UNSPECIFIC PERMEATION AND SPECIFIC UPTAKE OF SUBSTANCES IN SPINACH CHLOROPLASTS

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1. Introduction

Chloroplasts may be visualized as autonomous cell structures having a metabolism which is separated from that in the cytosol. By translocation of substances across the chloroplast membranes, these metabolic compartments are connected with each other.

Recently it was reported from our laboratory that chloroplasts have an adenine nucleotide translocation [1 – 3]. Our present investigation deals with the possible localisation of this translocation reaction in the chloroplast; furthermore the translocation of substrates, e.g. phosphoglycerate and malate, is investigated.

2. Materials and methods

2.1. *Chloroplasts*

Spinach, obtained from a local gardener, was usually harvested a few hours before the experiment. For preparation of chloroplasts see [4]. From phase contrast microscopy 70–90% of the chloroplasts appeared to have retained their outer membrane. CO_2-dependent oxygen evolution measured was 45–110 μmoles O_2 per mg chlorophyll per hour.

2.2. *Conditions of incubation*

The incubations were carried out at 4° in a medium containing 0.33 M sorbitol, 20 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethane sulphonic acid (HEPES) pH 7.6, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 2 mM EDTA, and chloroplasts equivalent to 0.1 mg chlorophyll per ml. In the experiments in table 1 and fig. 1, the time of incubation was 2 min. The hypotonic medium in fig. 1 contained half the concentration of the constitutents listed above, the concentration of the chloroplasts being unaltered. For disruption by osmotic shock, the chloroplasts were suspended for 30 sec in distilled water. An equal volume of double concentrated medium was added afterwards.

2.3. *Radioactive-labelled substances used*

The specific activities employed are given in brackets; the concentrations employed in the experiment are listed behind:

U^{14}C-3-Phosphoglyceric acid (Boehringer, 50 μCi/mmole), 6 mM.

U^{14}C-Fructose-1,6-diphosphate (Boehringer, 50 μCi/mmole) 6 mM.

U^{14}C-D-Glucose-6-phosphate (Boehringer, 50 μCi/mmole) 6 mM.

U^{14}C-Sucrose (Boehringer, 100 μCi/mmole) 6 mM.

U^{14}C-Malate (Amersham, 50 μCi/mmole) 6 mM.

8^{14}C-Guanosinediphosphate (Amersham, 600 μCi/mmole) 0.3 mM.

^3H_2O (Amersham, 3 μCi/ml).

Carboxyl^{14}C-dextrane (New England, 1.3 μCi/mg) 2 mg/ml.

The radioactivity measurements were carried out by liquid scintillation counting.

Table 1
Ion uptake into the sucrose-impermeable space of spinach chloroplasts.

	Intact chloroplasts (mM)	Osmotically shocked chloroplasts (mM)
PGA (6 mM)	13.7	1.5
Malate (6 mM)	13.0	0.5

For conditions see text.

3. Results and discussion

3.1. *Permeation of chloroplasts by water*

The permeability measurements were carried out by centrifugation filtration [5]. With this method the chloroplasts are separated from a medium containing ^3H_2O and ^{14}C-dextran by centrifugation through a layer of silicon oil into perchloric acid. The total amount of water which has been carried by the chloroplasts when passing through the silicon layer is determined from the ^3H activity in the perchloric acid layer. It must be taken into account, however, that part of this water is not actually within the chloroplast, but adheres to the outer surface during passage through the silicon layer. The adherent water can be corrected for by using ^{14}C-dextran, which does not permeate intact membranes, on the incubation medium. This correction has been used in all the following experiments. Fig. 1 shows the space in chloroplasts permeable to water under different conditions. In a hypotonic medium (0.16 M sorbitol) the water-permeable space is larger than in an isotonic medium (0.33 M sorbitol), indicating swelling of the chloroplast. It may be noted that with 0.16 M sorbitol there was no disruption of the chloroplasts observed with phase contrast microscopy. Disruption of the chloroplasts by osmotic shock renders part of the chloroplast space permeable to dextran, therefore the dextran-impermeable H_2O space decrease.

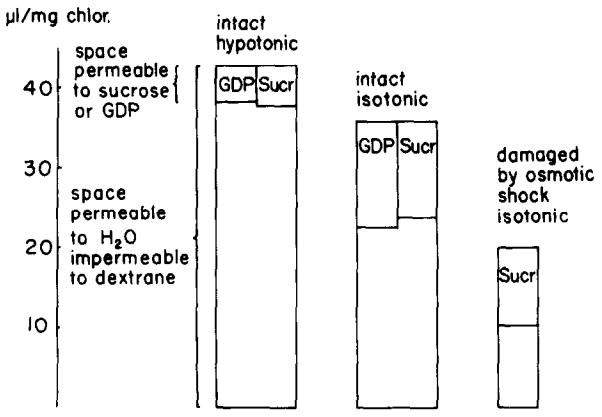

Fig. 1. Permeability of spinach chloroplasts. For conditions see text.

3.2. *Permeation of chloroplasts by sucrose and GDP*

Sucrose was selected as a molecule which does not freely permeate the osmotically active membrane of the chloroplasts, since it is widely used as an osmotic agent to maintain the tonicity of the medium for chloroplasts. GDP was chosen as a nucleotide which is not transported by the adenine nucleotide translocation [3]. As shown in fig. 1., part of the water-permeable space in chloroplasts is also permeated by sucrose and GDP. In experiments not shown here, the amount of permeated sucrose found in the chloroplasts was proportional to the sucrose concentration in the medium. These data indicate that in analogy to the mitochondria [6] part of the chloroplast space is accessible to free permeation by sucrose.

It may be feasible, however, that the sucrose-permeable space observed in our experiments is due to broken chloroplasts in the preparation. If this were so, a complete disruption of the chloroplasts as obtained by osmotic shock should enlarge the sucrose-permeable space. This is not the case, as shown in fig. 1. By osmotic shock the sucrose-permeable space is not increased, but its size remains constant. A decrease in the sucrose-permeable space is observed when the chloroplasts are kept in a hyptonic medium. Obviously, swelling of the chloroplasts results in a compression of this space.

Electronmicroscopic studies, which are presently being carried out in our laboratory, are required to correlate our experimental findings with the morphology of the chloroplast. However, it is already possible to conclude that of the two membranes surrounding the chloroplast, the outer membrane is permeable to sucrose and nucleotides. Therefore, specific translocation reactions (e.g. trans-

location of adenine nucleotides [3], PGA and malate (see below)) would have to be located behind this outer membrane. In this respect the chloroplasts seem to resemble the mitochondria, where the outer membrane was found to be freely permeable to molecules such as nucleotides and sucrose.

3.3. *Uptake of substrates into the sucrose-impermeable space of chloroplasts*

3-Phosphoglyceric acid (PGA) is an intermediate of the CO_2 fixation cycle located in the chloroplast and also of the glycolytic chain, which is situated in the cytostol. Kinetic studies on the distribution of PGA between the chloroplasts and the cytostol of leaf cells presented evidence for a transfer of PGA across the chloroplast membranes [7]. A transfer of PGA was also suggested from effects of PGA on CO_2 fixation and light induced oxygen uptake [4, 8] and from the accumulation of ^{14}C-PGA in the medium during photosynthesis [9] as observed with intact isolated chloroplasts. From similar studies (for references see [10]) it was concluded that hexose-monophosphates do not permeate the chloroplast, whereas there were uncertain data on a possible transfer of fructose-1,6-diphosphate (FDP).

These conclusions on the transfer of PGA, FDP and hexose-monophosphates have so far all been drawn from indirect evidence. Therefore, in order to obtain clear results, it was necessary to measure the transfer of these substances directly. The transfer of malate was included in our studies, because there are considerable activities of malate dehydrogenase found in the chloroplast and also in the cytostol [11, 12]. In analogy to the mitochondria, a transfer of malate had to be taken into consideration.

Fig. 2. shows the time course of the uptake of ^{14}C-labelled 3-phosphoglyceric acid (PGA), glucose-6-phosphate (G6P) and fructose-1,6-diphosphate (FDP) into the sucrose-impermeable space. These experiments were carried out by a modified method of centrifugation filtration using the Coleman centrifuge with 0.5 ml plastic tubes [13]. This method allows rapid mixing and centrifugation within a few seconds. The uptake of substrates into the sucrose-permeable space was corrected for by using ^{14}C-sucrose in control experiments. Whereas G6P and FDP are taken up rather slowly, there is an extremely rapid uptake of PGA. In other experiments, not shown here, malate

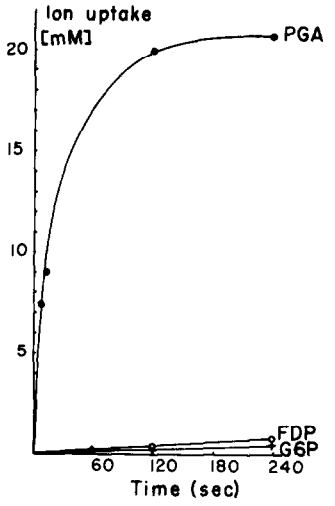

Fig. 2. Ion uptake into the sucrose-impermeable space of spinach chloroplasts. For conditions see text.

was taken up in a similar way. In comparison, the rate of citrate and acetate was less than 10% of that of PGA. In our experiments the rate of PGA uptake was calculated to be about 100 μmoles/mg of chlorophyll/hour at 4°. This high rate of PGA uptake is in line with recent results of Heber and Santarius (in preparation) who calculated, from PGA dependent oxygen evolution, PGA uptake rates as high as 300 μmoles/mg chlorophyll/hour (20°).

In the experiment shown in fig. 2, a concentration of 6 mM PGA in the medium yields a concentration of 20 mM in the sucrose-impermeable space, as calculated from ^{14}C activity measurements. Since these experiments were carried out at 4° in the dark, it is very unlikely that a major part of ^{14}C activity found in the chloroplasts is due to products being formed from PGA. Our results indicate therefore that PGA is being concentrated in the chloroplast. The same holds for malate, as shown in the experiment in table 1. The capability to take up PGA or malate is lost, if the chloroplasts are damaged by osmotic shock; this is also shown in table 1.

It appears from these data that chloroplasts contain specific mechanisms for the transport of PGA or malate. From its very high rate, the transport of PGA seems to be a very efficient connection between the pathways of glycolysis and CO_2 fixation. The transfer of FDP and G6P is surely negligible in this

respect. The transport of malate which has been observed in our experiments, may have an important function for the transfer of reducing equivalents across the chloroplast membranes.

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