ADENINE NUCLEOTIDE TRANSLOCATION IN SPINACH CHLOROPLASTS

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1. Introduction

It was recently reported from our laboratory that intact chloroplasts isolated from acetabularia mediterranea contained endogenous adenine nucleotides, which were exchanged specifically with external adenine nucleotides [1,2]. In analogy to the well established adenine nucleotide exchange in mitochondria [3], this reaction has been called adenine nucleotide (AdN) translocation.

For extensive investigations of this exchange reaction, the chloroplasts from acetabularia are not a favourable object, since they have a very low metabolic rate and the algae are difficult to grow. For these reasons, chloroplasts from spinach have been employed in our present investigations to characterize further the AdN translocation of chloroplasts and to evaluate its role in the cell metabolism of the plant.

2. Materials and methods

2.1. *Chloroplasts*

Spinach, grown by a local gardener, was harvested the evening before the experiment. The preparation of chloroplasts was carried out according to Cockburn et al. [4]. From phase contrast microscopy, about 70–80% of the chloroplasts appeared to have retained their outer envelope. Enzymatic assay of adenine nucleotides in these chloroplast preparations yielded 35–50 μmoles AMP + ADP + ATP per g chlorophyll. The CO_2 fixation rate was 1300 μmoles per g chlorophyll per min (20°).

2.2. *Exchange measurement*

The measurement of the exchange is carried out by back exchange [3]. First the AdN pool of the isolated chloroplasts (2 mg chlorophyll per ml) is labelled by incubation for 60 min at 0° with ^{14}C-ATP (100 μM, specific activity 10 μC/μmole) in a medium containing 0.33 M sorbitol, 20 mM N-tris (hydroxy-methyl) methylglycine (TRICINE) pH 8.4, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 2 mM EDTA. The labelled chloroplasts are washed twice afterwards. The amount of adenine nucleotides present in the chloroplasts is not altered by this treatment. The extent of labelling by exchange of the endogenous AdN with ^{14}C-ATP is checked by measuring the specific activity of the AdN in the supernatant of the preincubation and in the washed chloroplasts. Usually 70–80% of the endogenous AdN are found to have been exchanged with the label, the unexchanged portion consisting mainly of ADP, as shown by ion exchange chromatography. The labelled chloroplasts (6 μg chlorophyll per ml) are suspended in a medium as described above, containing either 20 mM TRICINE or 20 mM N-2-hydroxyethylpiperazine N'-2-ethane sulphonic acid (HEPES) and equilibrated for 6 min in a thermostated waterbath at 20°. The sample is illuminated by 2 focussed lamps (150 W each, distance 8 cm). The back exchange is started by addition of unlabelled nucleotides to the suspension. Samples of 0.5 ml each are removed and immediately centrifuged for 5 sec (Eppendorf centrifuge, 18000 rpm). Because of the very rapid acceleration of the centrifuge, the time of starting the centrifugation is taken as sampling time. Using 4 centrifuges, it is possible to cover the whole

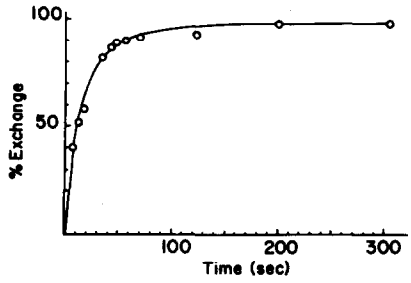

Fig. 1. Exchange of endogenous adenine nucleotides in spinach chloroplasts with ATP. For conditions see text.

exchange kinetic by one experiment. The exchange is calculated from the appearance of the ^{14}C-label in the supernatant, measured by liquid scintillation counting. For correction of unspecific leakage, a control sample without addition of nucleotides is run simultaneously.

2.3. *Evaluation of translocation activity*

Fig. 1 shows the time course of the exchange with ATP. It resembles the exchange kinetics observed with mitochondria. The activity of the AdN translocation is calculated in analogy to the mitochondrial AdN translocation [3]. It is assumed that there is a simple exchange of a pool, which we may call the active AdN pool, following a first order reaction. As in mitochondria, this pool may be smaller than the total amount of the ^{14}C-labelled endogenous AdN.

From the halftime of the exchange of this pool ($T\frac{1}{2}$ = 8 sec), the first order reaction constant (k) is obtained ($k = \ln 2/T\frac{1}{2}$). Multiplication of k with the size of the active AdN pool (A) yields the activity of translocation (v_T):

$$v_T = kA .$$

The value for A results from multiplication of the amount of AdN assayed enzymatically (35.7 μmoles per g of chlorophyll)) by correction factors for the extent of exchange during prelabelling (f_1 = 0.70), for unspecific leakage of adenine nucleotides (f_2 = 0.59) and for the size of the rapidly exchangeable pool (f_3 = 0.98). Thus the active pool in the experiment of fig. 1 amounts to $A = AdN \cdot f_1 \cdot f_2 \cdot f_3 = 35.7 \cdot 0.70 \cdot 0.59 \cdot 0.98 = 14.4$ μmoles per g chlorophyll.

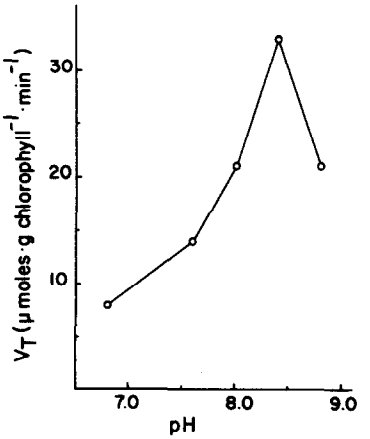

Fig. 2. pH dependence of the adenine nucleotide translocation in spinach chloroplasts with ATP. For conditions see text.

From these data a translocation activity v_T = 75 μmoles per g chlorophyll per min is calculated. In the same way, the translocation activities shown in fig. 2 and table 1 have been obtained. In these experiments lower activities have been found, probably due to different weather conditions during the growth of the spinach.

Although there has not been an exact evaluation of the AdN translocation in acetabularia [1,2], the activity of the AdN translocation in spinach chloroplasts appears to be considerably higher than in acetabularia chloroplasts.

2.4. *Properties of adenine nucleotide translocation*

There is a distinct peak for the activity of the AdN translocation at pH 8.4, as shown in fig. 2. For this reason, all our exchange measurements have been performed at this pH. The specificity of the translocation was studied as summarized in table 1. The reaction appears to be very specific for ATP, with ADP the activity is decreased to 12% and with AMP or nucleoside-triphosphates other than ATP to almost nothing. The difference in specificity for ADP and ATP was not markedly lowered by increasing the concentrations of added nucleotides to 0.8 mM. Thus we find in our experiments a much higher specificity for the ATP translocation than previously reported for chloroplasts from acetabularia [1,2].

Atractyloside, a strong inhibitor of mitochondrial AdN translocation [5], had no effect on the AdN

Table 1
Adenine nucleotide translocation in spinach chloroplasts. Nucleotide specificity and effect of m-chlorocarbonylcanide-phenyl-hydrazone (CCP).

Nucleotide (250 μM)	Activity of translocation (20°) (μmoles g chlorophyll^{-1} min^{-1})	
	No addition	+ CCP (100 μM)
ATP	34	6.5
ADP	4.1	$<$0.5
AMP	1.1	$<$0.2
CTP	$<$ 0.5	
UTP	$<$ 0.2	
GTP	$<$ 0.2	
ITP	$<$ 0.2	

translocation in chloroplasts (concentration used: 10^{-4} M). Chlorocarbonylcyanide-phenyl-hydrazone (CCP), an uncoupler of photophosphorylation, strongly inhibits the translocation of ATP, ADP and AMP, as shown in the second column of table 1. With 100 μM CCP, the inhibition of ATP translocation is more than 80%. In a similar experiment, 10 mM NH_4Cl yielded only 37% inhibition. In order to estimate the uncoupling effect of these substances in our experiments, the ATP/ADP ratio in the illuminated chloroplasts has been measured. Whereas by addition of 100 μM CCP the ATP/ADP ratio was decreased from 1.3 to 0.2, 10 mM NH_4Cl decreased this ratio from 1.5 to 0.6 only. These results indicate that there is some parallelism between uncoupler function and inhibition of AdN translocation. These findings may be explained in different ways: (a) The translocation requires energy in form of a high energy intermediate or a potential across the membrane, being dissipated by uncoupler. (b) The translocation requires internal ATP, which is hydrolysed by uncoupler. (c) NH_4Cl and CCP cause damage to the membrane in which the translocation is located.

Further investigations will be necessary to decide between these possibilities.

3. Discussion

Chloroplasts with intact envelope have the ability to fixate CO_2 without addition of nucleotides [6,7,8]. This finding implies that endogenous ADP acts as acceptor for the photophosphorylation reaction, the endogenous ATP thus formed supplying the energy for CO_2 fixation. It has been shown that the endogenous AdN is retained by the intact chloroplasts; furthermore, a specific exchange with external AdN was demonstrated. Assuming that there is only one site for photophosphorylation, utilizing only endogenous ADP, the photophosphorylation of external ADP would either involve a transport of ADP into the chloroplasts, which, according to the present data, would have to be mediated by the AdN translocation, or a transphosphorylation reaction, possibly facilitated by a shuttle of transport metabolites, e.g. dihydroxyacetone phosphate and phosphoglyceric acid [9].

The question whether a photophosphorylation of cytosolic ADP occurs in the cell has not yet been fully decided (for review see [9,10]). From the low activity of the ADP translocation in our experiments, it can be definitely concluded that the AdN translocation does not participate in the photophosphorylation of cytoplasmic ADP. The high specificity for external ATP suggests that the AdN translocation may act in the opposite direction, transporting ATP from the cytoplasm into the chloroplasts. The values for the activity of hexokinase located in the chloroplasts, as measured in intact chloroplasts using external ATP [11], are in agreement with our data. We may consider that the activity values measured in our experiments may be lower than the translocation activities *in vivo*, due to partial damage of the chloroplasts. Thus a fall in the level of cytosolic ATP, as observed with whole leaves during light-dark transitions [12,13] could be accounted for by a transport of cytoplasmic ATP into the chloroplasts, mediated by AdN translocation.

It may be concluded from our data that the main role of the AdN translocation in chloroplasts is to deliver ATP, synthesized by glycolysis or respiration, to the chloroplasts in order to secure some metabolism in the chloroplasts during the night phase.

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