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Review

Lipid Mobilization and Gluconeogenesis in Plants: Do Glyoxylate Cycle Enzyme Activities Constitute a Real Cycle? A Hypothesis

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Glyoxysomes are specialized peroxisomes present in various plant organs such as germinating cotyledons or senescing leaves. They are the site of β-oxidation and of the glyoxylate cycle. These consecutive pathways are essential to the maintenance of gluconeogenesis initiated by the degradation of reserve or structural lipids.

In contrast to mitochondrial β-oxidation, which is prevalent in animal cells, glyoxysomal β-oxidation and the glyoxylate cycle have no direct access to the mitochondrial respiratory chain because of the impermeability of the glyoxysomal membrane to the reduced cofactors. The necessity of NAD^+ regeneration can conceivably be fulfilled by membrane redox chains and/or by transmembrane shuttles.

Experimental evidence based on the active metabolic roles of higher plant glyoxysomes and yeast peroxisomes suggests the coexistence of two mechanisms, namely a reductase/peroxidase membrane redox chain and a malate/aspartate shuttle susceptible to transfer electrons to the mitochondrial ATP generating system.

Such a model interconnects β-oxidation, the glyoxylate cycle, the respiratory chain and gluconeogenesis in such a way that glyoxysomal malate dehydrogenase is an essential and exclusive component of β-oxidation (NAD^+ regeneration). Consequently, the classical view of the glyoxylate cycle is superseded by a tentative reactional scheme deprived of cyclic character.

Key words: Beta-oxidation / Gluconeogenesis / Glyoxylate cycle / Glyoxysomes / Lipids / Peroxisomes.

Introduction

Germination is a critical step in the lifetime of a plant, when growth is reinduced after a period of metabolic inactivity. Reserve macromolecules, stored as proteins, lipids or carbohydrates during the maturation of seeds, are catabolized in order to provide carbon and energy to the seedling. Since lipids do not migrate from cell to cell, oily stores are first converted into carbohydrates, which are then translocated to the growing root and shoot.

The conversion of lipids involves several consecutive pathways occurring in various cellular compartments:

(1) Hydrolysis of triglycerides in oleosomes (Huang, 1992; Garcia-Agustin *et al.*, 1992),
(2) β-oxidation (Cooper and Beevers, 1969b) and
(3) glyoxylate cycle (conversion of C2 units into C4 units) in glyoxysomes (Breidenbach and Beevers, 1967),
(4) partial citric acid cycle (conversion of succinate into malate) in mitochondria (Cooper and Beevers, 1969a), and finally
(5) gluconeogenesis in the cytosol (Nishimura and Beevers, 1979).

A comparable reactional scheme involving structural lipids has also been observed in various senescing plant tissues. Senescence, which is initiated by natural or experimentally triggered photosynthate starvation, is an active and genetically programmed process leading to the organized disassembly of biological functions at various levels, from individual cells or specific organs to entire plants (for a review, see Noodén, 1988). Reserve carbohydrates are rapidly depleted during senescence, so that lipids become the major energy source for the process, as substrates of the mitochondrial respiration (Dieuaide *et al.*, 1992, 1993; Hooks *et al.*, 1995). Galactolipids, which are predominant components of thylakoid membranes, are degraded during senescence (Gut and Matile, 1988a, b). Most of the acyl residues are released as CO_2, but a portion is converted into metabolites such as sucrose and glucose (Wanner *et al.*, 1991). This conversion implies reinitiation of gluconeogenesis. Reactivation of peroxisomal β-oxidation has indeed been observed in senescent petals (De Bellis *et al.*, 1991), and glyoxylate cycle activities have been measured in senescent tissues such as leaves (Godavari *et al.*, 1973; Gut and Matile, 1988b; Pistelli *et al.*, 1991; Graham *et al.*, 1992; Pastori and Del Rio, 1994), cotyledons (De Bellis *et al.*, 1990; Vincentini and Matile, 1993; Mc Laughlin and Smith, 1995; Pistelli *et al.*, 1995), or petals (De Bellis *et al.*, 1991). Gluconeogenesis enzyme activities have also been detected in senescent cotyledons (Kim and Smith, 1994).

During post-germinative growth, the conversion of lipids into carbohydrates via β-oxidation and the glyoxylate cycle is essential to the development of the seedling, whereas two situations can be described for the similar conversion characterizing senescing tissues. For monocarpic plants, where whole plant senescence is associated with the transition from vegetative to reproductive growth, gluconeogenesis based on structural lipids provides carbon and energy to the maturing seeds. In contrast, gluconeogenesis associated with the seasonal senescence of leaves of a perennial plant results in the temporary storage of carbohydrates (in the trunk and roots), which subsequently sustain the chemoheterotrophic metabolism of the organism at the onset of the next growing season, i.e. before photosynthesis is reinitiated.

Mitochondrial and Peroxisomal/Glyoxysomal Metabolism during Lipid Mobilization

Peroxisomes are ubiquitous small spherical one-membrane organelles that contain H_2O_2 producing enzymes, such as urate oxidase, acyl-CoA oxidase, glycolate oxidase or L-amino acid oxidase, as well as H_2O_2 scavenging enzymes such as catalase or ascorbate peroxidase. They contain neither DNA, nor ribosomes or internal membrane systems. Their granular matrix is amorphous but sometimes contains a paracrystalline dense core, or a protein crystal (for a review, see Beevers, 1979). Peroxisomes are bounded by a fragile membrane that is easily broken during isolation procedures (for reviews, see Lazarow and Fugiki, 1985; Van den Bosch *et al.*, 1992; Sulter *et al.*, 1993). Glyoxysomes are plant specific peroxisomes first observed in germinating castor bean (Breidenbach and Beevers, 1967). They are the sites of two major metabolic pathways that are active in growing seedlings as well as in senescing tissues, namely β-oxidation and the glyoxylate cycle. The glyoxysome diameter may reach 2–4 μm, whereas the peroxisome typical diameter ranges from 0.1 to 1.7 μm. The final size of peroxisomes/glyoxysomes appears to depend on the amount of imported matrix proteins (Mullen and Trelease, 1996), which in turn is correlated with the nature and intensity of the prevalent metabolic pathways.

Several reports on the biogenesis of yeast and mammal peroxisomes describe them as self-perpetuating organelles essentially characterized by processes of proliferation/enlargement or enlargement/proliferation. The corresponding mechanisms for higher plant peroxisomes have not yet been elucidated to a comparable extent (for a review, see Mullen and Trelease, 1996).

The β-oxidation of fatty acids is composed of a recurring sequence of four reactions: first oxidation (dehydrogenation), hydration, second oxidation, and finally thiolysis. Each round of catalysis produces one $FADH_2$, one $NADH_2$ and one acetyl-CoA. In animal cells, β-oxidation is active in mitochondria (where it depends on the carnitine acyltransferase system for the import of fatty acids), as well as in peroxisomes. The main function of mitochondrial β-oxidation is to provide the cell with ATP, since it completely degrades fatty acids and feeds electrons into the respiratory chain. By contrast, animal peroxisomal β-oxidation serves to shorten fatty acids to medium chain compounds ($\sim C \geq 12$), which can be channelled into various metabolic processes.

In plant cells, β-oxidation mainly occurs in peroxisomes, where degradation of fatty acids reaches completion (Cooper and Beevers, 1969b; Hutton and Stumpf, 1969). At the onset of seed germination as well as in senescing tissues, redirection of the ensuing carbon flow toward gluconeogenesis is observed.

The initial dehydrogenation step of β-oxidation is performed by an acyl-CoA dehydrogenase in mitochondria and by an acyl-CoA oxidase in peroxisomes. Acyl-CoA dehydrogenase transfers redox equivalents to the respiratory chain and initiates production of ATP, whereas acyl-CoA oxidase directly uses molecular oxygen as electron acceptor without recovery of chemical energy (Masterson *et al.*, 1992; Dieuaide *et al.*, 1993) (Figure 1). Various types of plant acyl-CoA oxidases that show specific affinities for long, medium or short acyl-CoA chain lengths have been identified and purified. Coordinate expression of these enzymes provides a control of the levels of substrates and products of peroxisomal β-oxidation (Hooks *et al.*, 1995).

In animal systems, multifunctional proteins are known to participate in the β-oxidation pathway in mitochondria (Carpenter *et al.*, 1992; Uchida *et al.*, 1992), as well as in peroxisomes (for a review, see Hiltunen *et al.*, 1996), where proteins exhibiting multifunctional enzyme activities are involved in the degradation of the low amounts of unsaturated fatty acids. It has also been noted that some activities of the β-oxidation pathway in plant systems are similarly carried out by multifunctional proteins, or show multiple locations on several distinct proteins (Kindl, 1992). For example, enoyl-CoA hydratase, hydroxyacyl-

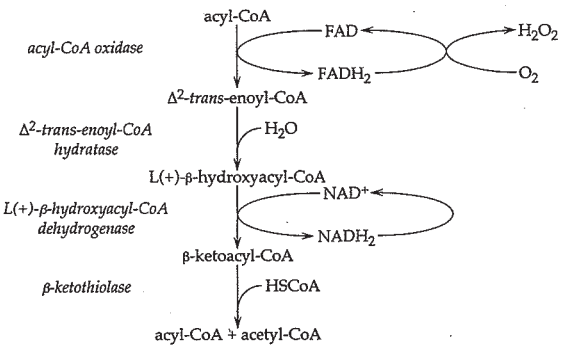

Fig. 1 The Peroxisomal β-oxidation Pathway (Saturated Fatty Acids), as Proposed by Cooper and Beevers (1969b).
In peroxisomes, the first oxidation is performed by an acyl-CoA oxidase, whereas the mitochondrial pathway is characterized by an acyl-CoA dehydrogenase (both enzymes are flavoproteins). It should also be noted that several activities of peroxisomal (as well as mitochondrial) β-oxidation are carried out by multifunctional proteins. The hydrogen peroxide produced by the oxidase can be degraded by catalase and/or peroxidase activities.

CoA dehydrogenase and hydroxyacyl-CoA epimerase activities are achieved by a trifunctional protein in the mold *Neurospora crassa* (Thieringer and Kunau, 1991) and in cucumber (Gühnemann-Schäfer and Kindl, 1995).

Plant β-oxidation is specifically characterized by the nature of its substrates, since saturated fatty acids, characteristic of animal triglycerides, are present in plants in very low amounts only. Degradation of unsaturated or polyunsaturated fatty acids therefore necessitates two additional enzymes, namely an isomerase and an epimerase that catalyze the conversions of the produced Δ^3-*cis*-enoyl-CoA and D(–)-β-hydroxyacyl-CoA to Δ^2-*trans*-enoyl-CoA and L(+)-β-hydroxyacyl-CoA respectively, the appropriate substrates of hydratase and hydroxyacyl-CoA dehydrogenase (Kindl, 1992, 1993).

Plant mitochondria are a minor site of β-oxidation, whose exact role is still unsolved (investigations on β-oxidation activities in plant tissues have shown that mitochondrial and peroxisomal enoyl-CoA hydratases have distinct kinetic and immunological properties; Miernyk *et al.*, 1991). The low capactiy of this mitochondrial pathway for long chain fatty acid degradation raises the question of its *in vivo* function. Compared to the corresponding peroxisomal activity, plant mitochondrial medium-chain fatty acid β-oxidation is however not negligible. This suggests some role for plant mitochondria in the oxidation of medium-chain fatty acids (Dieuaide *et al.*, 1993). These organelles possess carnitine acyltransferase activity (Masterson *et al.*, 1992) just as animal mitochondria do. This further suggests that fatty acids may be not entirely degraded in plant mitochondria since carnitine transferase would compete with β-oxidation enzymes for acyl-CoAs. This would induce export of shortened acyl-CoA chains. In other words, β-oxidation in plant mitochondria might not reach completion, the ensuing situation being the opposite of that assumed to prevail in animal cells.

At the onset of germination or senescence, acetyl-CoA produced by β-oxidation in plant peroxisomes (in this case more appropriately designated as glyoxysomes) is transferred in various proportions to the glyoxylate cycle, which can be considered as a short cut of the citric acid cycle. The glyoxylate cycle possesses two specific enzymes, isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2), and 'borrows' three enzyme activities (by gene duplication) from the citric acid cycle, namely citrate synthase (EC 4.1.3.7), aconitase (EC 4.2.1.3) and malate dehydrogenase (EC 1.1.1.37) (Figure 2). This pathway bypasses the two decarboxylative steps of the citric acid cycle, and redirects the 2-carbon units produced by β-oxidation toward gluconeogenesis. In the classical view, each turn of the cycle integrates two molecules of acetyl-CoA and produces one molecule of succinate, a citric acid cycle intermediate that is exported to the mitochondrion and subsequently initiates gluconeogenesis after oxidation. The glyoxylate cycle was initially described in *Pseudomonas* spp. (Saz and Hillary, 1956; Kornberg and Madsen, 1957), where it allows growth on acetate as the sole source of carbon. In plants, the pathway was first detected in the endosperm of germinating castor bean (Kornberg and Beevers, 1957).

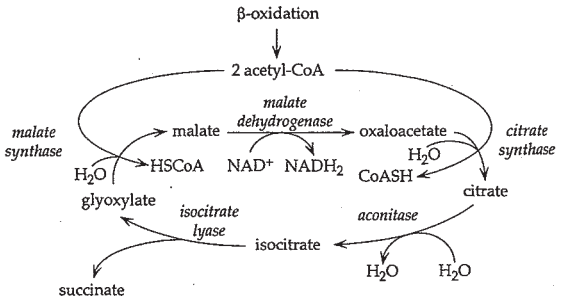

Fig. 2 Glyoxylate Cycle: Classical View.

The glyoxylate cycle has been localized in glyoxysomes (Breidenbach and Beevers, 1967), although aconitase activity has never been clearly measured in isolated organelles. The first interpretations assumed that aconitase is so soluble that the enzyme is lost during organelle isolation because of membrane disruption (Cooper and Beevers, 1969a). There is now evidence that glyoxysomes do not contain aconitase. The enzyme possesses an Fe-S cluster, and measurements by isotropic electronic paramagnetic resonance did not reveal any aconitase in the glyoxysomes (Courtois-Verniquet and Douce, 1993). One cytosolic aconitase isoform might participate in the glyoxylate cycle, since developmental changes in the abundance of this isoform are correlated with increases and decreases observed for other glyoxylate cycle enzymes during seedling growth (Hayashi *et al.*, 1995) and cotyledon senescence (De Bellis *et al.*, 1995). The absence of aconitase in glyoxysomes might be a necessity due to its strong sensitivity to H_2O_2 (Verniquet *et al.*, 1991). Since catalase shows a poor affinity for its substrate (Aebi, 1974), aconitase could not operate in peroxisomes/glyoxysomes, where continuous production of H_2O_2 occurs. The glyoxylate cycle thus requires a detour via the cytosol.

Glyoxylate cycle activities are also present in various animal tissues, such as the toad urinary bladder (Goodman *et al.*, 1980; Davis *et al.*, 1986), embryos, larvae, intestine and muscle of *Caenorhabditis elegans* (Kahn and McFadden, 1980; Liu *et al.*, 1995), marine bivalve mollusks (Benevides *et al.*, 1989), rat epiphyseal cartilage and liver (Davis *et al.*, 1989a, b), chicken liver (Davis *et al.*, 1990a), black bear brown adipose tissue (Davis *et al.*, 1990b), as well as human liver (Davis and Goodman, 1992). The presence of glyoxylate cycle activities in animal cells suggests that such organisms are able to convert fatty acids into carbohydrates. Particularly in the case of the bear brown fat tissue (a heat producing tissue), where peroxisomal β-oxidation activities increase and glyoxylate cycle activities appear during hibernation. Furthermore, incubation of the tissue in the presence of palmitate induces an increase of the glycogen content, which indicates that the complete gluconeogenesis pathway based on lipids is activated (Davis *et al.*, 1990b; by contrast, no such increase occurs in nonhibernating animals). What

might be the advantage of such a process versus a direct degradation of fatty acids by mitochondrial β-oxidation? Chemical energy initially contained in fatty acids would in the latter case be recovered as ATP in the mitochondrial matrix, via the electron transport chain and oxidative phosphorylation. In terms of ATP production, fatty acid degradation by peroxisomal β-oxidation is comparatively wasteful of chemical energy. It has been suggested that the heat directly produced by this loss of energy might participate in the thermoregulation of the organism (Kramar *et al.*, 1978) by a mechanism different from the dissipative proton pathway based on the mitochondrial uncoupling protein (thermogenin; for a review of the mitochondrial mechanism, see Wojtczak and Schonfeld, 1993).

Production of Reduced Cofactors

In peroxisomes, β-oxidation as well as the glyoxylate cycle (as postulated by the classical view; see Figure 2) produce reduced cofactors: two $FADH_2$ and three $NADH_2$ appear for each exported succinate molecule. These cofactors, which do not have direct access to the mitochondrial electron transport system, must nevertheless be reoxidized in order for both pathways to remain functional. Rat liver peroxisomes seem to be *in vitro* freely permeable to low molecular mass compounds (< 800 Da), such as $NADH_2$ or NAD^+ (Van Veldhoven *et al.*, 1983), as well as ATP, carnitine and HSCoA (Van Veldhoven *et al.*, 1987). This apparent permeability might however be due to the rupture of the membrane during organelle isolation, since yeast peroxisomes are *in vivo* closed compartments impermeable to $NADH_2$, acetyl-CoA or carnitine (Van Roermund *et al.*, 1995). These results suggest that regeneration of the oxidized cofactors must occur within the glyoxysomal matrix. The first oxidation step of peroxisomal β-oxidation is catalyzed by an acyl-CoA oxidase, and the redox equivalents are directly transferred to molecular oxygen (Figure 1). In contrast, $NADH_2$ produced by hydroxyacyl-CoA dehydrogenase and by malate dehydrogenase in the glyoxylate cycle accumulates in the matrix of isolated glyoxysomes (Cooper and Beevers, 1969b).

Two models have been proposed for the reoxidation of $NADH_2$:

(1) Oxidation by a membrane dehydrogenase, or
(2) transfer of redox equivalents to another cellular compartment via an appropriate shuttle.

These two systems will be explained and discussed in view of the overall equilibrium of the correlated metabolic pathways.

Regeneration of Oxidized Cofactors via a Membrane Redox Chain

The electron acceptors cyt c and ferricyanide have been instrumental in the characterization of the glyoxysomal redox system, in an approach similar to that already used in the investigation of the ER (endoplasmic reticulum) electron transport. $NADH_2$ and $NADPH_2$ dehydrogenase activities have thus been measured in glyoxysome membranes isolated on sucrose gradients (Hicks and Donaldson, 1982; Donaldson and Fang, 1987; Luster and Donaldson, 1987). The membranes were also shown to contain cyt b_5 and cyt P_{420}, a degradation product of cyt P_{450} (Fang *et al.*, 1987). These dehydrogenases would be capable of transferring reducing equivalents generated by the glyoxysomal matrix metabolism to an acceptor across the glyoxysomal membrane. The proposed model (Figure 3) is analogous to the electron transport chain found in mammalian liver microsomes, which consists of two components:

(1) A $NADH_2$ dehydrogenase flavoprotein able to reduce ferricyanide or to directly transfer electrons to cyt b_5, and
(2) a cyt b_5 which can reduce cyt c (Hicks and Donaldson, 1982; Luster and Donaldson, 1987; Struglics *et al.*, 1993).

Several tests were performed in order to validate the proposed system. Palmitoyl-CoA and malate oxidations can be coupled to ferricyanide or cyt c reduction, as demonstrated with the acceptors NAD^+, ferricyanide or cyt c (Donaldson and Fang, 1987). When membrane preparations (washed with 0.1 M Na_2CO_3 in order to completely remove the matrix) were combined with matrix fractions, matrix dehydrogenases were able to transfer reducing equivalents to membrane reductases ($NADH_2$ dehydrogenases; Fang *et al.*, 1987). The *in vitro* activities of these enzymes are sufficient to handle a considerable portion of the $NADH_2$ flux in glyoxysomes as evaluated by the activity of acyl-CoA oxidase, which catalyzes the β-oxidation rate-limiting step (Hicks and Donaldson, 1982). However, since the physiological acceptors of the redox components are not known, the actual capabilities of such a membrane electron transport system may be different from the rates measured with experimental acceptors.

Electron transport through the glyoxysomal membrane would require an appropriate orientation of the $NADH_2$ dehydrogenase in the membrane, and this has been evaluated using intact or deliberately broken glyoxysomes. Enzyme latency phenomena have been extensively used to assess the integrity of both outer and inner mitochondrial membranes, because these barriers prevent the immediacy of various enzymatic reactions. However, ma-

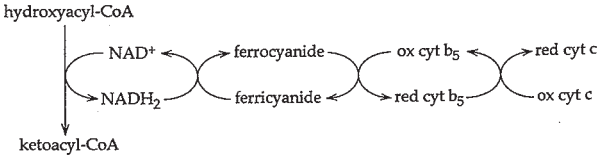

Fig. 3 Reoxidation of the Nicotinamide Coenzyme after its Reduction by the Second Redox Step of β-oxidation (Scheme 1). The reducing equivalents are transferred to an external acceptor by membrane redox compounds. This scheme is based on the $NADH_2$:ferricyanide and cyt c reductase activities measured *in vitro* in the glyoxysomal membrane.

trix enzymes can be organized into functional clusters (metabolons); this supposedly allows the direct interchange of metabolites, but might also create additional latency phenomena. Such 'reactional compartmentation' has for example been observed in leaf peroxisomes (Heupel *et al.*, 1991; Heupel and Heldt, 1994) and in cotyledon glyoxysomes (Guex *et al.*, 1995). In leaf peroxisomes, the suborganelle compartmentation remains functional after osmotic shock, so that isolated organelles lacking an intact boundary membrane can channel their photorespiratory metabolites within the remaining structures, and enzyme latency is still observed. In addition, it is well established that glyoxysomes are fragile organelles and that their membranes are damaged during isolation procedures. This could explain why electron donor and acceptor sites have been assumed to be located on both sides of the membrane, even when latency experiments suggested that the organelles were intact (Luster and Donaldson, 1987).

The evolution of $NADH_2$ dehydrogenase activities in the course of germination has already been determined. The results suggest that the ER and the glyoxysomal membranes are enriched with redox proteins during their development. It is observed that the increase and decrease of the redox activities are coordinated with the glyoxylate cycle activities, with a peak on the 5th day after germination (Alani *et al.*, 1990). This peak actually corresponds to a general maximum in the metabolism of the endosperm, which is strongly active at the onset of germination and then atrophies after depletion of its metabolic reserves.

More intriguing is the $NADPH_2$ dehydrogenase activity measured in the glyoxysomal membrane. Isocitrate dehydrogenase specific for $NADP^+$ is believed to exist in the matrix of animal peroxisomes (Masters and Crane, 1995). In plant glyoxysomes, the presence of such an enzyme concurrently with isocitrate lyase would be detrimental, since the organelle compartmentation of the glyoxylate and citric acid cycles is supposed to appropriately prevent the partition of isocitrate between nonhydrolytic cleavage and oxidative decarboxylation. The activity of a glyoxysomal isocitrate dehydrogenase would induce a net loss of organic carbon during germination, whereas the glyoxylate cycle precisely redirects the carbon flow to gluconeogenesis with a minimum loss. The very minor isocitrate dehydrogenase ($NADP^+$) activity measured in isolated glyoxysomes (Cooper and Beevers, 1969a) cannot actually be considered as significant, and the function of the high proportion (16%) of total cellular $NADP(H_2)$ in these organelles (Donaldson, 1982) therefore remains unclear.

All these experiments were performed on glyoxysomes isolated on density gradients. In order to control that the reductase activities measured in these membranes were not due to contamination by microsomal membranes, the rate of contamination was evaluated using marker enzymes. The purification of organelles does not result in the strict separation of organelles, but the observed level of contamination remains comparatively low. It appears in such a situation that the $NAD(P)H_2$ dehydrogenase activities measured in the glyoxysomal membranes were greater than what could be expected from contaminations by the ER (Fang *et al.*, 1987). However, when soybean glyoxysomes isolated on a sucrose gradient are gently resuspended and reloaded on a second gradient, residual cyt c oxidase activity (a mitochondrial marker) is detected as a sharp band corresponding to the density of mitochondria (C.L. Escher, unpublished results). This observation indicates that mitochondria contaminate the glyoxysomal fractions, even if their marker enzyme activity may first be overlooked. Similar contaminations were shown to be responsible for the mislocations of putative glyoxysomal membrane proteins (for a review, see Mullen and Trelease, 1996). Systematic cross-contamination of membrane material would of course mitigate the hypothesis of a glyoxysomal membrane redox chain involving cyt b_5, and might explain why the ER and glyoxysomal membrane redox systems appear to be similar, as observed after isolation on density gradients. Both membranes possess the same redox proteins [cyt b_5, cyt P_{420}, $NADH_2$ dehydrogenases; Hicks and Donaldson, 1982) and $NADPH_2$ dehydrogenases (Fang *et al.*, 1987)], although the ER is relatively deficient in flavin as compared to glyoxysomal membranes (Hicks and Donaldson, 1982). $NADH_2$:ferricyanide reductases isolated from both membranes are homologous (Luster *et al.*, 1988), and even immunologically indistinct (Struglics *et al.*, 1993). However, the glyoxysomal $NADH_2$:ferricyanide reductase is specific for the β-hydrogen of $NADH_2$ (Struglics *et al.*, 1993), whereas the ER enzyme is α-specific (You *et al.*, 1978). ER and glyoxysomal membrane proteins show similar resistance to carbonate extraction compared to KCl washing, similar elution profiles when submitted to reverse phase chromatography on a C-18 HPLC column, and comparable mobilities in SDS polyacrylamide gel electrophoresis (Donaldson and Gonzalez, 1989). Proteins recognized as common to glyoxysomal and ER membranes were found in similar amounts in both membranes. This observation is particularly intriguing in view of the postulated biogenesis of glyoxysomes, since the glyoxysomal membrane proteins are not synthesized on the ER but on cytosolic free polysomes (for reviews, see Trelease, 1984; Lazarow and Fugiki, 1985; Sulter *et al.*, 1993).

With respect to the supposed occurrence of reductase activities in the glyoxysomal membrane, it is sensible to consider that the performed experiments do not properly represent the functioning of the *in vivo* system, since ferricyanide is not a physiological compound, and cyt c is located *in vivo* within the mitochondrial membrane. A tentative hypothesis had previously suggested the transfer of reducing equivalents by primary electron acceptor(s) from glyoxysomes to secondary acceptor(s) in the cytosol, and finally to the mitochondrial respiratory chain. The ascorbate/dehydroascorbate pair would be a good candidate as an electron mediator, because of its apparent ubiquity in plant cells were it functions as redox buffer, and its favorable reduction potential with respect to the $NADH_2$/NAD^+ pair. $NADH_2$:dehydroascorbate reductase activity

has indeed been measured in glyoxysomal membranes, and this enzyme exhibits characteristics similar to those of the putative $NADH_2$:ferricyanide reductase, such as insensitivity to trypsin digestion or to inactivation by Triton X-100 (Bowditch and Donaldson, 1990). This suggests that dehydroascorbate may be reduced by the same enzyme that reduces ferricyanide *in vitro*. Reoxidation of ascorbate could conceivably be performed by an ascorbate peroxidase, but this would obviously preclude any electron transport to a respiratory chain. Very few membrane proteins have been proven to be peroxisomal/glyoxysomal, but immunogold labeling (using an antibody raised against a 31 kDa glyoxysomal membrane protein; Yamaguchi *et al.*, 1995a) definitely demonstrated the glyoxysomal nature of ascorbate peroxidase (Yamaguchi *et al.*, 1995b). Its active site is now predicted to be on the matrix side of the membrane, and it is assumed that all oilseed glyoxysomes possess such an enzyme (Bunkelmann and Trelease, 1996). The substrates of ascorbate peroxidase are ascorbate and H_2O_2; if the presence of ascorbate in the glyoxysomal matrix has yet not been reported, H_2O_2 is efficiently produced by oxidases and superoxide dismutases.

The latter enzymes have been immunocytochemically localized in the peroxisomal membrane (Del Rio *et al.*, 1983), where they constitute a protective mechanism against superoxide radicals (for a review, see Del Rio *et al.*, 1992). Superoxide radicals may be generated in these organelles by side reactions of the matrix xanthine oxidase and of membrane $NADH_2$ dehydrogenases (Sandalio *et al.*, 1988; Del Rio and Donaldson, 1995).

Dismutation of hydrogen peroxide by glyoxysomal catalase might not be very efficient, because of a K_m value in the 1–5 M range indicative of a low affinity between catalases of various origins and their substrate (Aebi, 1974; Huang *et al.*, 1983). This property mitigates the *in vivo* significance of the long established high molecular activity of such enzymes (Barman, 1969; Schonbaum and Chance, 1976). Consequently, low concentrations of hydrogen peroxide may be more effectively 'scavenged' by ascorbate peroxidase (Bunkelmann and Trelease, 1996), whose K_m value is in the 30–80 mM range (Chen and Asada, 1989). Moreover, since catalase is inhibited by semidehydroascorbate (Davison *et al.*, 1986), high concentrations of ascorbate would further favor the ascorbate peroxidase pathway for H_2O_2 degradation.

The coordinated effects of $NADH_2$:dehydroascorbate reductase and ascorbate peroxidase would thus simultaneously allow regeneration of NAD^+ and scavenging of residual H_2O_2 (Figure 4). This pathway does not involve any transfer of electrons across the glyoxysomal membrane toward an ATP producing system. Formally, the proposed mechanism is stoichiometrically correct if all the H_2O_2 produced by acyl-CoA oxidase is reduced by ascorbate peroxidase. However, when glyoxysomal β-oxidation is strongly active (resulting in the production of significant amounts of hydrogen peroxide), catalase efficiently competes with ascorbate peroxidase. The dehydroascorbate

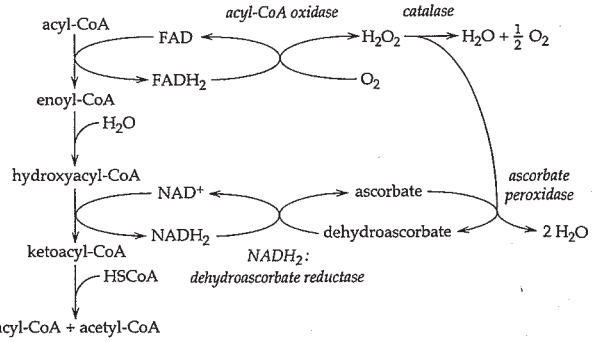

Fig. 4 Reoxidation of the Nicotinamide Coenzyme after its Reduction by the Second Redox Step of β-oxidation (Scheme 2). The reducing equivalents are transferred to matrix dehydroascorbate by an $NADH_2$:dehydroascorbate reductase. The produced ascorbate is then reoxidized by a membrane ascorbate peroxidase.

reductase/peroxidase system may not therefore constitute the primary mechanism for NAD^+ regeneration and H_2O_2 elimination in glyoxysomes, but it certainly provides an essential pathway for the protection of the glyoxysomal membrane against reactive oxygen species, and sustains the pool of NAD^+ required for postgerminative seedling growth (Bunkelmann and Trelease, 1996).

Regeneration of Oxidized Cofactors via a Transmembrane Shuttle

Another way to overcome a membrane barrier is to use a shuttle. Such systems have been described for the transfer of electrons from cytosol to mitochondria, which is mediated by a glycerol-3-phosphate/dihydroxyacetone-phosphate shuttle, and by a malate/aspartate shuttle. A similar system may be considered with regard to the necessity of NAD^+ regeneration sustaining glyoxysomal/peroxisomal β-oxidation. The observation that specific activities of the glyoxysomal forms of malate dehydrogenase and aspartate aminotransferase are 10 to 100-fold higher than those of other glyoxysomal enzymes (Cooper and Beevers, 1969a; Schnarrenberger *et al.*, 1971) suggested indeed that malate and aspartate might be involved in the transfer of electrons between glyoxysomes or leaf peroxisomes and other cellular compartments (Schnarrenberger *et al.*, 1971; Tolbert, 1971). This hypothesis postulates that reducing equivalents are exported as malate, which is oxidized elsewhere in the cell, e.g. in mitochondria, where high malate dehydrogenase and aspartate aminotransferase activities are observed. In order to maintain the required carbon balance and thus to ensure a shuttle mechanism, oxaloacetate returns to the glyoxysome as aspartate, which is reconverted into oxaloacetate by aspartate aminotransferase (Figure 5; Mettler and Beevers, 1980). In this scheme, glyoxysomal malate is not converted into oxaloacetate as previously assumed in the case of an 'independent' glyoxylate cycle (Figure 2), since glyoxysomal malate dehydrogenase would now

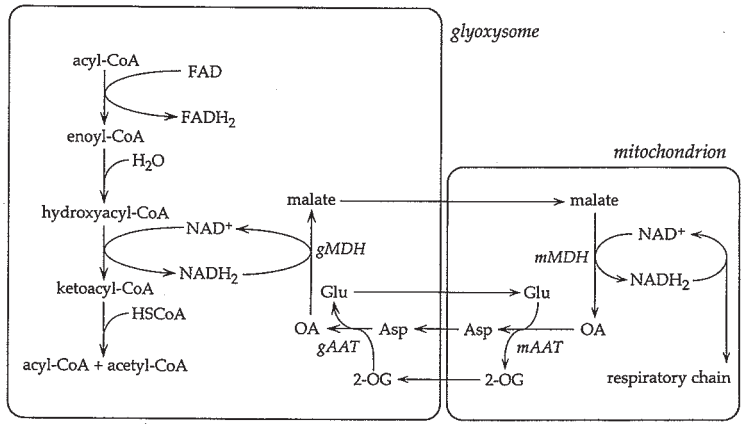

Fig. 5 Reoxidation of the Nicotinamide Coenzyme after Its Reduction by the Second Redox Step of β-oxidation (Scheme 3), as Proposed by Mettler and Beevers (1980).
The reducing equivalents are transferred to the mitochondrial matrix by a malate/aspartate shuttle. Nonstandard abbreviations: gAAT and mAAT, glyoxysomal and mitochondrial aspartate aminotransferases; gMDH and mMDH, glyoxysomal and mitochondrial malate dehydrogenases; OA, oxaloacetate, 2-OG, 2-oxoglutarate.

function in the reductive direction to consume $NADH_2$ and generate malate. Malate oxidation by malate dehydrogenase is a thermodynamically unfavorable reaction. Whether malate or oxaloacetate is formed by malate dehydrogenase actually depends on physiological parameters such as the $NADH_2/NAD^+$ balance. Since this ratio is maintained at a high level by glyoxysomal β-oxidation, oxaloacetate reduction to malate is favored over the reverse reaction. The opposite situation prevails in mitochondria because $NADH_2$ is readily reoxidized by an efficient electron transport chain.

Various tests were performed in order to assess the existence of this shuttle. Addition of aspartate and 2-oxoglutarate to purified glyoxysomes induces a rapid oxidation of accumulated $NADH_2$, and this oxidation is prevented by aminooxyacetate, which is an inhibitor of aminotransferase reactions (Mettler and Beevers, 1980).

The postulated shuttle requires regulated transports of malate, glutamate, aspartate and 2-oxoglutarate through the glyoxysomal and mitochondrial membranes. For transport studies, glyoxysomes purified on sucrose gradients at their buoyant density (1.25 g/cm^3; Huang, 1975) must first be resuspended, but since these organelles are very sensitive to osmotic shocks, no evidence could be obtained for specific transmembrane transport mechanisms for the shuttle intermediates (Mettler and Beevers, 1980). If the presence of transporters in the glyoxysomal membrane has not been proven, it has to be borne in mind that succinate transport from glyoxysomes to mitochondria is well accepted even if the corresponding transporter has not been found. Moreover, the photorespiratory pathway also involves intermediate transport through the membranes of different organelles, namely chloroplasts, peroxisomes and mitochondria (for a review, see Gietl, 1992). In this reactional mechanism, $NADH_2$ required for peroxisomal hydroxypyruvate reduction is equimolar to $NADH_2$ generated by mitochondrial glycine oxidation. Since malate dehydrogenase activity has been measured in leaf peroxisomes (Yamazaki and Tolbert, 1969), redox equivalents are assumed to be transferred from the mitochondrial matrix to the peroxisomal compartment by a malate/aspartate shuttle (Ebbighausen *et al.*, 1987; Reumann *et al.*, 1994). The transport of the shuttle intermediates across the peroxisomal membrane could be mediated by diffusion through specialized pores. The peroxisomal membrane contains a channel forming protein ('porin-like channel'), as demonstrated by purification from isolated organelles and reconstitution (Reumann *et al.*, 1995). The conductance of this channel is 10 to 20-fold lower than that of mitochondria or plastids, which are permeable to hydrophilic molecules up to 4–5 kDa (Fischer *et al.*, 1994). The diameter of peroxisomal channels has been estimated at about 1 nm, and thus appears to be just large enough to let photorespiratory metabolites pass through (Reumann *et al.*, 1995).

Isolation of glyoxysomes/peroxisomes is necessary in order to experimentally validate the hypothesis of a similar shuttle possibly required for NAD^+ regeneration (β-oxidation). In this process, partial tearing of the organelle membrane cannot be avoided, and the evolution of the observed reactions is further affected by the *in vitro* conditions, such as pH and intermediate concentrations. For example, malate oxidation by malate dehydrogenase will inevitably be detected (instead of the opposite reaction required by the shuttle) when electron acceptors are added in such a concentration that the overall equilibrium of the reaction is suitably altered (Donaldson and Fang, 1987). Therefore, the establishment of the *in vivo* pathway beyond any reasonable doubt definitely requires investigations on intact cells. Such experiments were carried out using transformed *Saccharomyces cerevisiae* strains. The inactivation of peroxisomal malate dehydrogenase by disruption of the corresponding gene results in an impaired β-oxidation capacity, which prevents cell growth on oleate and induces a strong accumulation of hydroxyacyl-CoA intermediates (Van Roermund *et al.*, 1995). In contrast,

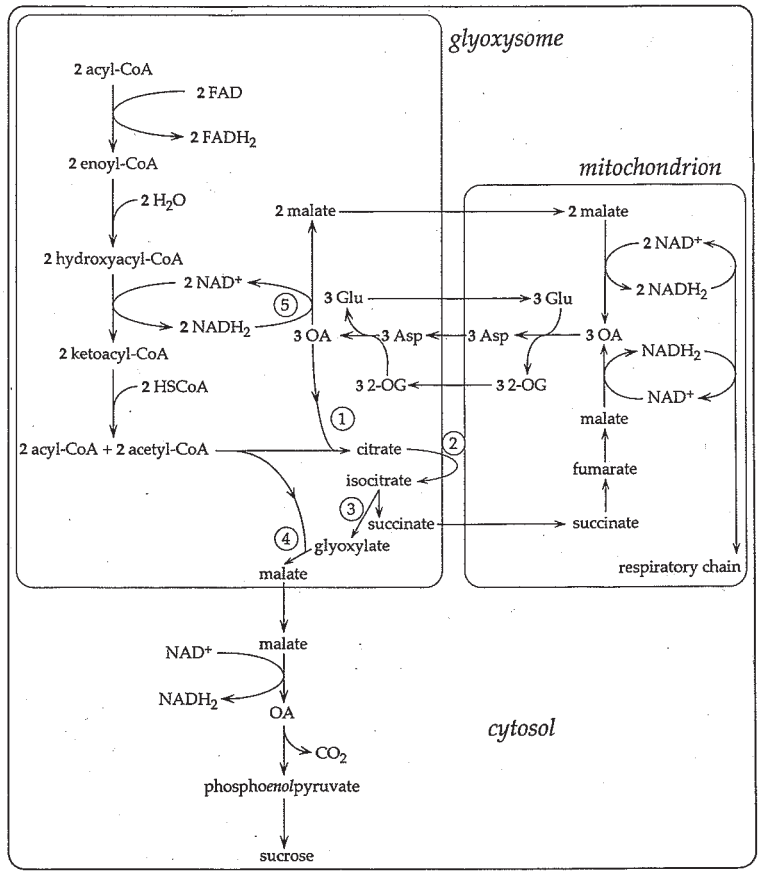

Fig. 6 Glyoxylate Cycle Activities as the Essential Intermediate between Fatty Acid Catabolism and Gluconeogenesis. The circled enzymes are: (1) citrate synthase, (2) aconitase (cytosolic isoform), (3) isocitrate lyase, (4) malate synthase, and (5) malate dehydrogenase (its reduction of oxaloacetate results in the regeneration of glyoxysomal NAD^+). Nonstandard abbreviations: OA, oxaloacetate, 2-OG, 2-oxoglutarate.

growth of transformed yeast on C2 or C3 compounds, such as acetate, ethanol or glycerol (known to be 'upstream substrates' of the glyoxylate cycle) is unaffected (Van Roermund *et al.*, 1995). These results suggest that glyoxysomal/peroxisomal malate dehydrogenase is essentially involved in the reoxidation of $NADH_2$ generated by fatty acid β-oxidation and does not therefore constitute a critical component of a 'classical' glyoxylate cycle.

Interplay between Glyoxysomal, Cytosolic and Mitochondrial Reactional Pathways

The occurrence of the putative malate/aspartate shuttle is supported by the thermodynamic parameters affecting oxaloacetate↔malate interconversions as well as by metabolic needs, and this imposes a reassessment of the current views on the glyoxylate cycle. The initiation of the cycle is considered to be the citrate synthase catalyzed production of citrate from acetyl-CoA and oxaloacetate. Acetyl-CoA can readily be formed by β-oxidation of fatty acids, and it has been assumed that oxaloacetate is produced from malate by the highly active glyoxysomal malate dehydrogenase. However, since it cannot now be ruled out that malate dehydrogenase would function *in vivo* in the reductive direction (thus catalyzing conversion of oxaloacetate into malate), the interconnections between β-oxidation, the glyoxylate cycle, the respiratory chain and gluconeogenesis may be different and/or more intricate than previously thought. The *in vivo* occurrence of a glyoxylate cycle *stricto sensu* might in particular be questioned. Figure 6 (Escher, 1996) proposes that glyoxysomal malate dehydrogenase is an essential and exclusive component of β-oxidation (NAD^+ regeneration). Its substrate oxaloacetate derives from the malate/aspartate shuttle, which also meets the need of citrate synthesis. The transport of succinate to the mitochondrion would equilibrate the shuttle. In this overall scheme, it is also considered that the C4 metabolite initiating gluconeogenesis would formally be malate resulting from the malate synthase catalyzed condensation of acetyl-CoA and glyoxylate.

Concluding Remarks

The sustenance of active gluconeogenesis initiated by lipid degradation during the germination and senescence processes presupposes that the glyoxysomal matrix is the

site of efficient β-oxidation and acetyl-CoA condensation reactions. Since the organelle membrane is not permeable to produced reduced cofactors, their *in situ* reoxidation is required. The readily available acceptor for the electrons generated by the flavine containing acyl-CoA oxidase is molecular oxygen,with subsequent elimination of the produced hydrogen peroxide by catalase and/or peroxidase activities. It is reasonable to assume that the reoxidation of $NADH_2$ produced by hydroxyacyl-CoA dehydrogenase is not independent of this reactional scheme, since the membrane dehydroascorbate reductase/peroxidase system is a likely candidate as a regeneration mechanism of NAD^+, concurrently with the malate/aspartate shuttle connected to the mitochondrial respiratory chain. Both the reductase/peroxidase system and the shuttle might coexist in a dynamic equilibrium depending on the simultaneous and varying necessities of $NADH_2$ reoxidation, protection against damages by hydrogen peroxide and demand for ATP.

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