Vol. 143, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 27, 1987 Pages 164-169

# IDENTIFICATION OF THE PHOSPHATE-TRANSLOCATOR FROM MAIZE MESOPHYLL CHLOROPLASTS

Andrew G. Thompson, Michael A. Brailsford, and R. Brian Beechey

Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Aberystwyth, Dyfed, Wales SY30 2DD

Received December 9, 1986

**SUMMARY:** Intact maize mesophyll chloroplasts have been isolated in yields of up to 10mg of chlorophyll per preparation. The chloroplasts were able to reduce 3-phosphoglycerate at a rate of 2.4μmol of oxygen/min/mg of chlorophyll. This activity was inhibited by preincubating the intact chloroplasts with pyridoxal 5-phosphate. Chloroplast envelopes have been prepared and the protein profile has been obtained on SDS-polyacrylamide gels. The phosphate-translocator from the chloroplast envelope has been identified as a 30kDa polypeptide. 

The phosphate-translocator from maize mesophyll chloroplasts (C4) is an important transport protein which is believed to facilitate the exchange of inorganic phosphate, 3-phosphoglycerate, dihydroxyacetone phosphate and phosphoenol pyruvate across the chloroplast envelope membrane (1). Maize mesophyll chloroplast envelopes have not been purified previously, nor have any transport proteins been identified in chloroplasts from C4 plants. Here we report the purification of chloroplast envelopes and the identification of the phosphate translocator from maize mesophyll chloroplasts.

## MATERIALS AND METHODS

Unless otherwise stated chemicals were obtained from BDH, Poole, Dorset, U.K. Catalase (Bovine liver) was obtained from Sigma, Poole, Dorset, U.K. Miracloth was from Calbiochem U.K. and Percoll was from Pharmacia U.K. [$^3$H]-sodium borohydride was from New England Nuclear, Stevenage, Herts, U.K.

**Abbreviations:** HEPES, N-2-Hydroxyethylpiperazine-N-2-ethane-sulfonic acid; EDTA, ethylene-diamine-tetra-acetic acid; DHAP, Dihydroxyacetone phosphate; 3-PGA, 3-phosphoglyceric acid; Pi, inorganic phosphate; PEP, phospho(enol)pyruvate.

0006-291X/87 $1.50

## The Preparation of Intact Maize Mesophyll Chloroplasts

Intact maize mesophyll chloroplasts were purified by a procedure based on that of Jenkins and Russ (2). Maize leaves (200g) were harvested from three week old plants which had been grown from seed (var. Brutus, Nickerson RPB, Boston, Lincs. U.K.) in a glasshouse maintained at 27°C. Natural light was supplemented for 16h each day with 400 watt metal halide lighting. The leaves were chopped into 0.5cm pieces and 50g batches were added to 600ml of medium A which had been frozen to an icy slush. Medium A comprised 0.35M sorbitol, 25mM HEPES, 1mM $MgCl_2$, 5mM EDTA, 0.2% (w/v) BSA, pH 7.8 with KOH. Isoascorbate (2mM) and DTT (5mM) were added immediately prior to use. All procedures after this point were carried out on ice or at 4°C. The leaf pieces were homogenised in a 7.5x7.5x30cm Perspex container for 1s at full speed with a Polytron 45/2M. The homogenate was filtered through Miracloth and centrifuged at 3500g max for 5s (4500rpm, Sorvall 6X250 rotor). The pellets were resuspended combined into 2x50ml tubes and centrifuged at 2000g max for 1min (3500rpm, Sorvall 4X50 swing out). Each pellet was resuspended in 10ml of buffer B (0.3M sorbitol, 25mM HEPES, 5mM EDTA, pH 8.0 with KOH) and each 10ml aliquot was layered onto 20ml of Percoll [32% (v/v) Percoll, made up as buffer B with 0.1% (w/v) BSA]. The layers were centrifuged at 2000g max for 3.5min (3500rpm, Sorvall 4X50 swing out). The intact maize mesophyll chloroplasts pelleted at the bottom of the tube whilst the lysed chloroplasts remained at the Percoll/medium interface.

## The Inhibition of 3-Phosphoglycerate Reduction by Pyridoxal 5-Phosphate.

Intact maize mesophyll chloroplasts (30μg of chlorophyll, in 0.5ml of buffer B) were incubated in the dark and on ice with 1mM pyridoxal 5- phosphate for varying times. The chloroplasts were then pelleted (MSE microfuge, high speed 5s) washed by resuspension in 0.5ml of buffer B, pelleted and resuspended in assay medium. The rate of 3-PGA dependent oxygen evolution was assayed in a total volume of 1ml of buffer B containing 1mM 3-PGA and 0.5mM phosphate in an oxygen electrode (Hansatech, Cambridge U.K.). Illumination was provided by a slide projector and was 800 e/s at the surface of the cuvette.

## Labelling of the Phosphate Translocator Protein with Pyridoxal 5-Phosphate and [$^3$H]-Sodium Tetraborohydride.

The labelling procedure was based on that used for the identification of the C3 phosphate translocator (3). Intact maize mesophyll chloroplasts (10mg of chlorophyll) were suspended in 5ml of buffer B containing 1mM pyridoxal 5-phosphate. The suspension was incubated on ice for 30min and [$^3$H]-sodium borohydride (sp. act. 360Ci/mol) was added to 3.2mM. After a further 10min, the chloroplasts were pelleted by centrifugation at 2000g for 2min (3500rpm, 4X50ml Sorvall swing out rotor). The chloroplasts were washed by resuspension in 20ml of buffer B, re-pelleted and used for the preparation of envelope membranes.

## The Preparation of Maize Chloroplast Envelope Membranes.

Chloroplast envelopes were purified using a procedure based on that of Douce (4). The intact chloroplasts (10mg of chlorophyll) were resuspended in 2ml of buffer B and diluted to 0.5mg of

chlorophyll/ml with buffer C (50mM HEPES, 2mM $MgCl_2$, 1mM bemzamidine, 1mM phenylmethyl-sulfonyl fluoride, pH 7.5 with KOH). After a 2min incubation on ice the lysed chloroplasts were layered onto a step sucrose gradient composed 31.8% (w/v) sucrose (20ml) and 20.5% (w/v) sucrose (20ml) in buffer C. The gradient was centrifuged at 70,000g max (20,000rpm, Sorvall 3X65ml swing out rotor) for 1h. Thylakoid membranes pelleted at the bottom of the tube and envelope membrane collected at the interface of the sucrose density layers. The envelopes were removed with a syringe. They were diluted with 50ml of buffer C and pelleted by centrifugation for 1h at 70,000g max. (20,000rpm, Sorvall 3X65ml swing out). The pellet was resuspended in a minimum volume of buffer and frozen at -80°C.

Protein Determination and SDS-Polyacrylamide Gel Electrophoresis.

Protein was determined by the modified Lowry procedure as described by Kaplan and Pedersen (5). Bovine serum albumin was used as a standard.
SDS-polyacrylamide gel electrophoresis was performed in slab gels according to Laemmli (6). The running gel consisted of a 10-16% linear gradient of acrylamide. For labelling studies gels were sliced and each slice was placed in a glass scintilation vial. The gel was digested with NCS tissue solubliser (Amersham U.K.) at 40°C for 2h before the addition of scintilant and radio-counting. Quench correction was by internal standard.

## RESULTS AND DISCUSSION

Characterisation of the chloroplasts

The procedure described purified intact maize mesophyll chloroplasts in yields of up to 10mg of chlorophyll per preparation. The chloroplasts were routinely more than 85% intact by the ferricyanide exclusion test (7). They did not exhibit bicarbonate dependent oxygen evolution which suggests that they were free from contamination by bundle sheath chloroplasts.
In the light and in the presence of 0.5mM phosphate and 1mM 3-PGA the rate of oxygen evolution was 2.4μmol/mg of chlorophyll.

The inhibition of 3-PGA dependent oxygen evolution by pyridoxal 5-phosphate.

The uptake of 3-PGA, its reduction to DHAP with concomitant production of oxygen, and the export of DHAP involves three envelope membrane dependent processes. These are the import of 3-PGA and inorganic phosphate, and the export of DHAP. These processes are thought to be carried out by a single protein, the

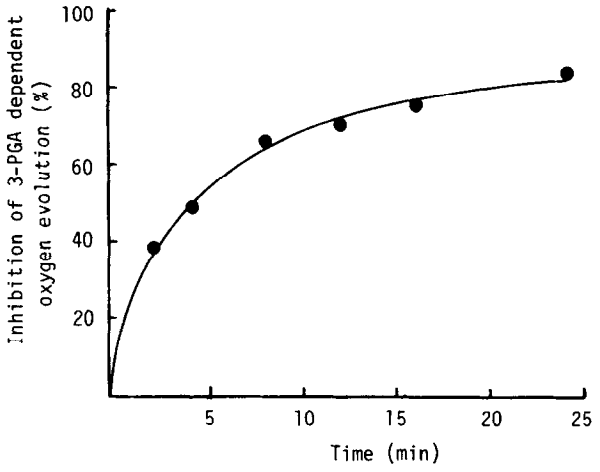


**Fig. 1. The inhibition of 3-PGA dependent oxygen evolution by pyrydoxal 5-phosphate.** Intact chloroplasts were preincubated with 1mM pyridoxal 5-phosphate and 3-PGA dependent oxygen evolution was assayed as in the methods.

phosphate translocator (1,8). The inhibition of 3-PGA dependent oxygen evolution by non-permeant molecules therefore provides an assay for inhibitors of phosphate-translocator activity.

Pyridoxal 5-phosphate is known to react with lysine residues to form a Schiffs base. The base can be reduced by borohydride to form a stable adduct. The phosphate-translocator from C3 plants has been shown to be inhibited by pyridoxal 5-phosphate and the translocator was identified after reduction with [$^3$H]-sodium borohydride (3).

When intact maize mesophyll chloroplasts were incubated with the non-permeant probe pyridoxal 5-phosphate, the activity of the phosphate-translocator was reduced (Fig.1). The inhibition of phosphate-translocator activity was dependent on incubation time and inhibitor concentration. With 1mM pyridoxal 5-phosphate, maximal inhibition (80%) was achieved after a 25min incubation (Fig.1).

Radiolabelling of the chloroplast envelopes.

Intact chloroplasts were radiolabelled by incubation with pyridoxal 5-phosphate and reduction of the resulting Schiffs

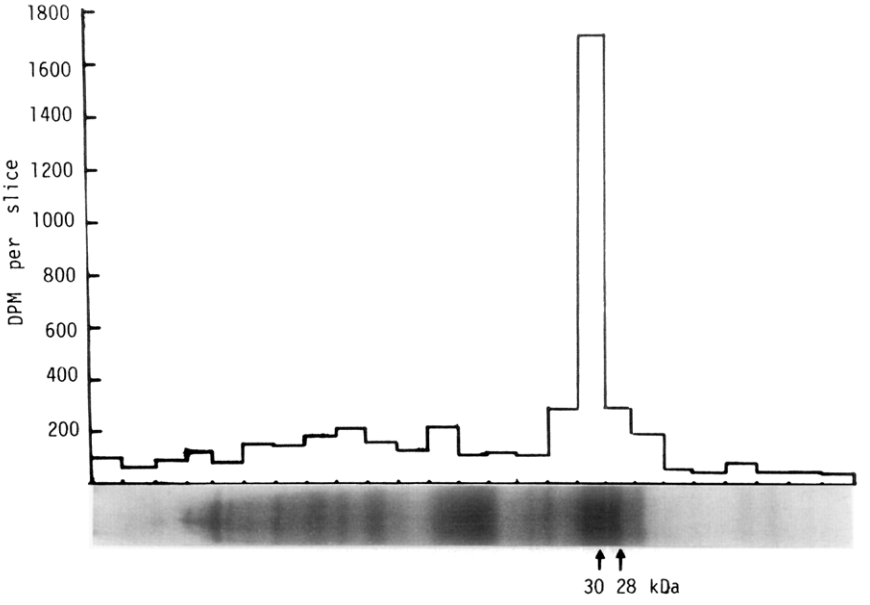


**Fig. 2. The labelling of a 30kDa envelope membrane polypeptide by [$^3$H]-sodium borohydride.** Chloroplasts were prepared and labelled as in the methods. Envelope membranes (200µg) were run on a 10-16% gradient SDS-polyacrylamide gel and stained with coomassie blue. Radiolabelled envelopes (8µg) were run on the same gel and the distribution of radiactivity was determined.

base with [$^3$H]-sodium borohydride. The chloroplasts were lysed and after centrifugation on the stepped sucrose gradient chloroplast envelopes were visible as a yellow band at the interface of the sucrose layers. After collection dilution and centrifugation, the envelopes formed a loose yellow pellet. The yield of chloroplast envelope membranes was 22µg of protein/mg of chlorophyll. The coomassie stained protein profile of envelope membranes on 10-16% gradient SDS-polyacrylamide gels is shown in Fig.2. The same protein profile was obtained from both non-labelled and radiolabelled envelope membranes after gels had been silver stained.

To determine the distribution of radiolabel in the envelopes radiolabelled and non-radiolabelled envelope membranes were run on the same gel. The gel was destained, sliced, digested in NCS tissue solubliser (Amersham U.K.) and radiocounted. The profile

revealed a single major peak in a band which corresponded to a polypeptide of 30kDa (Fig.2). The neighbouring band at 28kDa contained only a low level of radioactivity. These data suggest that the maize mesophyll chloroplast phosphate translocator is a protein with one or more 30kDa monomers. The labelling pattern is similar to that described for envelopes from C3 plants, where the phosphate translocator was identified as a 29kDa polypeptide (3). The C3 chloroplast phosphate-translocator transports Pi, triose phosphate and 3-PGA whereas the maize mesophyll chloroplast phosphate-translocator is postulated to transport Pi, triose phosphate, 3-PGA and PEP. Changes in substrate specificity may have occured between C3 and C4 chloroplast phosphate-translocators. These changes would represent a major factor in the evolution of C4 plants. Identification of the phosphate-translocator protein is a first step in the elucidation of the nature and mechanism of transport of phosphorylated compounds in the C4 mesophyll chloroplast.

## REFERENCES

1) Day, D.A. & Hatch, M.D. (1981) Arch. Biochem. Biophys. 211, 743-749
2) Jenkins, C.L.D. & Russ, V.J. (1984) Plant Sci. Lett. 35, 19-24
3) Flugge, U.I. & Heldt, H.W. (1977) FEBS Lett.82, 29-33
4) Douce, R. & Joyard, J. (1980) in Methods in Enzymol. 69 (Colowick, S.P. & Caplan, N.O., eds.) p.290, Academic Press, N.Y.
5) Kaplan, R.S. & Pederson, P.L. (1985) J. Biol. Chem. 260, 10293
6) Laemmli, U.K. (1970) Nature 227, 680-685
7) Lilley, R.Mc.C., Fitzgerald, M.P., Rients, K.G. & Walker, D.A. (1985) New Phytol. 75, 1
8) Fliege, R., Flugge, U.I., Werdan, K. & Heldt, H.W. (1978) Biochim. Biophys. Acta. 502 232-247