate is a good substrate for the phosphate translocator in C_4 mesophyll chloroplasts [21] somewhat mitigates against the essentiality of an adenine nucleotide-phospho*enol*pyruvate counter exchange. In this regard, it may, therefore, be of interest to make some comparisons in the properties of the adenine nucleotide and phosphate carriers in different plants. In spinach chloroplasts with a low activity ATP/ADP carrier, phospho*enol*pyruvate transport on the phosphate carrier is also slow and there is a high K_m for phospho*enol*pyruvate [1,19]. By contrast, in C_4 chloroplasts with a high activity ADP/ATP carrier [6] the transport of phospho*enol*pyruvate on the phosphate carrier is more rapid and the K_m for phospho*enol*pyruvate lower [21]. There may, therefore, be some functional relationship between the phosphate and adenine nucleotide transporter in plant chloroplasts. A similar suggestion has been proposed for an interaction between the adenine nucleotide translocase and tricarboxylate carrier in mammalian mitochondria [18].

Phospho*enol*pyruvate [18] and pyrophosphate [22,23] adenine nucleotide counter transport in mammalian mitochondria have been implicated in mitochondrial calcium cycling [22,24]. The fact that the phospho*enol*pyruvate and pyrophosphate exchange with adenine nucleotides could be inhibited by carboxyatractylate and bongkrekic acid was considered evidence that their transport was dependent upon the adenine nucleotide translocase.

In mammalian mitochondria the inhibition of the ADP/ATP carrier by carboxyatractylate and bongkrekic acid is asymmetric, the former inhibiting on the cytosolic side and the latter from the matrix side of the inner mitochondrial membrane [25,26]. In the present study with plant chloroplasts the experiments do not distinguish whether bongkrekic acid is inhibiting from the external or internal side of the chloroplast membrane, nor is it even known whether the orientation of the ADP/ATP carrier in mammalian mitochondria and plant chloroplasts are similar. It is, however, now apparent that the ATP/ADP carrier from chloroplasts is not as previously considered, resistant to the classical inhibitors of adenine nucleotide transport, but responds to the inhibitors in a somewhat different manner than does the translocator in mammalian mitochondria. This may be due to intrinsic properties of the plant ATP/ADP carrier itself or to the lipid environment of the envelope membrane which, in mammalian mitochondria, is considered to play a role in modulating the kinetics of adenine nucleotide transport [24,27].

In the present study additional properties and kinetics of the ADP/ATP carrier from pea chloroplasts have been defined, but it will be necessary to obtain more quantitative data on phospho*enol*pyruvate and pyrophosphate transport before a definite function can be assigned to this carrier. It may, however, be relevant to consider a physiological role for an adenine nucleotidephospho*enol*pyruvate and pyrophosphate counter exchange in vivo because of the potential importance of these metabolites to the activity of the photosynthetic enzymes pyruvate P_i dikinase, [28] phospho*enol*pyruvate carboxylase [29] and the recently identified pyrophoshate dependent phosphofructokinase [30].

Acknowledgement

This work was supported by USDA Competitive Grant 82-CRCR-1-1031 and USPHS NIH Grant GM-14033.

References

1 Fliege, R., Flügge, U.I., Werdan, K. and Heldt, H.W. (1978) Biochim. Biophys. Acta 502, 232–247
2 Heber, U. and Heldt, H.W. (1981) Annu. Rev. Plant Physiol. 32, 139–168
3 Heldt, H.W. (1969) FEBS Lett. 5, 11–14
4 Robinson, S.P. and Wiskich, J.T. (1976) Plant Physiol. 58, 156–162
5 Robinson, S.P. and Wiskich, J.T. (1977) Plant Physiol. 59, 422–427
6 Huber, S.C. and Edwards, G.E. (1976) Biochim. Biophys. Acta 410, 675–687
7 Woldegiorgis, G., Voss, S., Shrago, E., Werner-Washburne, M. and Keegstra, K. (1983) Biochoem. Biophys. Res. Commun. 116, 945–951
8 Cline, K., Andrews, J., Mersey, B., Newcomb, E.H. and Keegstra, K. (1981) Proc. Natl. Acad. Sci. USA 78, 3595–3599
9 Pfaff, E. and Klingenberg, M. (1968) Eur. J. Biochem. 6, 66–79
10 Shrago, E., Shug, A., Elson, C., Spennetta, T. and Crosby, C. (1974) J. Biol. Chem. 249, 5269–5274
11 Bray, G.A. (1960) Anal. Biochem. 1, 279–285
12 Kleineke, J., Sauer, H. and Soling, H.D. (1973) FEBS Lett. 29, 82–86
13 Arnon, D.I. (1949) Plant Physiol. 24, 1–5
14 Lilley, R.McC., Fitzgerald, M.P., Rients, K.G. and Walker, D.A. (1975) New Phytol. 75, 1–10
15 Heldt, H.W. (1980) Methods Enzymol. 69, 604–613
16 Vignais, P.V., Douce, R., Lauquin, G.J.M. and Vignais, P.M. (1976) Biochim. Biophys. Acta 440, 688–696
17 Passam, H.C. and Coleman, J.O.D. (1975) J. Exp. Bot. 26, 536–543
18 Shug, A.L. and Shrago, E. (1973) Biochem. Biophys. Res. Commun. 53, 659–665
19 Heldt, Heldt, H.W. and Ropley, L. (1970) FEBS Lett. 10, 143–148
20 Robinson, S.P. and Wiskich, J.T. (1977) Arch. Biochem. Biophys. 181, 546–554
21 Huber, S.C. and Edwards, G.E. (1977) Biochim. Biophys. Acta 462, 603–612
22 Vercesi, A. and Lehninger, A.L. (1984) Biochem. Biophys. Res. Commun. 118, 147–153
23 Krämer, R. (1985) Biochem. Biophys. Res. Commun. 127, 129–135
24 Sul, H.S., Shrago, E., Goldfarb, S. and Rose, F. (1979) Biochim. Biophys. Acta 551, 148–156
25 Chua, B.H. and Shrago, E. (1977) J. Biol. Chem. 252, 6711–6714
26 Lauquin, G.J.M., Villiers, C., Michejda, J.W., Hryniewiecka, L.V. and Vignais, P.V. (1977) Biochim. Biophys. Acta 460, 331–345
27 Woldegiorgis, G. and Shrago, E. (1985) J. Biol. Chem. 260, 7585–7590
28 Burnell, J.N. and Hatch, M.D. (1984) Biochem. Biophys. Res. Commun. 118, 65–72
29 Hatch, M.D. and Slack, C.R. (1968) Biochem. J. 106, 141–146
30 Carnal, N.W. and Black, C.C. (1979) Biochem. Biophys. Res. Commun. 86, 20–26