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# On the Function of Mitochondrial Metabolism during Photosynthesis in Spinach (*Spinacia oleracea* L.) Leaves[1]

## Partitioning between Respiration and Export of Redox Equivalents and Precursors for Nitrate Assimilation Products

**Iris Hanning and Hans W. Heldt***

Institut für Biochemie der Pflanze, Universität Göttingen, Untere Karspüle 2, 37073 Göttingen, Germany

**The functioning of isolated spinach (*Spinacia oleracea* L.) leaf mitochondria has been studied in the presence of metabolite concentrations similar to those that occur in the cytosol in vivo. From measurements of the concentration dependence of the oxidation of the main substrates, glycine and malate, we have concluded that the state 3 oxidation rate of these substrates in vivo is less than half of the maximal rates due to substrate limitation. Analogously, we conclude that under steady-state conditions of photosynthesis, the oxidation of cytosolic NADH by the mitochondria does not contribute to mitochondrial respiration. Measurements of mitochondrial respiration with glycine and malate as substrates and in the presence of a defined malate:oxaloacetate ratio indicated that about 25% of the NADH formed in vivo during the oxidation of these metabolites inside the mitochondria is oxidized by a malate-oxaloacetate shuttle to serve extramitochondrial processes, e.g. reduction of nitrate in the cytosol or of hydroxypyruvate in the peroxisomes. The analysis of the products of the oxidation of malate indicates that in the steady state of photosynthesis the activity of the tricarboxylic acid cycle is very low. Therefore, we have concluded that the mitochondrial oxidation of malate in illuminated leaves produces mainly citrate, which is converted via cytosolic aconitase and NADP-isocitrate dehydrogenase to yield 2-oxoglutarate as the precursor for the formation of glutamate and glutamine, which are the main products of photosynthetic nitrate assimilation.**

During photosynthetic metabolism in leaf mesophyll cells, mitochondria provide the cytosol with ATP (Krömer and Heldt, 1991b). The substrates of mitochondrial respiration may derive from glycolysis and may be metabolized further by the TCA cycle, although it is still debatable whether this metabolic pathway is fully active during photosynthesis (Graham, 1980; Turpin et al., 1988). A special feature of plant mitochondria is their ability to oxidize external pyridine nucleotides (Douce and Neuburger, 1989), but the metabolic role of this process is obscure. The main substrate for mitochondrial respiration in the light is probably Gly, which is produced at high rates during photorespiration.

Instead of being oxidized in the respiratory chain for ATP production, the reducing equivalents that are derived from the oxidation of Gly or TCA cycle intermediates may be exported from the mitochondria by metabolite shuttles, to serve the reduction of nitrate in the cytosol (Weger and Turpin, 1989) or the reduction of hydroxypyruvate in the peroxisomes (Tolbert, 1982). From studies with spinach leaf mitochondria, Journet et al. (1981) postulated that export of reducing equivalents from mitochondria proceeds by a malate-Asp shuttle. In work with pea leaf mitochondria, Dry et al. (1987) and Krömer and Heldt (1991a) could not detect any major activity of a malate-Asp shuttle, but they found a high activity of a malate-OAA shuttle. Alternatively, cytosolic nitrate reductase and peroxisomal hydroxypyruvate reductase can be served via a chloroplastic malate-OAA shuttle with reducing equivalents generated from photosynthetic electron transport (Hatch et al., 1984; Heineke et al., 1991). The question, therefore, arises as to what extent is the NADH derived from mitochondrial substrate oxidation exported into the cytosol in vivo, either via a malate-OAA or a malate-Asp shuttle?

Nitrate assimilation requires another mitochondrial function: production and export of carbon skeletons for the synthesis of the primary amino acids Glu and Gln. According to Miflin and Lea (1982), mitochondria synthesize and export 2-OG, which is then converted to Glu via the chloroplastic Gln synthetase/Glu synthase system. Chen and Gadal (1990) proposed an alternative path to 2-OG. Citrate synthesized inside the mitochondria is transferred into the cytosol for further conversion to 2-OG via cytosolic aconitase (Brouquisse et al., 1987) and NADP-isocitrate dehydrogenase (Randall and Givan, 1981). It remains to be elucidated whether this proposed metabolic sequence operates in vivo.

To assess the in vivo significance of the results of in vitro studies, it is necessary to perform these studies under conditions simulating the situation in vivo. A prerequisite is to know the metabolite concentrations in a compartment of an intact cell. Using nonaqueous fractionation of frozen leaves, the concentrations of various metabolites in the cytosol of

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* Corresponding author; fax 49–551–395749.

Abbreviations: GOT, glutamate-oxaloacetate transaminase; OAA, oxaloacetate; 2-OG, 2-oxoglutarate; PEP, phospho*enol*pyruvate; TCA cycle, tricarboxylic acid cycle.

illuminated spinach leaves have been determined (Winter et al., 1993). In the present report we describe specific studies with mitochondria from the same spinach cultivar as had been employed for the above-mentioned subcellular metabolite analyses to assess the in vivo significance of various mitochondrial functions.

## MATERIALS AND METHODS

Mitochondria were prepared from leaves of spinach (*Spinacia oleracea* L., U.S. hybrid 424, Ferry Morse Seed Co., Mountain View, CA) and pea seedlings (*Pisum sativum* L. var Kleine Rheinländerin) grown hydroponically (Krömer and Heldt, 1991a; Riens et al., 1991). Respiration was measured as described by Krömer and Heldt (1991a). Measurements of NADH and NADPH oxidation by mitochondria were carried out in the same medium that was used for respiration measurements, and the reaction was monitored by a dual-wavelength spectrophotometer (Sigma ZFP 22, Eppendorf, Germany). For the measurements of product formation, mitochondria (0.15 mg protein/mL) were incubated in a standard incubation medium, pH 7.2 (Krömer and Heldt, 1991a), containing 0.05 mM CoA, 0.1 mM thiamine pyrophosphate, 0.5 mM $NAD^+$, 1 mM ADP, and/or 1 mM OAA, 1 mM pyruvate, 3 mM Gly, 10 mM malate as substrates at 20°C. The reaction was started by addition of mitochondria and samples were withdrawn immediately after the start of the reaction and 5 min later. The samples were centrifuged and the supernatants were deproteinized by perchloric acid. The resulting extracts were neutralized by the addition of KOH, and metabolite (pyruvate, OAA, 2-OG, citrate, isocitrate, and malate) levels were determined by standard procedures (Bergmeyer, 1983) using a dual-wavelength spectrophotometer (Sigma ZFP 22). Exported succinate and fumarate were detected by UV absorbance at 210 nm (UV detector: Hitachi 655A-22, Hitachi, Ltd., Japan) after separation by ion-exchange HPLC (column: Aminex HPX-87h, Bio-Rad).

Sufficient concentrations of ADP (1 mM) and Pi (10 mM) in the experiments ensured that the respiration rate was constant in state 3 during the 5-min incubation, as shown by parallel polarographic measurements of $O_2$ consumption. Throughout this presentation, the term protein means mitochondrial protein.

## RESULTS AND DISCUSSION

### Respiratory Substrates of Spinach Leaf Mitochondria

One characteristic of plant mitochondria is their ability to oxidize many different substrates. Table I shows state 3 respiration rates of isolated spinach leaf mitochondria with various substrates. The experiments were carried out at pH 7.2.

As shown in Table I, Gly is oxidized with the highest rate, followed by malate, NADH, 2-OG, succinate, and Glu. Citrate and isocitrate yield only low respiration rates. The very low rate of NADPH oxidation results from the chosen pH of 7.2. Unlike NADH oxidation having an optimum at pH 7.4, NADPH oxidation is maximal at pH 6 and shows little activity at pH 7.2 (Arron and Edwards, 1979; Edman et al., 1985). This has also been shown for mitochondria from *Helianthus tuberosus* L. and *Arum maculatum* L. (Møller and Palmer, 1981a, 1981b), whereas with mitochondria from *P. sativum* L. (Krömer and Heldt, 1991a) and *Helianthus annuus* L. (Arron and Edwards, 1979) high respiratory rates with NADPH as substrate at pH 7.2 have been observed.

**Table I.** *Respiration rates of isolated spinach leaf mitochondria*

Measurements were carried out with a Clark-type electrode at pH 7.2 in the presence of 0.05 mM CoA, 0.1 mM thiamine pyrophosphate, 0.5 mM NAD, 0.2 mM ADP, and 0.05 to 0.3 mg mitochondrial protein/mL. The values are mean values from at least three separate experiments ± SD.

| Substrate | Respiration (State 3) |
|---|---|
| | *natom [O] $min^{-1}$ $mg^{-1}$ protein* |
| Gly (10 mM) | 505 ± 86 |
| Malate (10 mM) | 441 ± 61 |
| NADH (1 mM) | 428 ± 74 |
| NADPH (1 mM) | 46 ± 20 |
| 2-OG (1 mM) | 340 ± 87 |
| Glu (40 mM) | 251 ± 53 |
| Succinate (10 mM) | 298 ± 91 |
| Citrate (10 mM) | 145 ± 31 |
| Isocitrate (10 mM) | 129 ± 33 |
| Gly (10 mM) + malate (10 mM) | 700 ± 177 |
| Gly (10 mM) + Glu (40 mM) | 464 ± 86 |
| Malate (10 mM) + Glu (40 mM) | 445 ± 101 |
| Gly (10 mM) + 2-OG (1 mM) | 527 ± 121 |
| Malate (10 mM) + 2-OG (1 mM) | 408 ± 112 |

Whereas the mitochondria oxidizing either Gly or malate did not show a major change in respiration rate upon addition of a second substrate, e.g. Glu or 2-OG, addition of malate to mitochondria oxidizing Gly resulted in a much higher respiration rate than with either substrate alone (Table I). Wiskich et al. (1990) made a similar observation with pea leaf mitochondria. These authors found that the increase in respiration with Gly upon addition of malate was actually due to an increase in Gly oxidation, followed by an increased NADH production by malate dehydrogenase, due to the removal of OAA, the inhibiting reaction product. They concluded that there are metabolic domains in the mitochondrial matrix that enable malate dehydrogenase to operate simultaneously in opposite directions within the same mitochondrial compartment.

The question that arises is which of the metabolites supporting mitochondrial respiration in vitro have a major function as respiratory substrates in vivo? In illuminated spinach leaves the following metabolite concentrations in the cytosolic compartment have been found: NADH, 0.7 μM (Heineke et al., 1991); malate, 0.8 mM; 2-OG, 0.4 mM; Glu, 21 mM; Gly, 1.8 mM (Winter et al., 1993). Because Gly and malate are regarded as the main substrates of mitochondrial respiration, we determined the concentration dependence of Gly and malate-supported respiration with spinach leaf mitochondria to assess the capacity of respiration under in vivo conditions (Fig. 1, A and B). Table II lists mean values of the $K_m$ and $V_{max}$ as evaluated from double-reciprocal plots according to Lineweaver-Burk (experiments as described in the legend to Fig. 1). It may be noted that the difference between

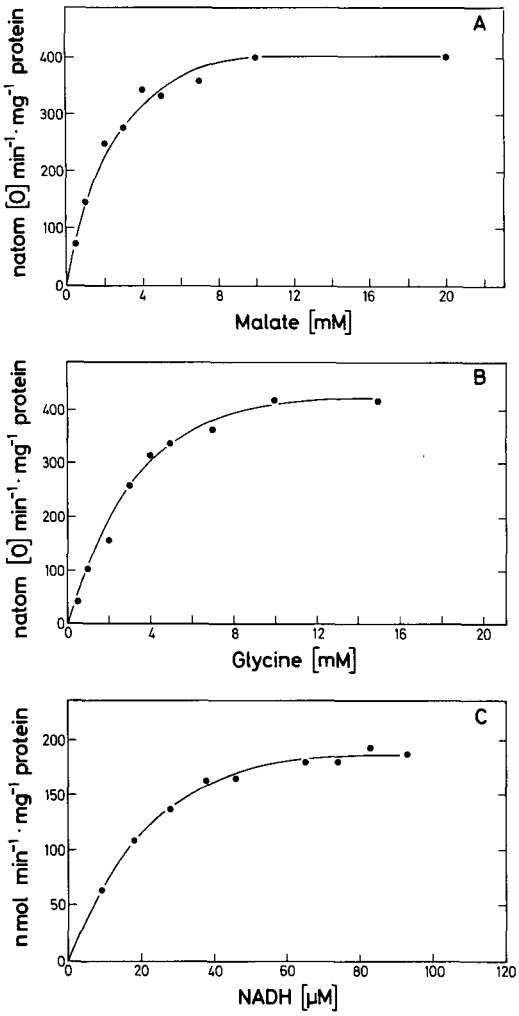


**Figure 1.** Concentration dependence of mitochondrial respiration. The measurements were carried out at pH 7.2 in the presence of 0.5 mM NAD. A and B, 2 mg; C, 1 μg mitochondrial protein/mL. A and B, Polarographic assay; C, spectrophotometric assay.

the $V_{max}$ values of Table II and the respiratory rates of Table I reflects the usual fluctuation in activities between different mitochondrial preparations.

A comparison of the $K_m$ values with the corresponding cytosolic concentrations indicates that in an illuminated spinach leaf cell, respiration with Gly and malate is saturated less than half with each substrate. Apparently, mitochondrial respiration is substrate limited in vivo. In fact, during photosynthesis there is less Gly formed than can be oxidized by the mitochondrial electron transport chain. The spinach plants, from which the mitochondria were isolated, showed a net rate of photosynthesis of 80 μmol $O_2$ $h^{-1}$ $mg^{-1}$ Chl under growth conditions. Assuming a ratio of carboxylation to oxygenation of 2.5 (Sharkey, 1988), the photosynthetic Gly production by these leaves would be 40 μmol $h^{-1}$ $mg^{-1}$ Chl. The complete oxidation of this Gly by the respiratory chain would result in a respiration rate of 20 μatom [O] $h^{-1}$ $mg^{-1}$ Chl. Since spinach leaves contain 1.5 mg of mitochondrial protein per mg of Chl (Ebbighausen et al., 1985), this translates to a rate of 220 natom [O] $min^{-1}$ $mg^{-1}$ protein.

Taking into account that part of the reducing equivalents derived from Gly oxidation do not seem to be available to mitochondrial electron transport, as will be discussed below, Gly-driven respiration may only proceed at about one-third of its capacity in a leaf under steady-state conditions of photosynthesis (Table I). An even lower degree of saturation is found for malate-driven respiration. Assuming that the 2-OG needed for nitrate assimilation derives solely from the conversion of malate via mitochondrial malate dehydrogenase, malic enzyme, pyruvate dehydrogenase, and citrate synthase (see below), a nitrate assimilation rate of 2 to 4 μmol $NO_3^-$ $h^{-1}$ $mg^{-1}$ Chl necessitates a malate conversion rate of 1.8 to 3.6 μmol $h^{-1}$ $mg^{-1}$ Chl. Total respiration of the NADH produced during this conversion would result in a respiration rate of 30 to 60 natom [O] $min^{-1}$ $mg^{-1}$ protein. From a comparison with the respiration rate documented in Table I, it appears that malate-driven respiration in the light maximally occurs at about one-seventh of its capacity.

## Respiration with External Pyridine Nucleotides as Substrates

Using a dual-wavelength spectrophotometer, we measured the concentration dependence of NADH-supported respiration (Fig. 1C). To simulate physiological conditions, the measurements were carried out in the presence of 0.5 mM NAD (Heineke et al., 1991). Table II lists mean values of the $K_m$ and $V_{max}$ for NADH oxidation. The presence of Gly and malate as additional substrates did not change the $K_m$ for NADH, but markedly decreased the $V_{max}$. It appears from these results that the capacity of spinach leaf mitochondria to oxidize external NADH is reduced when internal NADH, as produced by dehydrogenation of Gly and malate, is available to the mitochondria. A decrease of NADH oxidation when Gly was present as second substrate has been observed by Bergman and Ericson (1983).

It is also important to consider the extent to which external NADH oxidized by the mitochondria in vivo contributes to ATP synthesis during illumination. The rate of NADH oxidation during steady-state photosynthesis has been evaluated according to the equation $V = V_{max} \times (S/S + K_m)$ using the $K_m$ and $V_{max}$ values measured in the presence of malate plus Gly (Table II) and a cytosolic NADH concentration of 0.7 μM

**Table II.** *Apparent $K_m$ and $V_{max}$ values for the oxidation of malate, Gly, and NADH by isolated spinach leaf mitochondria*

Mean values from different experiments ± SD. The number of different experimental series is given in parentheses.

| Substrate | $K_m$ | $V_{max}$ |
|---|---|---|
| | *mM* | *natom [O] $min^{-1}$ $mg^{-1}$ protein* |
| Malate (3) | 1.45 ± 0.17 | 319 ± 94 |
| Gly (3) | 2.73 ± 0.20 | 397 ± 28 |
| NADH (5) | 0.014 ± 0.003 | 254 ± 90 |
| NADH + 10 mM Gly + 10 mM malate (5) | 0.013 ± 0.006 | 142 ± 26 |

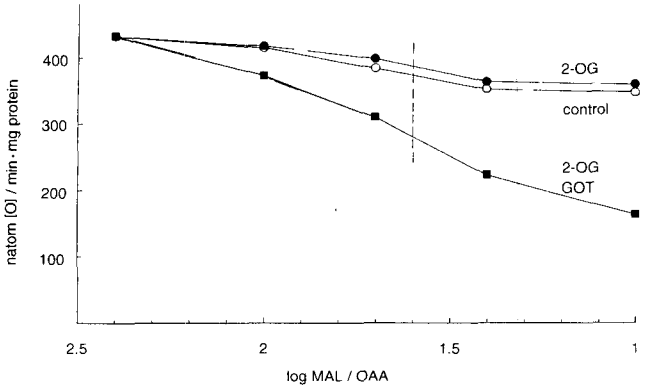


**Figure 2.** Respiration of spinach leaf mitochondria (state 3) in the presence of 10 mM Gly, 5 mM malate, and various concentrations of Asp and Glu. The incubation medium (pH 7.2) contained (from left to right; concentration in mM): Asp (10), Glu (45); Asp (25), Glu (45); Asp (40), Glu (40); Asp (40), Glu (18); Asp (40), Glu (7). After addition of 0.1 mg of mitochondrial protein/mL, 0.66 mM 2-OG and 0.066 μkat GOT were added as indicated above (Krömer et al., 1992).

(Heineke et al., 1991). The rate of NADH oxidation thus evaluated is 7 nmol $min^{-1}$ $mg^{-1}$ protein, which is 2 orders of magnitude lower than the respiratory rate with Gly plus malate (Table I). This clearly indicates that in spinach leaves in the steady state of photosynthesis, mitochondrial oxidation of external NADH does not occur to any appreciable extent. The cytosolic NADH concentration in pea leaves is not known; assuming that it is roughly the same as in spinach, the estimated rate of external NADH oxidation by pea leaf mitochondria under in vivo conditions was also very low (Krömer and Heldt, 1991a).

### Transfer of Redox Equivalents to Extramitochondrial Compartments

With isolated leaf mitochondria, respiration with Gly can almost be eliminated totally by addition of OAA, which enters the matrix and sequesters the NADH generated from substrate oxidation for the formation of malate (Woo and Osmond, 1976; Day and Wiskich, 1981; Journet et al., 1981).

To determine the activity of both a malate-Asp and a malate-OAA shuttle, we measured the state 3 respiration rates of isolated spinach leaf mitochondria in the presence of 10 mM Gly, 5 mM malate, and various concentrations of OAA. To maintain a steady-state concentration of OAA during the entire experiment, it was regenerated from 2-OG, Asp, and Glu via added GOT. The concentrations of Glu and Asp were varied to yield a defined equilibrium concentration of OAA (see legend to Fig. 2).

To the control assay, which already contained Asp and Glu, 2-OG and GOT were added successively. This enabled us to determine which portion of the redox equivalents produced as NADH during Gly oxidation is oxidized by the respiratory chain and which is exported into the cytosol via a malate-Asp shuttle (after the addition of 2-OG) or via a malate-OAA shuttle (after addition of GOT) as dependent on the malate:OAA ratio in the assay.

The measured respiration rates in Figure 2 have been plotted against the logarithm ($log_{10}$) of the malate:OAA ratios. The figure illustrates that after addition of 2-OG a slight increase of the respiratory activity can be observed, probably due to the additional oxidation of the added substrate. Since the curve obtained after addition of 2-OG represents the activity of the malate-Asp shuttle, it can be concluded that under simulated in vivo conditions a malate-Asp shuttle does not operate at any substantial rate. Upon addition of GOT, which enabled a malate-OAA shuttle to operate, a clear decrease of the respiratory rate can be seen. In experiments not shown here, the addition of 0.1 mM phthalonate, a powerful inhibitor of OAA transport in plant mitochondria (Day and Wiskich, 1981; Ebbighausen et al., 1985), totally reversed this decrease in respiration caused by Asp plus malate plus GOT. These results clearly indicate that the observed decrease in respiration in the presence of Asp, malate, and GOT was due to a malate-OAA shuttle withdrawing reducing equivalents from the matrix.

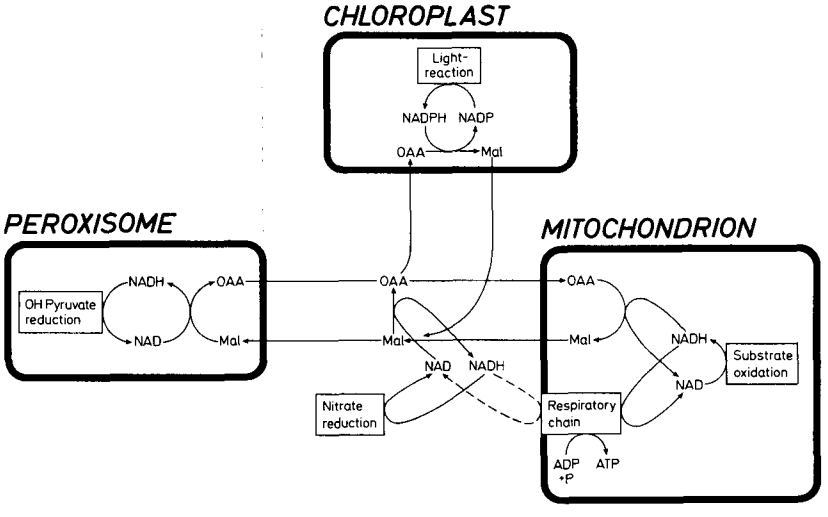


**Figure 3.** Intracellular redox transfer between mitochondria, chloroplasts, and peroxisomes.

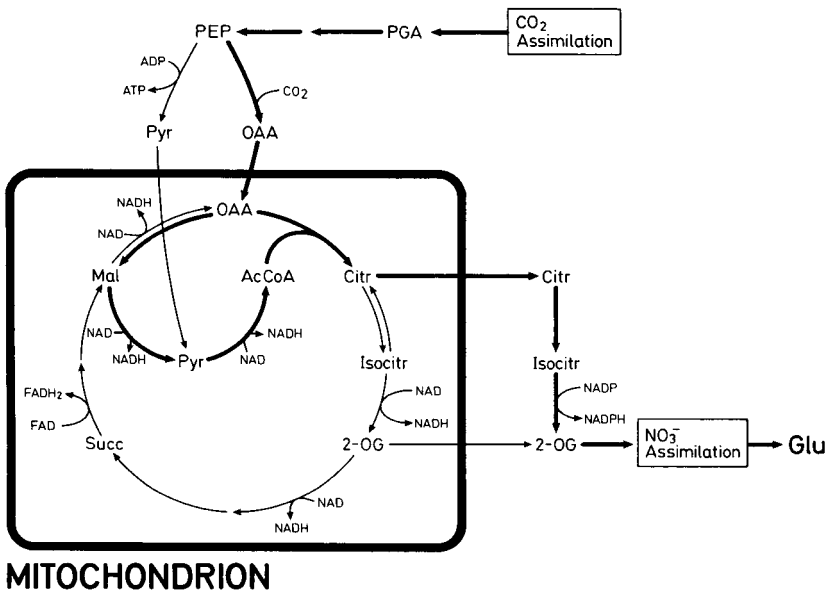


**Figure 4.** Reaction scheme of the TCA cycle and for the synthesis of 2-OG for Glu formation.

It became important to determine the extent to which such a transport occurs under in vivo conditions. In spinach leaves during steady-state photosynthesis, the NADH and NAD concentrations in the cytosol were evaluated as 0.7 $\mu$M and 0.6 mM, respectively, yielding a cytosolic NADH:NAD ratio of $10^{-3}$ (Heineke et al., 1991). In Figure 2, a malate:OAA ratio of 40 (log 1.6), which is in equilibrium with the above NADH:NAD ratio ($K_{EQ}$ malate dehydrogenase $2.8 \times 10^{-5}$ [Veech et al., 1969]), decreased the rate of respiration by 25%. This result shows that under simulated steady-state conditions (Fig. 2, dashed line), 25% of the redox equivalents formed in the mitochondria from the oxidation of Gly and malate are exported into the cytosol, whereas the remaining 75% are oxidized by the respiratory chain. Similarly, with pea leaf mitochondria at an external malate:OAA ratio of 40, 50% of the reducing equivalents formed during oxidation of Gly and malate were found to be exported by the malate-OAA shuttle (Krömer and Heldt, 1991a).

At an NADH:NAD ratio in the mitochondrial matrix as low as $10^{-3}$, the matrix NADH dehydrogenase of the respiratory chain is not expected to be operative. Our results show that at a set NADH:NAD ratio of $10^{-3}$ outside the mitochondria, mitochondrial respiration is only partially inhibited. The malate-OAA shuttle across the mitochondrial inner membrane, although capable of operating at very high rates (Ebbighausen et al., 1985), apparently does not equilibrate the NADH:NAD ratios inside and outside the mitochondria. The redox gradient between the NADH:NAD systems in the mitochondrial matrix and the cytosol might be achieved by a control of malate transport by OAA, as reported earlier (Zoglowek et al., 1988).

During photorespiration an equivalent amount of the NADH generated from Gly oxidation is required for the reduction of hydroxypyruvate, which occurs predominantly in the peroxisomes (Heupel et al., 1991). Our results indicate that in the steady state of photosynthesis, only part of the reducing equivalents required in the peroxisomes are provided by the mitochondria via the malate-OAA shuttle. To a large extent the NADH demand for peroxisomal hydroxypyruvate reduction and also for nitrate reduction in the cytosol might be met by chloroplast electron transport via a malate-OAA shuttle (Hatch et al., 1984; Fig. 3). This redox transfer from the chloroplasts to the peroxisomes also seems to be important to prevent an overreduction of the photosynthetic electron transport chain (S. Krömer, personal communication). Additionally, it enables the NADH formed by Gly oxidation in the mitochondria to remain in the matrix to be utilized by the respiratory chain for ATP generation. As was recently demonstrated (Krömer and Heldt, 1991b), mitochondrial ATP synthesis plays an important role in photosynthetic metabolism of a leaf cell.

In the oxidation of external NADH, only two coupling sites are involved (Møller and Lin, 1986). Since the oxidation of mitochondrial NADH by the respiratory chain involves three coupling sites, the existence of a malate-OAA shuttle for the reduction of external NAD at the expense of internal NADH, and its subsequent oxidation by the external NADH dehydrogenase, might constitute a bypass of the first coupling site (Fig. 3, dashed line). Our results indicate that this does not occur. At the low NADH:NAD ratio required in the cytosol for the malate-OAA shuttle to operate (Fig. 2), the rate of external NADH oxidation is almost zero (see above).

In summary, our results indicate that a malate-OAA shuttle functions only to export reducing equivalents from the mitochondria. A redox transfer in the opposite direction, e.g. for an oxidation of reducing equivalents derived from photosynthetic electron transport by the mitochondrial respiratory chain, might proceed via the external NADH dehydrogenase, but only when the cytosolic NADH/NAD system is much more reduced than in the steady state of photosyn-

**Table III.** *Metabolite export by spinach leaf mitochondria during substrate oxidation*

The values from series A and B are mean values from three and two separate experiments, respectively, carried out as described in "Materials and Methods" at pH 7.2 in the presence of 0.15 mg of mitochondrial protein/mL. n.d., Not determined.

| Series | Substrates | Products | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Pyruvate | OAA | Citrate | Isocitrate | 2-OG | Succinate | Fumarate | Malate |
| | | *nmol min$^{-1}$ mg$^{-1}$ protein* | | | | | | | |
| A | Malate (10 mM) | 59 | 5 | 41 | <1 | 15 | <5 | $\leq 10^{-3}$ | n.d. |
| | Malate + Gly (3 mM) | 47 | <1 | 25 | <1 | 12 | <5 | $\leq 10^{-3}$ | n.d. |
| B | Pyruvate (1 mM) | n.d. | n.d. | <1 | <1 | 1 | <5 | $\leq 10^{-3}$ | <1 |
| | OAA (1 mM) | 58 | n.d. | 22 | <1 | 13 | <5 | $\leq 10^{-3}$ | 66 |
| | OAA + pyruvate | n.d. | n.d. | 47 | <1 | 18 | <5 | 1 | 77 |
| | OAA + Gly (3 mM) | 16 | n.d. | 18 | <1 | 19 | <5 | 1 | 109 |
| | OAA + pyruvate + Gly | n.d. | n.d. | 57 | <1 | 19 | <5 | 1 | 158 |

thesis. The external NADH dehydrogenase may thus represent a safety device to eliminate excessive reducing equivalents derived from photosynthesis.

### Nitrogen Assimilation: Do Mitochondria Provide 2-OG or Citrate?

In a plant cell, mitochondria not only provide energy (in the form of ATP) and redox equivalents, but also carbon skeletons for the synthesis of amino acids as end products of nitrate assimilation. In spinach leaves, Glu and Gln represent about 60% of the products of nitrate assimilation that are translocated to other parts of the plant via the sieve tubes (Riens et al., 1991). It has been assumed that the 2-OG required as a precursor for the synthesis of Glu and Gln is provided by the mitochondria via a partial sequence of the TCA cycle, involving pyruvate dehydrogenase, citrate synthase, aconitase, and isocitrate dehydrogenase. In contrast, Chen and Gadal (1990), based on the high activities of aconitase and NADP-isocitrate dehydrogenase present in the cytosol (Randall and Givan, 1981; ap Rees, 1990), have proposed that the mitochondria need only to synthesize citrate, which is further converted to 2-OG by cytosolic aconitase and NADP-isocitrate dehydrogenase (Fig. 4).

To decide whether citrate or 2-OG is formed as the main product by the mitochondria, we studied the efflux of intermediates of the TCA cycle during the oxidation of malate, malate plus Gly, pyruvate, OAA, OAA plus Gly and/or plus pyruvate by spinach leaf and pea leaf mitochondria. As shown in Table III, during respiration with malate as substrate relatively high amounts of pyruvate and citrate, a much lower amount of 2-OG, and only a minimal amount of OAA are exported. In the presence of malate plus Gly no release of OAA occurred, probably due to a reduction of the OAA within the mitochondria at the expense of NADH generated from Gly oxidation (Wiskich et al., 1990).

To evaluate the metabolite fluxes through the various enzymic steps, the $O_2$ consumption connected with the production of the various intermediates during malate oxidation (Table III, first line) was calculated by multiplying the rate of pyruvate and OAA export by 1, the rate of citrate by 3, and the rate of 2-OG by 4. These factors correspond to the moles of NADH produced during synthesis of the various metabolites using malate as the substrate (Fig. 4), which is equal to the atoms of oxygen consumed by the oxidation of this NADH via the respiratory chain. The respiration rate thus calculated is lower than the measured respiration rate (Table IV). Since there are no appreciable amounts of succinate and fumarate released from the mitochondria, the difference between the measured and the calculated rate can be attributed to the conversion of 2 mol of malate to 1 mol of malate via pyruvate dehydrogenase, malic enzyme, and all the enzymes of the TCA cycle (Fig. 4). In this reaction NADH and reduced flavin adenine dinucleotide ($FADH_2$) equivalent to 6 atoms of oxygen are formed. Therefore, the difference between the measured and the calculated respiration rate in Table IV,

**Table IV.** *Metabolite fluxes during respiration of spinach leaf mitochondria in the presence of malate (10 mM)*

Data from Table III, first line. For details of evaluation, see text.

| | |
|---|---|
| Respiration | |
| Measured | 366 natom [O] min$^{-1}$ mg$^{-1}$ protein |
| Calculated | 247 natom [O] min$^{-1}$ mg$^{-1}$ protein |
| | Δ 119 natom [O] min$^{-1}$ mg$^{-1}$ protein |
| Metabolite fluxes | |
| Malic enzyme | 135 nmol min$^{-1}$ mg$^{-1}$ protein |
| Malate dehydrogenase | 81 nmol min$^{-1}$ mg$^{-1}$ protein |
| Pyruvate dehydrogenase, citrate synthase | 76 nmol min$^{-1}$ mg$^{-1}$ protein |
| Isocitrate dehydrogenase | 35 nmol min$^{-1}$ mg$^{-1}$ protein |
| 2-OG dehydrogenase, succinate dehydrogenase | 20 nmol min$^{-1}$ mg$^{-1}$ protein |

**Table V.** *Metabolite export by isolated pea leaf mitochondria during substrate oxidation*

The values are mean values from three separate experiments carried out according to "Materials and Methods" at pH 7.2 in the presence of 0.15 mg of mitochondrial protein/mL. n.d., Not determined.

| Substrates | Products | | | | | | |
|---|---|---|---|---|---|---|---|
| | Pyruvate | Citrate | Isocitrate | 2-OG | Succinate | Fumarate | Malate |
| | *nmol min$^{-1}$ mg$^{-1}$ protein* | | | | | | |
| OAA (1 mM) | 7 | 18 | <1 | <1 | <5 | $\leq 10^{-3}$ | 19 |
| OAA + pyruvate (1 mM) | n.d. | 57 | <1 | 5 | <5 | 1 | 55 |
| OAA + Gly (3 mM) | 1 | 21 | <1 | 1 | <5 | 2 | 114 |
| OAA + pyruvate + Gly | n.d. | 58 | <1 | 5 | <5 | 1 | 123 |

divided by 6, yields the metabolite flux through the entire TCA cycle (20 nmol min$^{-1}$ mg$^{-1}$ protein). The total flux through each enzymic step of malate metabolism, as depicted in Figure 4, is calculated by adding the flux through the TCA cycle to the flux connected with the efflux of metabolites, mentioned above.

The results of these evaluations, as presented in Table IV, show that with malate as substrate, 80% of the total respiration is due to the oxidation of NADH derived from reactions preceding the TCA cycle, namely, the reactions catalyzed by malate dehydrogenase, malic enzyme, and pyruvate dehydrogenase. Isocitrate dehydrogenase and, even more so, 2-OG dehydrogenase appear to limit the metabolite flux through the TCA cycle. These results are in agreement with the earlier finding of Wiskich and Dry (1985) that the mitochondrial isocitrate dehydrogenase has a lower activity than citrate synthase. These results also explain why the respiration rate of mitochondria with citrate or isocitrate as substrates is much lower than with 2-OG (Table I).

With respect to the efflux of intermediates, similar results are obtained when OAA instead of malate is added to the mitochondria as a substrate. Besides the efflux of malate and pyruvate, formed by malate dehydrogenase and malic enzyme, citrate and 2-OG are exported. With OAA plus pyruvate, the efflux of citrate was increased, probably due to the activation of pyruvate dehydrogenase by pyruvate (Budde et al., 1988). With Gly present as a third substrate, the export of citrate was three times higher than that of 2-OG, very similar to the results in the experiment with malate alone as the substrate. In pea leaf mitochondria, which like spinach leaf mitochondria show very high respiratory rates coupled to ATP synthesis (Krömer and Heldt, 1991a), with OAA plus pyruvate and with OAA plus pyruvate plus Gly, the efflux of citrate (Table V) was strikingly similar to that measured with spinach leaf mitochondria, whereas the efflux of 2-OG was comparatively low.

In experiments with mitochondria from spinach and pea leaves (results not shown), the pattern of metabolite efflux with OAA plus pyruvate plus Gly as substrates was not altered by the presence of nitrate, ammonium ions, or dihydroxyacetone phosphate added at concentrations of 1 mM.

For the provision of carbon skeletons, OAA, formed from PEP by PEP carboxylase, seems to be the main substrate. It has been shown recently that the activity of leaf PEP carboxylase is controlled by nitrate assimilation, probably via a protein kinase (Van Quy and Champigny, 1992). The provision of pyruvate to the mitochondria may not be essential, since it was shown that transgenic tobacco lacking cytosolic pyruvate kinase showed normal growth (Hugh et al., 1992). Our results indicate that citrate is a major product of OAA metabolism in the mitochondria.

The spinach plants, from which the mitochondria were prepared, showed a net rate of photosynthesis of 80 μmol h$^{-1}$ mg$^{-1}$ Chl and a nitrate assimilation rate of 2 to 4 μmol h$^{-1}$ mg$^{-1}$ Chl (Riens, 1992; Riens and Heldt, 1992). Analysis of the phloem sap from these plants showed that 39% of the total amino nitrogen herein was fixed as Glu and 10% as Gln (Riens et al., 1991). Assuming that the products of nitrate assimilation are totally transferred into the sieve tubes, a net rate of 2-OG synthesis of 0.9 to 1.8 μmol h$^{-1}$ mg$^{-1}$ Chl, equivalent to 10 to 20 nmol min$^{-1}$ mg$^{-1}$ protein (see above), would be necessary to meet the demand for carbon skeletons. Compared with the results shown in Table III, the 2-OG export rate in spinach leaf mitochondria would cover the requirements for carbon skeletons for nitrate assimilation only under some conditions, whereas the citrate export always exceeds the demand. In agreement with Chen and Gadal (1990) we conclude that mitochondria primarily produce citrate (Fig. 4) to supply carbon skeletons for Glu and Gln synthesis, which is further converted to 2-OG via cytosolic aconitase and NADP-isocitrate dehydrogenase. Thus, only the first step of the TCA cycle, catalyzed by citrate synthase, appears to be required for the synthesis of Glu and Gln as main products of photosynthetic nitrate assimilation.

The problem of whether the TCA cycle operates in illuminated leaves was mentioned earlier. Gemel and Randall (1992) reported a light dependence of pyruvate dehydrogenase inactivation, probably due to Gly production during photorespiration, and they concluded that a reduction of TCA cycle activity occurred during the light to about 30 to 40% of the dark activity. They also showed that pyruvate is the most effective activator of pyruvate dehydrogenase. The data in Table IV show that during oxidation of malate, only 20% of the respiration results from the operation of the TCA cycle. With Gly plus malate, as present under photosynthetic conditions and according to a calculation analogous to that employed for the data in Table IV, less than 10% of the respiration can be accounted for by the operation of the TCA cycle (results not shown). This indicates that in illuminated spinach leaves the flux of metabolites through the TCA cycle is very low when compared with the metabolite fluxes from malate to citrate and Gly to Ser.

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