A specific translocator for oxaloacetate transport in chloroplasts

M.D. Hatch[+], L. Dröscher, U.I. Flügge and H.W. Heldt*

Institut für Biochemie der Pflanze, Universität Göttingen, D-3400 Göttingen, FRG

Received 1 October 1984

Evidence is provided that oxaloacetate is taken up by chloroplasts from maize and spinach leaves by a high-affinity carrier. Apparent K_m values for oxaloacetate are about 45 and 9 μM, respectively. Uptake was measured by following [4-^{14}C]oxaloacetate reduction in illuminated chloroplasts. With maize chloroplasts the rates of uptake were sufficient to account for photosynthesis. This carrier had a very low affinity for malate and 2-oxoglutarate. Ratios of the K_i for malate or 2-oxoglutarate to the K_m for oxaloacetate were about 150 and 50, respectively.

Oxaloacetate transport *Chloroplast envelope* *Malate dehydrogenase* *Photosynthesis* *Spinach* *Maize*

1. INTRODUCTION

Chloroplasts isolated from spinach [1], pea [2] and the mesophyll cells of maize and other C_4 plants [3,4] photoreduce oxaloacetate via NADP malate dehydrogenase located in the chloroplast stroma. In C_3 plants this process may be part of a mechanism for transferring reducing equivalents across the chloroplast envelope [5]. However, in NADP-malic enzyme-type C_4 plants such as maize the reduction of oxaloacetate is a critical step in the path of carbon assimilation [3]. Both these processes imply a shuttle of oxaloacetate and malate across the chloroplast envelope and for C_4 plants this transport must match the rate of photosynthesis. In spinach chloroplasts a dicarboxylate exchange carrier was identified which transports compounds such as malate, succinate and fumarate [6]. These substances exchange for each other and also compete for transport in the same direction; for a particular dicarboxylate the K_m for transport is similar to the K_i for inhibition of uptake of other compounds. Notably, the K_i for oxaloacetate inhibition of malate transport in spinach chloroplasts was about 0.3 mM, similar to the K_m for malate uptake (0.4 mM) [7]. Similar results and kinetic constants were obtained for maize mesophyll chloroplasts [8]. This system appears unsuitable for catalysing oxaloacetate uptake in a situation where the malate concentration may be orders of magnitude higher. This would be the case if these metabolites were in equilibrium for the malate dehydrogenase reaction (K_{eq} 3×10^{-5}, [9]). Notably, measurements of the relative sizes of dicarboxylate pools in several C_4 species, based on early labelling from $^{14}CO_2$ indicate that oxaloacetate was between 1 and 10% of the combined concentration of the malate plus aspartate pools involved in photosynthesis [10]. Our aim was to examine possible alternative mechanisms for oxaloacetate transport in maize mesophyll chloroplasts that would be sufficient to account for observed rates of photosynthesis. Evidence is provided for a very active OA carrier which had a high affinity for OA but was very little affected by high concentrations of malate.

2. MATERIALS AND METHODS

Maize (*Zea mays* var. Dekalb 805A or Mutin) was grown in a glasshouse (24°C day, 15°C night)

[+] Permanent address: Division of Plant Industry, CSIRO GPO Box 1600, Canberra A.C.T. 2601, Australia

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.

with supplementary lighting to give a 14 h light period. Chloroplasts were prepared from the last fully expanded leaves taken 2–4 weeks after germination, according to [11]. Spinach (*Spinacia oleracea*, var. Atlanta) was grown in a hydroponic culture [12] and chloroplast prepared as in [13].

For measuring the conversion of [4-^{14}C]oxaloacetate to products, chloroplasts (10 μg chl/ml) were suspended in a medium containing 0.3 M sorbitol, 25 mM Hepes-K$^+$ (pH 7.8), 1 mM EDTA and further additions as indicated. Samples of 200 μl of suspended chloroplasts in 400 μl polypropylene tubes were preilluminated at 20°C for 2 min in white light (tungsten halogen lamp) of about 100 W/m^2. The reaction was started by the addition of 10 μl of [4-^{14}C]oxaloacetate and terminated after 30 s by the addition of 20 μl 20% perchloric acid. From this an aliquot of 100 μl was transferred to a scintillation counting vial with 200 μl of a mixture containing 0.12 M KH_2PO_4 and 0.12 M $CuSO_4$ and the vial heated for 10 min at 80°C. This procedure completely degrades oxaloacetate yielding the C-4 carboxyl as CO_2 (see [14]). The $^{14}CO_2$ produced was removed and the residual radioactivity (representing products formed from [4-^{14}C]OA) was counted as in [14]. The results were corrected for the ^{14}C remaining after heating a blank reaction containing [4-^{14}C]oxaloacetate (usually about 1% of the total radioactivity of the untreated sample).

The procedures for preparing, storing, and determining the specific activity of [4-^{14}C]OA [14], and for the silicone oil filtering centrifugation of chloroplasts [15] were as described.

3. RESULTS AND DISCUSSION

3.1. *Direct measurement of [4-^{14}C]OA uptake*

Experiments were conducted to directly follow the uptake of [4-^{14}C]OA into chloroplasts by the silicone oil centrifugation technique (see section 2). The results indicated a very rapid phase of uptake which was too fast to resolve kinetics even at 0°C (not shown). OA appeared to accumulate with respect to the concentration of supplied substrate but subsequent analysis showed most of the radioactivity in the chloroplasts was associated with products formed from [4-^{14}C]OA (see section 2 for details). When chloroplasts were illuminated, essentially all the radioactivity was in products, presumably malate formed by OA reduction. In the dark, transamination from endogenous glutamate to give aspartate was probably the major reaction. After correcting for radioactivity in products, the steady-state level [4-^{14}C]OA in chloroplasts in the dark was generally similar to the external concentration of OA. Notably, the very low internal steady-state concentration of OA in the light would be consistent with the rate of metabolism of OA being limited by its rate of entry into chloroplasts. Because OA transport into chloroplasts was very rapid, and because OA was not accumulated and was transformed to products, the silicone oil centrifugation method was not a useful procedure for studying this process. In the following sections we describe alternative procedures.

3.2. *Oxaloacetate-dependent O_2 evolution*

Some information about OA transport was obtained by measuring OA-dependent O_2 evolution at limiting OA concentration with NH_4Cl to uncouple electron transport. With maize chloroplasts the apparent K_m for OA was about 70 μM and, in the experiment shown, the V_{max} expressed as the rate of OA reduction was about 250 μmol·mg^{-1} Chl·h^{-1} (fig.1); generally rates varied between 30 and 50% of those obtained with chloroplasts from

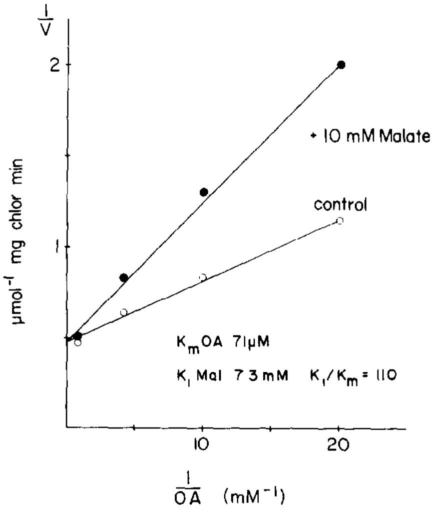

Fig.1. Effect of varying OA on OA-dependent O_2 evolution and inhibition by malate. All reactions contained 2.5 mM NH_4Cl. For other details see section 2.

maize plants grown in higher light intensities in Australia. Malate inhibited OA-dependent O_2 evolution but the K_i for malate was more than 100-times higher than the K_m for OA (fig.1). Similar results were obtained with spinach chloroplasts (K_m OAA, 60 μM; K_i malate, 8 mM) but the V_{max} was much lower. Notably, authors in [2] found a similar K_m for OA using pea chloroplasts. These studies clearly demonstrate that OA may be rapidly transported into chloroplasts and that this process is only substantially inhibited by very high concentrations of malate. This transport is clearly not being mediated by the dicarboxylate exchange carrier demonstrated earlier [6,8]. This polarographic procedure presents several limitations to the more detailed study of OA transport into chloroplasts. An alternative procedure, described below, was therefore adopted.

3.3. *Measurement of OA transport by product formation*

Mesophyll chloroplasts from maize and related C_4 species contain particularly high levels of NADP malate dehydrogenase (600–1200 μmol · mg^{-1} chl · h^{-1}) and somewhat less aspartate aminotransferase [3,11]. We set out to develop a system for assaying OA transport into chloroplasts by measuring either reduction of OA to malate in the light via NADP malate dehydrogenase or conversion to aspartate via aspartate aminotransferase. When reduction of added [4-^{14}C]OA was followed in illuminated chloroplasts the initial rate of conversion was readily measured over a 30 s period at 20°C even with very low concentrations of OA (fig.2). When OA was varied in the absence of NH_4Cl, half saturation was observed at about 50 μM (fig.3). In a series of 14 similar experiments the apparent K_m ranged between 30 and 70 μM. Malate and 2-oxoglutarate competitively inhibited OA transport but the high K_i values indicated that the OA carrier system had a low affinity for these compounds (fig.3). At least with limiting OA the addition of NH_4Cl either had no effect or significantly reduced the rate of OA reduction.

In another experiment the kinetic characteristics of light-dependent OA reduction in intact maize chloroplasts were compared with the kinetics for NADP-dependent [4-^{14}C]OA reduction by malate dehydrogenase released from the same chloroplasts and measured under essentially identical conditions (table 1). The V_{max} for released NADP malate dehydrogenase was more than 4-times the maximum rate of OA reduction in intact chloroplasts and the K_m for OA was about half that measured for chloroplast-mediated metabolism. Furthermore, the inhibitory response to

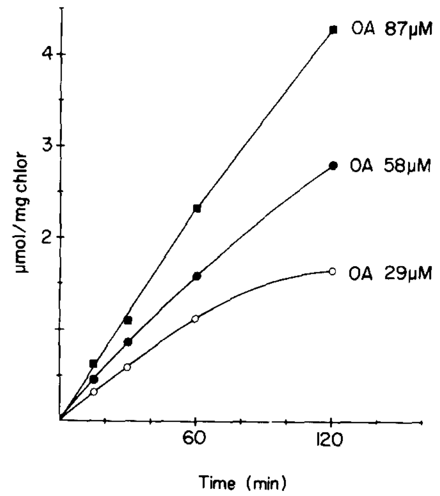

Fig.2. Time course for the conversion of [4-^{14}C]OA to products by illuminated maize mesophyll chloroplasts. The light intensity used (> 100 W/m^2) was saturating for this process.

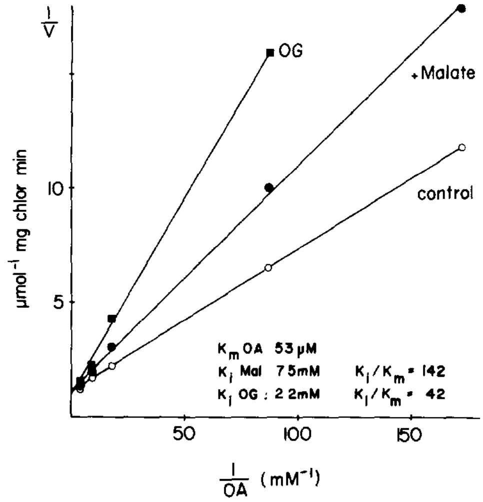

Fig.3. Effect of varying OA on the conversion of OA to products by illuminated maize chloroplasts and inhibition by malate and 2-oxoglutarate (OG).

Table 1

Concentration dependence of the conversion of [^{14}C]oxaloacetate by maize mesophyll chloroplasts

Expt	System	Kinetic constants
1A	Intact chloroplasts, light:	V_{max}: 84 μmol/mg chl·h K_m OA: 0.032 mM K_i OG[a]: 1.8 mM K_i OG/K_m OA = 56
B	Broken chloroplasts, dark: 0.3 mM NADPH, 10 mM DTT, 50 mM Tris (pH 8.5), 0.25% Triton X-100	V_{max}: 360 μmol/mg chl·h K_m OA: 0.016 mM K_i OG: 8.2 mM K_i OG/K_m OA = 513
2A	Intact chloroplasts, light:	V_{max}: 258 μmol/mg chl·h K_m OA: 0.060 mM
B	Dark + 10 mM glutamate	V_{max}: 255 μmol/mg chl·h K_m OA: 0.058 mM
C	Broken chloroplasts, 50 mM Tris (pH 8.5), 0.25% Triton X-100, 10 mM glutamate	V_{max}: 608 μmol/mg chl·h K_m OA: 0.001 mM

[a] OG, 2-oxoglutarate

2-oxoglutarate was markedly different with ratios for the K_i for 2-oxoglutarate to the K_m for OA of about 50 for intact chloroplasts (table 1, fig.3) but about 10-fold higher for the enzyme released from chloroplasts (table 1). These results support the view that with the conditions used, OA reduction in chloroplasts is at least largely limited by OA transport into the chloroplasts and that the kinetics should therefore reflect the properties of the transport process.

With chloroplast preparations (summer-grown) showing generally higher rates for all activities, OA uptake was followed by measuring conversion to aspartate in chloroplasts incubated in the dark with glutamate (table 1). The K_m for OA (60 μM) and V_{max} measured in this way were very similar to the values obtained by measuring OA reduction in the light with the same chloroplasts. Notably, for the aspartate aminotransferase released from these chloroplasts by osmotic shock, the V_{max} was higher but the K_m was extremely low (table 1).

Similar studies of OA transport into spinach chloroplasts were conducted and qualitatively similar results were obtained (fig.4). Compared with maize the K_m for OA (average about 8 μM) was lower for spinach chloroplasts and the V_{max} values were less (fig.4, table 2). Again, malate and 2-oxoglutarate were inhibitory and the ratio of the K_i to the K_m for OA (fig.4) were very similar to the values obtained with maize chloroplasts (see fig.3).

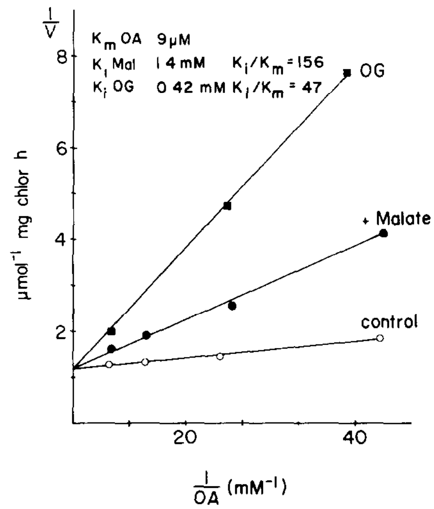

Fig.4. Effect of varying OA on the conversion of OA to products by spinach chloroplasts and inhibition by malate and 2-oxoglutarate (OG).

4. CONCLUDING REMARKS

The evidence presented here suggests that oxaloacetate reduction in chloroplasts illuminated in high light is at least largely limited by OA transport. The results obtained indicate the operation of a high affinity carrier for OA which is particularly active in maize where it would have to operate at rates comparable to photosynthesis (up to 250 μmol $CO_2 \cdot mg^{-1}$ $Chl \cdot h^{-1}$ depending on growth conditions, see [3]). This system has an affinity for OA more than 100-times higher than that of malate. Such a feature is critical when, for C_4 plants at least, the malate pool in the mesophyll cytosol may be between 10- and 100-times higher than the OA pool (see section 1).

Recent evidence that light activation of NADP malate dehydrogenase in spinach is inhibited by NADP [16] would mean that the enzyme may only be activated when the ratio of NADPH to NADP is very high. This would be consistent with NADP-malate dehydrogenase, and the OA translocator, being involved in the transfer of excess reducing equivalents to the cytosol. However, there is a comparable effect of NADP on the light-dark regulation of malate dehydrogenase in maize which is reversed by NADPH [17]; since the maize enzyme is directly involved in the path of carbon assimilation, the significance of this effect is not as readily apparent.

ACKNOWLEDGEMENT

Supported by grant from the Deutsche Forschungsgemeinschaft. M.D.H. was the recipient of a guest professorship by the Deutsche Forschungsgemeinschaft.

REFERENCES

[1] Heber, U. and Krause, G.H. (1971) in: Photosynthesis and Photorespiration (Hatch, M.D. et al. eds) pp.218–225, Wiley-Interscience, New York.

[2] Anderson, J.W. and House, C.M. (1979) Plant Physiol. 64, 1058–1063.

[3] Hatch, M.D. and Osmond, C.B. (1976) in: Encyclopedia of Plant Physiology, vol.3, Transport (Stocking, C.R. and Heber, V. eds) pp.144–154, Springer-Verlag, Berlin.

[4] Day, D.A., Jenkins, C.L.D. and Hatch, M.D. (1981) Aust. J. Plant Physiol. 8, 21–29.

[5] Heber, U. and Heldt, H.W. (1981) Annu. Rev. Plant Physiol. 32, 139–168.

[6] Heldt, H.W. and Rapley, L. (1970) FEBS Lett. 10, 143–148.

[7] Lehner, K. and Heldt, H.W. (1978) Biochim. Biophys. Acta 501, 531–544.

[8] Day, D.A. and Hatch, M.D. (1981) Arch. Biochem. Biophys. 211, 738–742.

[9] Stern, J.R., Ochoa, S. and Lynen, F. (1952) J. Biol. Chem. 198, 313–320.

[10] Hatch, M.D., Slack, C.R. and Johnson, H.S. (1967) Biochem. J. 102, 417–422.

[11] Jenkins, C.L.D. and Russ, V.J. (1984) Plant Sci. Lett. 35, 19–24.

[12] Lilley, R.McC. and Walker, D.A. (1974) Biochim. Biophys. Acta 368, 269–278.

[13] Heldt, H.W. and Sauer, F. (1971) Biochim. Biophys. Acta 234, 83–91.

[14] Hatch, M.D. and Heldt, H.W. (1984) Analyt. Biochem., submitted.

[15] Heldt, H.W. (1980) Methods Enzymol. 69, 604–613.

[16] Scheibe, R. and Jaquot, J.-P. (1983) Planta 157, 548–553.

[17] Ashton, A.R. and Hatch, M.D. (1983) Arch. Biochem. Biophys. 227, 416–424.