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Review

The role of peroxisomes in the integration of metabolism and evolutionary diversity of photosynthetic organisms

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Abstract

The peroxisome is a metabolic compartment serving for the rapid oxidation of substrates, a process that is not coupled to energy conservation. In plants and algae, peroxisomes connect biosynthetic and oxidative metabolic routes and compartmentalize potentially lethal steps of metabolism such as the formation of reactive oxygen species and glyoxylate, thus preventing poisoning of the cell and futile recycling. Peroxisomes exhibit properties resembling inside-out vesicles and possess special systems for the import of specific proteins, which form multi-enzyme complexes (metabolons) linking numerous reactions to flavin-dependent oxidation, coupled to the decomposition of hydrogen peroxide by catalase. Hydrogen peroxide and superoxide originating in peroxisomes are important mediators in signal transduction pathways, particularly those involving salicylic acid. By contributing to the synthesis of oxalate, formate and other organic acids, peroxisomes regulate major fluxes of primary and secondary metabolism. The evolutionary diversity of algae has led to the presence of a wide range of enzymes in the peroxisomes that are only similar to higher plants in their direct predecessors, the Charophyceae. The appearance of seed plants was connected to the acquirement by storage tissues, of a peroxisomal fatty acid oxidation function linked to the glyoxylate cycle, which is induced during seed germination and maturation. Rearrangement of the peroxisomal photorespiratory function between different tissues of higher plants led to the appearance of different types of photosynthetic metabolism. The peroxisome may therefore have played a key role in the evolutionary formation of metabolic networks, via establishing interconnections between different metabolic compartments.

Keywords: Peroxisome; Diversity; Evolution; Photorespiration; Reactive oxygen species; Metabolic networks

Contents

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1. Introduction: plasmatic and non-plasmatic compartments

The organization of the eukaryotic cell includes different nucleic acid containing (plasmatic) compartments of endosymbiotic origin (cytosol plus nucleus, matrix space of mitochondria, stroma of plastids) separated by the non-plasmatic (exoplasmatic) space characterized by the absence of nucleic acids. In addition, the non-plasmatic compartments lack ATP, NAD(P)H, and a cytoskeleton, but have a higher [Ca^{2+}] and a lower pH (Schnepf, 1966, 1984; Friedrich, 1986). In plants, the non-plasmatic compartment generally occupies the largest part of the cellular volume, which is connected with its special role in the evolution and adaptation of photosynthetic land plants to a non-aqueous environment.

The non-plasmatic compartment can be viewed as an internalized outer space, a part of the aqueous environment captured during the process of going from water to land. The non-plasmatic compartments carry out different crucial functions: lytic (vacuole in plants, lysosome in animal cells), sorting (Golgi), transporting (endoplasmic reticulum and plasmodesmata). It was proposed that all non-plasmatic compartments are interconnected and that the symbiotic organelles are really located in the endoplasmic reticulum (ER), which is percolating between cells as the plasmodesmata system (Gamalei, 1997). The lumens of the ER, Golgi complex and vacuole are topologically equivalent to the cell exterior (Vitale and Denecke, 1999). Vacuoles and complex Golgi are homologous, since there is an underlying similarity in targeting determinants in the cytoplasmic tails of Golgi-based receptors (Robinson and Hinz, 1997).

Peroxisomes, organelles possessing an oxidative function and separated from the cytosol by one membrane, are not completely equivalent to the non-plasmatic compartments. During the biogenesis of peroxisomes, proteins are directed into them mostly via recognition of the terminus that is opposite to that recognized by other organelles. The transported proteins are assembled in the cytosol and in oligomeric complexes with specific peroxisomal membrane proteins, are incorporated into peroxisomes (McNew and Goodman, 1996; Titorenko et al., 2000; Titorenko and Rachubinski, 2001; Mullen et al., 2001a,b). The direct interconnection between peroxisomes and other non-plasmatic compartments has not been shown. However, an interconnection net between individual peroxisomes (peroxisomal reticulum) has been proposed (Lazarow and Fujiki, 1985). Both the C and N termini of the peroxisomal membrane protein PMP34 are directed to the cytosol in human cells (Fujiki, 2000; Honsho and Fujiki, 2001). However, data for the plant peroxisomal membrane protein, ascorbate peroxidase, indicates that the N terminus faces the cytosol and the C terminus faces the matrix (Mullen et al., 2001b), i.e. it has an orientation opposite to other non-plasmatic compartments. The internal pH of peroxisomes is usually 7 or even higher, similar to plasmatic compartments (Dansen et al., 2001), peroxisomes carry out the turnover of NADH (Givan and Kleczkowski, 1992) and NADPH (Corpas et al., 1998). Thus, the peroxisomal compartment is not fully homologous to the other non-plasmatic compartments, encompassing the properties of both plasmatic and non-plasmatic constituents of the cell. These unusual properties of peroxisomes together with the data on the sequences of peroxisomal enzymes and their genes, led to a hypothesis proposing the endosymbiotic origin of peroxisomes, as for mitochondria and plastids (Latruffe and Vamecq, 2000), this however still lacks definite substantiation.

In this review, we have analyzed the role of the peroxisomal compartment (as a separate compartment differing from both plasmatic and non-plasmatic compartments) in plant metabolism and have outlined the ontogenetic and evolutionary consequences of peroxisomal function. In particular, we have discussed the participation of peroxisomes in signal transducton pathways and the adaptation of plants to changing levels of O_2 and CO_2 in the atmosphere.

2. Metabolic significance of peroxisomes

Various types of peroxisomes can be classified based on the nature of the prevalent substrates that they oxidize. Hydrolysis of proteins by proteases is common for different types of peroxisomes, and the liberated amino acids are transaminated to organic acids prior to oxidation.

Ureide-type peroxisomes of symbiotic nodules of legumes and other plants, perform the oxidation of nucleotides, xanthine and uric acid (Smith and Atkins, 2002). The glyoxysome type of peroxisomes carries out the oxidation of fatty acids in germinating seeds coupled to the operation of the glyoxylate cycle (Eastmond and Graham, 2001). Oxidation of glycolate is the main function of photorespiratory (leaf) peroxisomes (Heupel and Heldt, 1994). Oxidations of methanol, alkanes, amines and D-amino acids are the other functions of certain types of peroxisomes, mostly in primitive organisms (Huang et al., 1983). To a greater or lesser extent all peroxisomes possess most (or even all) of these functions, but one function may be amplified many times; this corresponds to the specialization of peroxisomes (for review see Huang et al., 1983). Peroxisomes are really the 'organelles at the crossroad' (Erdmann et al., 1997): different cell compartments are linked via peroxisomal reactions. There are also unique functions for peroxisomes, like bioluminescence in algae and in insects (Morse et al., 1990). In lower organisms, peroxisomes are involved in many terminal oxidations, whereas at higher evolutionary levels, they catalyze intermediate steps of oxidative pathways coupled to biosynthetic reactions.

Most of the peroxisomal functions mentioned above are also present in other cell compartments, where they may be coupled to ATP synthesis (although mitochondria of plants and fungi also carry out oxidation reactions not coupled to energy conservation). This means that oxidative processes are duplicated in plasmatic and peroxisomal compartments. Peroxisomes are metabolically located in the midway position towards mitochondria: they are involved in the 'intermediate' oxidation of biosynthetically formed products, such as fatty acids during the germination of fat-storing seeds, glycolate in photosynthetic tissues or urates in the legume nodules. In all cases, the peroxisomal pathway connects different organelles including those having endosymbiotic origin: chloroplasts and mitochondria in photosynthetic tissues, bacteroids, plastids and mitochondria in nitrogen-fixing nodules, oleosomes (utilizing triacylglycerols from fatty acids synthesized in plastids) and mitochondria in fat-utilizing seedlings. The peroxisome can be regarded as having developed during evolution as an 'internalized outer space' for these organelles, e.g. photorespiratory glycolate is excreted into the environment by algae (some of them, like *Chlamydomonas*, may even lack or almost lack peroxisomes); in higher plants glycolate is metabolized inside the peroxisome.

The metabolic flux through peroxisomes is determined by the concentrations of substrates, by the amount of the enzymes present in the matrix and membrane and (for optimal channeling) by supramolecular organization of enzymes (catalase cores, metabolons). Many peroxisomal enzymes possess relatively broad specificities, including flavin-dependent oxidases and aminotransferases (Liepman and Olsen, 2001). On the other hand, mitochondrial metabolism has many control points possessing complex modes of regulation. In animals, mitochondria fulfill mostly the function of oxidative phosphorylation; in plants this function is a part of a main regulatory process required to prevent over-reduction, balancing NAD(H)/NADP(H) ratios, and supplying ATP to the cytosol (Gardeström and Wigge, 1988; Gardeström and Lernmark, 1995; Noctor and Foyer, 1998, 2000; Mackenzie and McIntosh, 1999).

Plant cells contain pairs of enzymes fulfilling similar functions but differing in the energy coupling of their oxidative reactions. These are acyl-CoA dehydrogenase in mitochondria and a corresponding oxidase in peroxisomes; glycolate dehydrogenase characteristic of algal mitochondria and also probably chloroplasts of higher plants (Goyal and Tolbert, 1996) and glycolate oxidase in the peroxisomes. Another complementary pair is xanthine dehydrogenase–xanthine oxidase. It was demonstrated that non-coupled superoxide-generating xanthine oxidase appears in senescent leaves as a result of proteolytic cleavage of NAD^+ dependent xanthine dehydrogenase by a specific peroxisomal endoprotease (Distefano et al., 1999), indicating that peroxisomal endoproteases are involved in the regulated modification of proteins. Citrate synthase in glyoxysomes differs from the mitochondrial isoform by the absence of allosteric regulation by ATP, i.e., its function is not coupled to the ATP pool (Barbareschi et al., 1974).

Thus, the peroxisome is an organelle complementary to a mitochondrion (in many functions) and sometimes to other compartments. The previously mentioned peroxisomal oxidases are not coupled to energy conservation, and they are also less specific for their substrates than their mitochondrial counterparts. This leads to the appearance of metabolic bifurcations in peroxisomes (Igamberdiev, 1989, 1999). However, the duplication of oxidative pathways, one that is potentially coupled and the other not coupled to ATP synthesis, provides a robustness of metabolic organization, important for adaptation to a changing environment (Barkai and Leibler, 1997). Peroxisome metabolism provides mechanisms of bypassing certain pathways without the requirement for redox control (fatty acid oxidation, glycolate oxidase, NADH oxidation). Similarly there is no fine-tuned metabolic control in peroxisomes when electron transport is passing via catalase. Also, the oxidation of peroxisomal derived substrates (e.g. glycine from photorespiration and succinate from the glyoxylate cycle) in mitochondria is always less coupled than of substrates synthesised directly from mitochondrial metabolism (Millhouse and Wiskich, 1986; Igamberdiev et al., 1997). The increase of NAD and/or NADP

reduction level and turnover in peroxisomes leads to the formation of signalling molecules, while the increase of flux through peroxisomes may lead to leakage of glyoxylate, formate and oxalate — strong regulators of metabolism. The interconnections between plasmatic and non-plasmatic compartments in plants are crucial for the understanding of organization and evolution of metabolism. The eukaryotic plant cell can be viewed as a system of embedded endosymbiotic compartments (Herrmann, 1997). In this system, the peroxisome (as a compartment having properties of both plasmatic and non-plasmatic parts) fulfills an important interconnecting role. It can be regarded as a unique non-plasmatic-type compartment with incorporated plasmatic content and unique direction of membrane proteins (inside-out orientation).

3. Specialization of peroxisomes: intracellular heterotopy

Peroxisomes possess complex import machinery differing significantly from that in other organelles (Mullen and Trelease, 1996; Mullen et al., 2001a). This machinery allows peroxisomes to acquire specialization that results in realizing different functions at different stages of ontogenesis and in different tissues. It is based on a special templating (chaperone) function of peroxisomal membranes resulting in intracellular transfer (heterotopy) of newly synthesized proteins into peroxisomes.

It is now evident that peroxisomes possess their own specific mechanism of protein import distinct from that of the endoplasmic reticulum (ER) and other compartments. In these other compartments, the signal for import is an alkaline N-terminal sequence that is recognized and cleaved during translocation through the membrane. In contrast, most peroxisomal proteins are recognized by a C-terminus of SKL (serine-lysine-leucine) or similar sequence of three amino acids which is not cleaved in most cases. Sometimes it can be extended to 11 or even 20 amino acids (Hoop and Ab, 1992). A C-terminal position of the microbody import signal implies that microbody proteins will have become folded before the signal emerges from the ribosome. This clearly indicates that the peroxisomal membrane has different chaperone properties from the ER membrane, possessing some characteristics of an inside-out membrane, compared to other non-plasmatic compartments.

Those peroxisomal proteins that are recognized from the N-terminus have such a sequence that can function internally (even being fused into the C-terminus) (Rehling et al., 1996). Import and cleavage in these cases are not coupled, contrary to import into other organelles (Hettema et al., 1999). At least four peroxisomal enzymes—malate dehydrogenase, citrate synthase, acyl-CoA oxidase and 3-ketoacyl-CoA-thiolase are imported via recognition of the N-terminus (Kato et al., 2000). Thus, two main different types of targeting signals direct proteins into the peroxisome matrix. Each targeting signal interacts with a different receptor protein, which may also form a complex with the peroxisomal protein before it docks on the membrane. Peroxisomes have the unusual capacity of being able to import proteins that are fully folded or assembled into oligomers.

Some translocators in the peroxisomal membrane resemble their mitochondrial counterparts, e.g. a Ca^{2+}-dependent translocator from rabbit intestine exhibits a 67% homology to the human ADP/ATP mitochondrial translocator. The Ca^{2+}-binding N-terminal half of the peroxisomal translocator faces the cytosol (Weber et al., 1997). This property resembles the orientation of a membrane, which is similar to plasmatic compartments and indicates that peroxisomes being non-plasmatic vesicles, nevertheless possess some features of inside-out orientation. The import machinery of peroxisomes may also include similar chaperone proteins as identified in chloroplasts and mitochondria. It was found that the 72-kDa molecular chaperone (Hsp70) is encoded by a single gene, but targeted alternatively into two organelles (plastids and peroxisomes) by the modulation of its presequence (Wimmer et al., 1997).

Other evidence for an inside-out operation of peroxisomes, is the oxidation of NAD(P)H inside the organelle, whereas in the ER it is oxidized outside. The energy may be needed for membrane internalization during import of oligomers, i.e. for providing endocytosis-like import of peroxisomal proteins. There is ample evidence for internal peroxisomal membrane structures, and the peroxisomal membrane might be a very dynamic system undergoing rapid changes in conformation (McNew and Goodman, 1996). If membranes are internalized, this may allow a sorted protein to enter the matrix. These observations confirm the hypothesis concerning the inside-out properties of peroxisomes, which distinguish these organelles from other non-plasmatic compartments. In this case, translocating proteins are located in plasmatic vesicles which are subsequently released into the peroxisomal compartment (McNew and Goodman, 1996). Another possibility is the presence of a putative structure similar to a nuclear pore (which connects the two plasmatic compartments), but which assembles only transiently and is therefore hard to detect (McNew and Goodman, 1996).

Most peroxisome biogenesis disorders including those in humans, are caused by a failure to target peroxisomal proteins to the organellar matrix or membrane (Hettema et al., 1999). Peroxisomal protein import does not require unfolding of the targeted protein. However, the molecular chaperones Hsp70 and Hsp40 may be important for translocation (Crookes and Olsen, 1998). Many of the proteins (peroxins) that play critical roles

in peroxisome biogenesis have been identified, some of the interacting partners include molecular chaperones (Crookes and Olsen, 1999). For human cells, it was proposed that peroxisomes may be formed by either of two pathways: one that involves Pex11-mediated division of preexisting peroxisomes, and another that involves Pex16-mediated formation of peroxisomes de novo, in the absence of preexisting peroxisomes (South and Gould, 1999). Peroxisome assembly includes both ATP-independent and ATP-dependent steps (López-Huertas et al., 1999a). The membrane protein Pex14p functions at the first stage, before the ATP-dependent step of peroxisome assembly.

There is no substantial difference in the properties of glyoxysomal and leaf-type-peroxisomal membranes involved in protein import. Experiments showed that glyoxysomal proteins are transported into several classes of peroxisomes using a common targeting determinant, suggesting that protein import does not play a regulatory role in determining peroxisomal function. Rather, the specific metabolic role of peroxisomes appears to be determined primarily by processes that regulate the synthesis and/or stability of its constituent proteins. These processes are specified by the differentiated state of the cells in which the organelles are found (Olsen et al., 1993).

The uptake of glycolate oxidase into peroxisomes has been studied using an in vitro import system. Import of glycolate oxidase was found to be ATP-dependent and temperature-dependent and specific for peroxisomes. In these respects, it resembles the import of isocitrate lyase into both glyoxysomes and leaf-type peroxisomes; thus the ATP-dependence and temperature dependence appear to be general properties of plant microbody protein import (Horng et al., 1995). At present, the C-terminal motif is a well-established peroxisome-targeting signal. In cotton and tobacco cells, antiserum raised against SKL peptides recognized proteins only in the peroxisomes. In *Arabidopsis thaliana* it was demonstrated that SKL-COOH or its modification KSRM-COOH is necessary for in vivo targeting of proteins to plant peroxisomes (Trelease et al., 1996).

Twenty-three proteins termed peroxins are required for peroxisome biogenesis, the roles of some of these have been determined (Olsen, 1998). Some peroxins can act as cycling receptors, which pick up proteins in the cytosol and deliver them to the peroxisomal membrane. After penetrating into peroxisomes, they are exported out for another round of targeting (Hettema et al., 1999). Peroxisomal membrane proteins are themselves incorporated into peroxisomal membranes by specific targeting signals, which include at least two non-overlapping sets of targeting information (Jones et al., 2001). One sequence is a short positively charged intervening loop sequence, whilst the other includes flanking hydrophobic segments (Honsho and Fujiki, 2001). In this process, incorporation of some peroxins precedes incorporation of the others, ensuring the solubility of integral peroxisomal membrane proteins (Jones et al., 2001). The peroxisomal ascorbate peroxidase was shown to undergo membrane "zippering", i.e. oligomerization via formation of cytosol facing membrane vesicles, which are then integrally inserted into the organelles (Mullen et al., 2001b).

The cytoskeleton participates in the formation of peroxisomes and the dynamics of the peroxisomal reticulum, by facilitating contacts between peroxisomes and newly formed peroxisomal vesicles with oligomerized proteins (Schrader et al., 2000). Plant peroxisomes exhibit movement within cells driven by the actin-polymerization. This movement, contrary to animal cells, does not involve myosin microtubule motors and it could be responsible for the coordinated motility of peroxisomes, chloroplasts and mitochondria (Mathur et al., 2002).

4. Metabolon organization and metabolite transport in peroxisomes

Peroxisomes are organized in such a way that the peroxisomal membrane does not play a significant role as a barrier, but surrounds the matrix core organized as a metabolon. Peroxisomal multienzyme complexes allow efficient metabolic channelling with high flux rates and minimum leakage of reactive oxygen species from the organelle. The membrane is a very important site of electron transport that is characterized by special organization, intrinsic for peroxisomes.

In plants, the fatty acid oxidation enzyme apparatus is located mostly within glyoxysomes or peroxisomes, the mitochondrial β-oxidation pathway is minor, if present (Masterson and Wood, 2000). Following the formation of an acyl-CoA-ester, the machinery for the degradation of endogenous fatty acids consists of a family of acyl-CoA oxidase isozymes with distinct fatty acyl-CoA chain-length specificities (Hayashi et al., 1999; Eastmond et al., 2000a). It is followed by L-3-hydroxyacyl-CoA hydrolyase, L-3-hydroxyacyl-CoA dehydrogenase, D-3-hydroxyacyl-CoA epimerase and 2,3-enoyl-CoA isomerase activities, all four being confined to a single multifunctional protein, and a separate 3-ketoacyl-CoA thiolase (Preisigmüller et al., 2000). During the oxidation of CoA esters of branched chain fatty acids (e.g. formed by the deamination of leucine) in peroxisomes, free acid intermediates are formed which include 2-hydroxyisovalerate. The latter is oxidized by 2-hydroxyacid oxidase producing H_2O_2 (as opposed to the mitochondrial dehydrogenase), which is similar to glycolate oxidase but differs from it in specificity (Gerbling, 1993).

The specific structure of the peroxisomal matrix shows signs of disorganization, if peroxisomes are incubated in a

medium which contains a substrate for a key oxidase (e.g. glycolate) but is devoid of a substrate for coupled transamination (Heupel and Heldt, 1994). These data imply that a complete set of peroxisomal enzymes is necessary for the formation of a standard organelle. The integrity of peroxisomes is associated with their functioning (Luzikov, 1999). Research on intact and osmotically shocked peroxisomes revealed that the specificity of redox transfer into the peroxisomes is not due to selectivity of the peroxisomal boundary membrane but to the multienzyme structure of the peroxisomal matrix (Reumann et al., 1994). The required reducing equivalents are provided by the chloroplasts and mitochondria, via the malate-oxaloacetate shuttle, in which oxaloacetate is reduced in these organelles by NAD(P)H generated during photosynthesis and glycine oxidation (Krömer, 1995). In the photorespiratory cycle in a leaf during photosynthesis, the transfer of redox equivalents from the mitochondria to peroxisomes via the malate-oxaloacetate shuttle can operate under conditions of the very low reductive state of the NADH/NAD system prevailing in the cytosol of mesophyll cells (Raghavendra et al., 1998).

Evidence was obtained that the high permeability of the peroxisomal membrane is caused by pore-forming protein (VanVeldhoven et al., 1987; Corpas et al., 2000). It represents a relatively unspecific but highly efficient transport system (Reumann, 2000). In the photorespiratory pathway, numerous shuttles exist to support transamination, ammonia refixation and the supply or export of reductants generated or consumed (via malate-oxaloacetate shuttles) (Douce and Neuburger, 1989). A porin-like channel that is anion selective represents the major permeability pathway of the peroxisomal membrane. The membrane of spinach leaf peroxisomes contains an anion-selective channel that does not form a general diffusion pore similarly to known eukaryotic porins, but has properties comparable to specific and inducible porins, which have been characterized in some gram-negative bacteria (Reumann et al., 1998). The single channel conductance was found to be only moderately dependent on the salt concentration in the aqueous phase. This may be explained by the presence of positive point net charges in or near the channel, or by the presence of a saturable binding site inside the channel (Reumann et al., 1995).

Glyoxysomes of castor bean endosperm contain a similar porin-like channel (Reumann et al., 1997). The porin of glyoxysomes was shown to have a relatively small single-channel and to be strongly anion selective. Thus, the glyoxysomal porin differs from the other previously characterized porins in the outer membrane of mitochondria or plastids, but is similar to the porin of spinach leaf peroxisomes. By analogy to the porin of leaf peroxisomes, the glyoxysomal porin facilitates the passage of small metabolites, such as succinate, citrate, malate, and aspartate, through the membrane.

Crystalline structures termed cores are frequently seen inside peroxisomes (Fig. 1). Three-dimensional reconstruction of peroxisomal and glyoxysomal cores from sunflower suggested that they are quadrangular blocks. Ultrastructural analysis revealed a regular periodic arrangement of repeating units, which are probably cubes with 20 nm long edges (Tenberge et al., 1997). Isolated peroxisomal cores from potato tubers were regular rhomboidal prisms. The cores appear to consist solely of enzymically active catalase, but the substructure may vary from species to species (Tenberge et al., 1997). The catalase in peroxisomal cores was shown to be a 59 kDa form which differs in amino acid sequence from the 55 kDa form found in the peroxisomal matrix (Kleff et al., 1997). Three cDNA clones for catalase were isolated from pumpkin cotyledons. The expression pattern of the first was similar to that of malate synthase, a characteristic enzyme of glyoxysomes suggesting that it might encode a catalase associated with glyoxysomal functions. The mRNA of the second was present at high levels in green cotyledons and the mature leaf, being characteristic for leaf peroxisomes. The third was abundant in both green and etiolated tissues (Esaka et al., 1997). These data suggest that besides

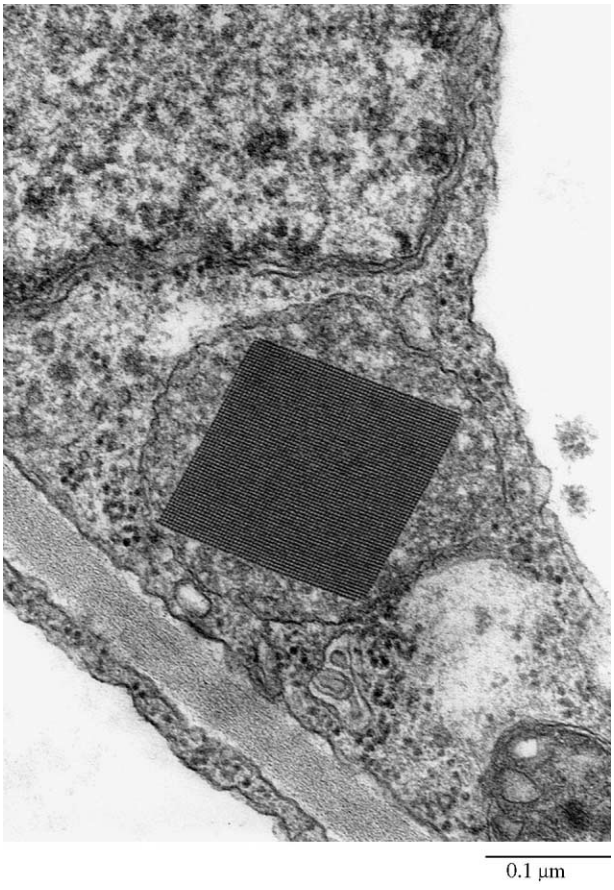

Fig. 1. Electron micrograph of a crystal-containing peroxisome in a leaf cell of potato *Solanum tuberosum* L. (generously provided by Dr. M.L. Parker, Institute of Food Research, Norwich, UK).

a common enzyme, there are specialized catalases that can provide the most efficient flux through the different specialized peroxisomes. In barley, light-inducible and light-repressible catalase genes and proteins were found (Acevedo et al., 1996; Holtman et al., 1998) which corresponds to the presence of isoenzymes associated with leaf peroxisomes and glyoxysomes (Skadsen et al., 1995).

It has been proposed that the channeling of metabolites through multienzyme clusters or metabolons in the peroxisome provide the possibility of passing intermediates rapidly through a pathway without delivering them first to the bulk phase, thereby circumventing diffusion and escape from the organelle. The following metabolons have been isolated from different types of peroxisomes: isocitrate lyase - malate synthase complex (Beeckmans et al., 1994), acyl-CoA oxidizing metabolon containing a tetrafunctional protein (Kindl, 1993), leaf peroxisomal metabolon (Heupel and Heldt, 1994). Malate dehydrogenase and citrate synthase have appeared in peroxisomes probably at later stages of evolution. They are observed in these organelles mostly in higher plants. The different mechanism of their import (via N-targeting signal) compared to other peroxisomal proteins is in agreement with this suggestion.

5. Peroxisomes and signal transduction pathways involving reactive oxygen species

Peroxisomes regulate redox level in cells, thus it is reasonable that they may also be involved in signal transduction pathways connected with redox regulation. Signal messengers produced in peroxisomes are the reactive species, hydrogen peroxide, superoxide radical and nitric oxide (Fig. 2). Also organic acids like glyoxylate, oxalate and formate are involved in the chains of regulation of major metabolic fluxes and of NAD(P)H/NAD(P) ratios in cytosol and chloroplasts. A high redox state in peroxisomes is maintained by an increased influx of isocitrate and to a lesser extent of glucose-6-phosphate (Corpas et al., 1998), generating NADPH (Fig. 3) and by intensive oxidation of malate generating NADH (Fig. 4). Association of malate dehydrogenase with the peroxisomal membrane in potato tubers was observed, generating NADH oxidized by a short electron transport chain in the membrane containing β-specific NADH-ferricyanide reductase and a *b*-type cytochrome (Struglics et al., 1993). It has also been demonstrated that NADPH is involved in the production of NO and both NADH and NADPH are responsible for the production of superoxide (del Río and Donaldson, 1995; Corpas et al., 2001). Superoxide anion is generated by the membrane proteins PMP18, PMP56 and PMP61 during oxidation of NADH and by PMP29 during oxidation of NADPH (López-Huertas et al., 1999b).

Hydrogen peroxide has been implicated in the signalling and response to many stress conditions (Levine et al., 1994). Mutants and transformed plants with specific decreases in key components have been used to dissect the complex system that maintains redox homeostasis. Since H_2O_2 is a signal-transducing molecule relaying information on intracellular redox state, its pool size must be rigorously controlled within each compartment of the cell (Noctor et al., 2000). A mutant of barley deficient in catalase (10% of wild type), showed that high rate of production of H_2O_2 occurred in normal air, while in 0.2% CO_2 it was very low (Kendall et al., 1983; Azevedo et al., 1998). In normal air, accumulation of hydrogen peroxide resulted in disruption of granal membranes and other damage of the chloroplasts (Parker and Lea, 1983). By using transgenic tobacco with 10% wild-type catalase activity, it was also shown that in elevated light plants rapidly developed white necrotic lesions on the leaves. Leaf necrosis correlated with accumulation of oxidized glutathione and a 4-fold decrease in ascorbate, indicating that catalase is critical for maintaining the redox balance during oxidative stress (Smith et al., 1984, 1985; Willekens et al., 1997). Stress analysis revealed increased susceptibility of catalase-deficient plants to paraquat, salt and ozone, but not to chilling.

The production of another key signaling reactive oxygen species superoxide is a common metabolic property of peroxisomal membranes (del Río and Donaldson, 1995; del Río et al., 1998). Several integral membrane polypeptides have been shown to be involved in superoxide radical production (López-Huertas et al., 1997,1999b). Close relationships exist between peroxisomal metabolism and the control of functional damage caused by reactive oxygen species, which can act as second messengers (Masters, 1996, 1998). The signalling systems include those governing peroxisome proliferation and regulating the permeability of the peroxisomal membrane.

Since many stress conditions are accompanied by an increase of redox state, a role of the peroxisome compartment may be to assist in restoration of the cellular redox balance. The import of proteins into glyoxysomes is under the control of the cytosolic NADPH/NADP ratio, elevation of which inhibits it, however leaf peroxisomes lose this property (Pool et al., 1998). NADPH (which can be generated in peroxisomes via oxidation of isocitrate and glucose-6-phosphate) is required for the generation of NO inside peroxisomes, for the protection of catalase from oxidative damage and for increasing the level of reduced glutathione in peroxisomes, thus protecting glycolate oxidase from photoinhibition (Corpas et al., 2001 and references therein).

Different stresses induce peroxisome biogenesis genes by the action of hydrogen peroxide (López-Huertas et al., 2000). The redox level is modulated by peroxisomes

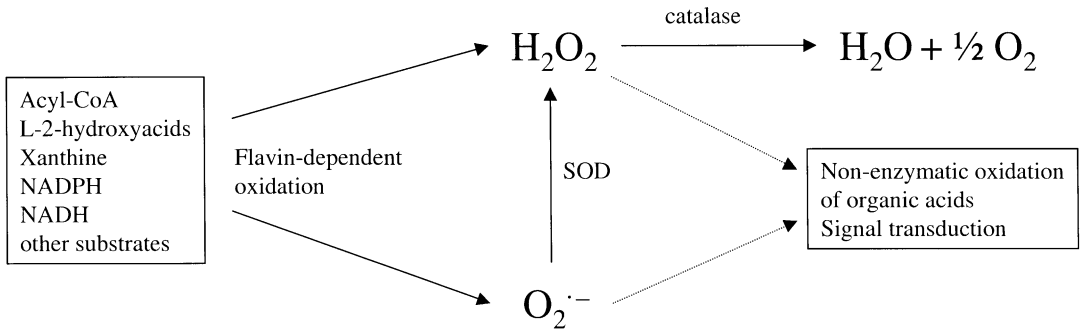

Fig. 2. Flavin-dependent oxidation of peroxisomal substrates and the formation of reactive oxygen species; SOD—superoxide dismutase.

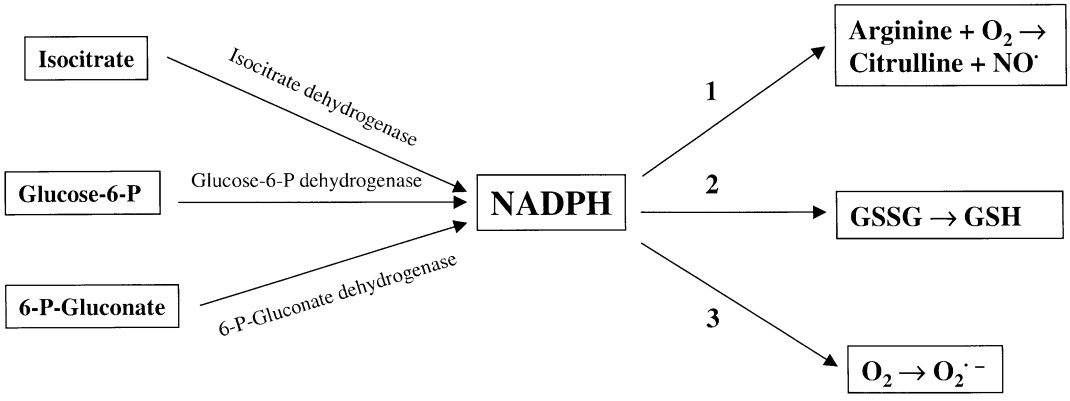

Fig. 3. NADPH turnover in peroxisomes. NADPH is formed by NADP-isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. It is used for: (1) the production of NO from arginine by NO-synthase; (2) the reduction of glutathione and (3) the production of superoxide by PMP29.

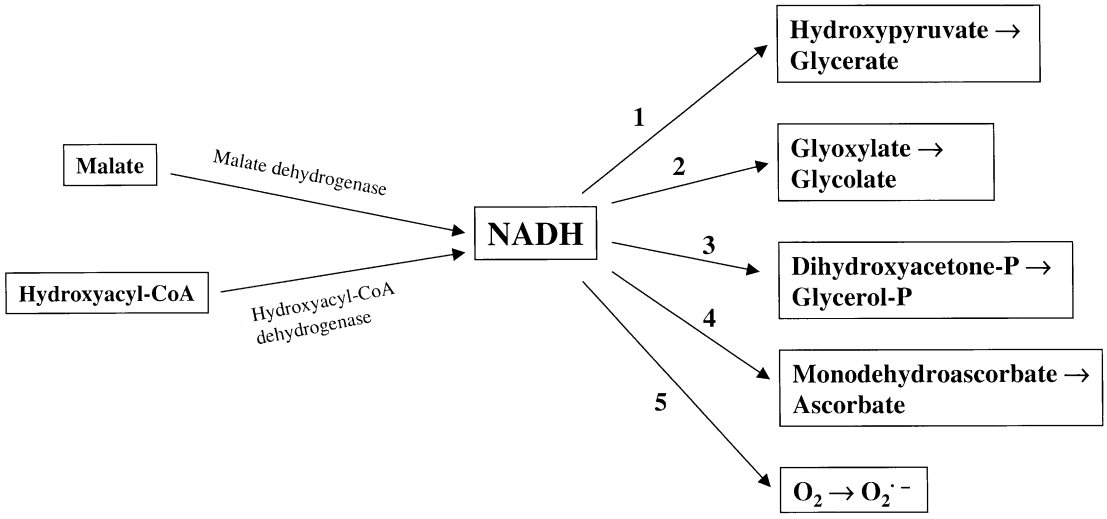

Fig. 4. NADH turnover in peroxisomes. NADH is formed mainly by malate dehydrogenase and hydroxyacyl-CoA dehydrogenase. It is used for: (1) the reduction of hydroxypyruvate; (2) the reduction of glyoxylate; (3) the reduction of dihydroxyacetone phosphate; (4) the reduction of monodehydroascorbate by monodehydroascorbate reductase (PMP32); (5) the reduction of flavins/ cytochromes by membrane proteins PMP18, PMP56 and PMP61 with the production of superoxide.

via changes in the levels of ascorbate-glutathione cycle enzymes, although, apart from ascorbate peroxidase, the enzyme activities are lower than those detected in mitochondria (Jiménez et al., 1997, 1998). During senescence, the membrane-bound ascorbate peroxidase and monodehydroascorbate reductase activities decrease, dehydroascorbate reductase activity in the matrix increases, while glutathione reductase remains unchanged (del Río et al., 1998; Jiménez et al., 1998). As a result, the ratios of ascorbate to dehydroascorbate and of reduced to oxidized glutathione are altered. The mitochondria are affected by oxidative damage earlier than peroxisomes, which participate in the cellular oxidative mechanism longer than mitochondria, under senescence and stress (del Río et al., 1998).

The redox state of peroxisomes is controlled by the rates of synthesis and degradation of reactive oxygen species. Superoxide generation takes place both in the matrix of peroxisome via a xanthine oxidase system and in the membrane via oxidation of NAD(P)H, whilst peroxide generation takes place via the operation of flavin-containing oxidases present in the matrix and in the membranes. Plant peroxisomes contain Cu/Zn-containing superoxide dismutase in the matrix (Sandalio et al., 1997) and some peroxisomes (e.g. leaf peroxisomes) also contain a membrane-bound Mn-containing superoxide dismutase (Sandalio et al., 1987; Sandalio and del Río, 1988; Palma et al., 1998). Besides catalase, the H_2O_2 level is also controlled by ascorbate peroxidase participating in the ascorbate-glutathione cycle. Under normal physiological conditions, this cycle maintains an appreciable level of H_2O_2 and O_2^-, while under stress when production of reactive oxygen species is increased their concentration will be established at higher levels. Superoxide radicals inhibit catalase activity (Kono and Fridovich, 1982), while NO and peroxynitrite (a powerful oxidant formed by interaction between NO and O_2^-) inhibit both ascorbate peroxidase and catalase (Clark et al., 2000). In animal peroxisomes, glutathione peroxidase exhibits inhibition by NO while β-oxidation is enhanced by this radical (Dobashi et al., 1997).

A role of photorespiration and peroxisomal metabolism in signal transduction pathways can also be inferred from the fact that photorespiratory glycine enhances glutathione accumulation in both chloroplastic and cytosolic compartments (Noctor et al., 1999). Glutathione and ascorbate are part of a redox signalling system activating many redox-dependent enzymes and participating in stress response. Glutathione protects glycolate oxidase against photoinactivation (Schäfer and Feierabend, 2000). Ascorbate, glutathione and active oxygen species are metabolic indicators of redox status, acting as sensors and signal molecules leading to acclimatory responses (Foyer and Noctor, 2000). The *A. thaliana* gene APX3 that encodes a putative peroxisomal membrane-bound ascorbate peroxidase was expressed in transgenic tobacco plants and was shown to protect leaves against oxidative stress damage (Wang et al., 1999).

Plant defence mechanisms include two independent signal transduction chains, one involving salicylic acid and another involving nitric oxide. Salicylic acid related oxidative damage requires H_2O_2 (Rao et al., 1997). Thus it was suggested that it inhibits catalase via direct binding to the enzyme (Takahashi et al., 1997). However, salicylic acid was later shown not to act as an inhibitor of either catalase or ascorbate peroxidase in soybean (Tenhaken and Rubel, 1997). It was shown nevertheless that the signal transduction chain requiring salicylic acid, involves many factors including catalase and ascorbate peroxidase, and that peroxisomes play an important role in this process (Klessig et al., 2000). During the infection process in soybean, an attack of the bacterial parasite (*Bacillus* and *Erwinia*) induced peroxisomal ureide oxidizing enzymes involved in steps of decomposition of nucleic acids. The glyoxylate cycle enzymes were also induced, participating in the utilization of lipids liberated from necrotized cells (Cots and Widmer, 1999). It is assumed that in such a case, the glyoxylate cycle is reinitiated as a part of a carbon reallocation system feeding on the diseased tissue cellular components. Produced during the "oxidative burst", reactive oxygen species activate programmed cell death and induce antimicrobial defences such as pathogenesis-related proteins. During the interaction of plants with pathogens, the expression of reactive oxygen-detoxifying enzymes such as ascorbate peroxidase and catalase is suppressed. This suppression, occurring upon pathogen recognition, plays a key role in elevating cellular levels of reactive oxygen species. Transgenic antisense ascorbate peroxidase and catalase tobacco plants were found to be hyperresponsive to the pathogen attack caused by inoculation with *Pseudomonas syringae* (Mittler et al., 1999). Since catalase is located mostly in the peroxisomes, and ascorbate peroxidase is also present in these organelles, the role of peroxisomes in the plant immune response is clearly very important.

It has been shown recently that nitric oxide synthase is located in peroxisomes (Barroso et al., 1999). Thus peroxisomes are a cellular source of nitric oxide, a molecule important in the cellular signal transduction mechanisms, playing a key role in plant disease resistance (Delledonne et al., 1998). Nitric oxide functions independently from H_2O_2 or superoxide and induces genes for the synthesis of protective natural products (Bolwell, 1999). An increase in nitric oxide synthase activity was shown in pathogen-resistant lines of tobacco after infection with tobacco mosaic virus (Klessig et al., 2000). Nitric oxide has been shown to induce a dramatic transient increase in the concentration of cGMP, which in turn triggers the induction of chalcone synthase, ferredoxin $NADP^+$ oxidoreductase,

phenylalanine ammonia lyase and pathogenesis-related proteins (Wendehenne et al., 2001). There is thus a clear relationship between peroxisomal metabolism and the responses of plants to pathogen attack.

6. Formation of organic acids in peroxisomes and regulation of cellular metabolism

In photosynthetic plant cells glyoxylate, oxalate, formate and other photorespiratory intermediates can be involved in regulatory and signal transduction mechanisms. Phosphoglycolate is a potent inhibitor of triose-P isomerase in chloroplasts (Anderson, 1971), thus strongly inhibiting photosynthesis. A mutant of barley lacking phosphoglycolate phosphatase was shown to be non-viable in normal air, but which would grow in elevated CO_2 (Hall et al., 1987). Glyoxylate is an inhibitor, regulating both the activity of Rubisco itself and of its activation process (Igamberdiev and Kleczkowski, 1997 and references therein). There is a clear indication that in photorespiratory mutants, the activation state of Rubisco decreases via the effects of glyoxylate (Häusler et al., 1996). Glyoxylate also regulates mitochondrial enzymes, e.g. it activates the alternative oxidase in the same manner as pyruvate (Day and Wiskich, 1995), inhibits isocitrate dehydrogenase especially in combination with oxaloacetate forming non-enzymatically oxalomalate (Omran and Dennis, 1971) and also inhibits the glycine decarboxylase complex in a non-competitive manner (Peterson, 1982). Glyoxylate is also known to inhibit the glycolate/glycerate carrier in the inner chloroplast membrane, which mediates photorespiratory fluxes between chloroplasts and cytosol (Robinson, 1982). In peroxisomes, glyoxylate can inactivate aminotransferases, especially in the presence of ammonium ions (Havir, 1986) with which it forms carbinolamine, a serine analog binding irreversibly to the aminotransferases. Hydroxypyruvate is a reactive compound, the effect of which may be similar to glyoxylate, but its concentration in the cell is probably much lower because of effective scavenging via NADH- and NADPH-dependent reductases (Givan and Kleczkowski, 1992).

Various possible pathways of glyoxylate metabolism are summarized in Fig. 5. The major pathway is transamination of glyoxylate to glycine, conversion to formate (Grodzinski, 1979) and reduction to glycolate (Kleczkowski and Edwards, 1989) can also operate to some extent in green leaves, increasing under stress. Glyoxylate can be condensed with succinate in the cytosol in the reverse reaction of the non-peroxisomal form of isocitrate lyase, forming isocitrate (Igamberdiev et al., 1986). Although the activity of this enzyme is low, it can provide some metabolic flux in green leaves (Zelitch, 1988). Glyoxylate can enter the malate synthase reaction in green leaves, but only during senescence. Other possible pathways of glyoxylate metabolism are non-enzymatic condensation with oxaloacetate forming oxalomalate (which is further decarboxylated to 2-oxo-4-hydroxyglutarate), condensation with pyruvate by 2-oxo-4-hydroxyglutarate aldolase resulting in the formation of 2-oxo-4-hydroxyglutarate, condensation of two glyoxylate molecules with following decarboxylation, forming tartronic semialdehyde which can be reduced to glycerate directly or via isomerization to hydroxypyruvate. Some of the pathways, like synthesis of tartronic semialdehyde, oxalyl-CoA or 2-oxo-4-hydroxyglutarate are present in microorganisms, but probably do not function in higher plants. However, the isotopic labelling has indicated the possibility of these pathways in the green leaf and their quantitative contribution to organic acid metabolism has to be elucidated (Igamberdiev, 1989 and references therein).

A deficiency in the glycine decarboxylase complex in photorespiratory mutants, leads to a several-fold increase of glyoxylate, formate and oxalate (Wingler et al., 1997, 1999c). This may occur in normal plants in stress conditions, when the mitochondrial redox level is increased (the glycine decarboxylase complex is strongly inhibited by NADH—Oliver, 1994). Glyoxylate and phosphoglycolate under these conditions will inhibit photosynthesis, while formate will be a source of C_1 units for the biosynthesis of proteins, nucleic acids, pectins and for various secondary metabolites including lignin, glycine betaine and nicotine (Hanson and Roje, 2001). Formate is produced in the reaction of hydrogen peroxide with glyoxylate, and this can reduce the flow of the carbon through the normal photorespiratory pathway (Igamberdiev et al., 1999 and references therein; Wingler et al., 1999a). This is especially important at high temperatures, in transgenic plants with elevated catalase levels grown at 38 °C, the loss of CO_2 through the oxidation of glyoxylate was minimized (Brisson et al., 1998). Formate and related C_1 compounds are involved in signal transduction pathways leading to the induction of formate dehydrogenase (Hourton-Cabassa et al., 1998; Igamberdiev et al., 1999).

Oxalate arises as a side product of glycolate oxidase reaction (the enzyme utilizes also glyoxylate). It can also be formed from oxaloacetate (Chang and Beevers, 1968) or from ascorbate, however radiolabelling data indicate that in green leaves glycolate is the main source of oxalate (Fujii et al., 1993). The rate of oxalic acid formation depends on the relative affinities of glycolate oxidase for glycolate and glyoxylate, which differ in various plants, and on the degree of oxalate inhibition of the enzymes (Davies and Asker, 1983; Havir, 1983; Igamberdiev et al., 1988a; Watanabe et al., 1995). Differences in glycolate oxidase affinity for glyoxylate were found for C_3 and C_4 species (Devi et al., 1996) and for oxalate accumulating

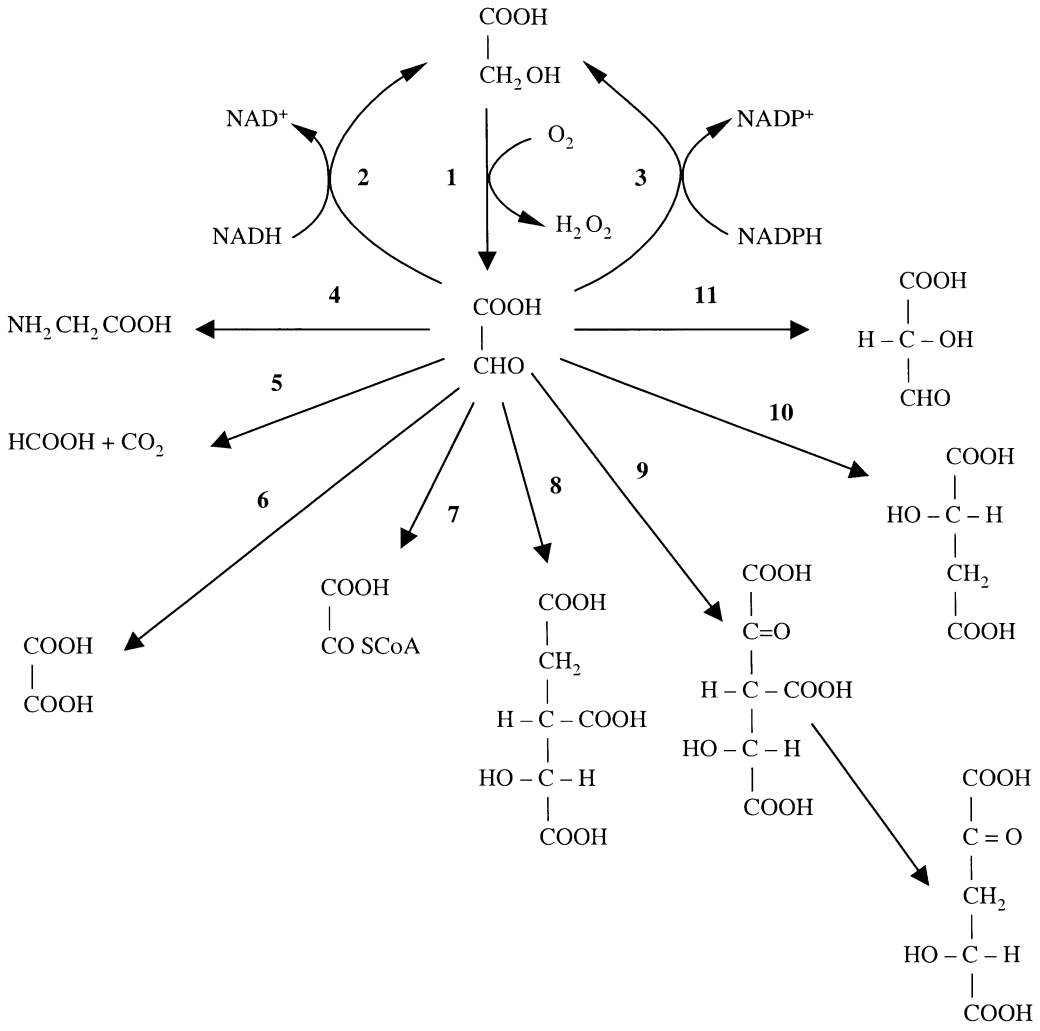

Fig. 5. Pathways of glyoxylate conversion in plants. 1—Oxidation of glycolate by glycolate oxidase in peroxisomes. 2, 3—Reduction of glyoxylate by NADPH-dependent glyoxylate reductase or by NADPH-dependent hydroxypyruvate reductase in the cytosol and by NADH-dependent hydroxypyruvate reductase in peroxisomes (the latter at high physiological concentrations of glyoxylate). 4—Transamination to glycine. 5—Non-enzymatic conversion of glyoxylate by H_2O_2 to formate and CO_2. 6—Oxidation of glyoxylate to oxalate in the side reaction of glycolate oxidase. 7—Synthesis of oxalyl-CoA (may be absent from higher plants). 8—Formation of isocitrate in the synthase reaction of isocitrate lyase, in green leaves, this reaction may occur in the cytosol. 9—Non-enzymatic (in reaction with oxaloacetate) formation of oxalomalate, a strong inhibitor of isocitrate dehydrogenase, with following decarboxylation and formation of 2-oxo-4-hydroxyglutarate (the latter can also be formed enzymatically via condensation of glyoxylate and pyruvate). 10—Formation of malate in malate synthase reaction (besides the glyoxysomes of fat-storing tissues this reaction takes place in senescing leaves). 11—Formation of tartronate semialdehyde in reaction of condensation of two glyoxylate molecules with following decarboxylation (likely absent from higher plants).

and non-accumulating plants (Igamberdiev et al., 1988a). In sugar beet leaves, glycolate oxidase is less inhibited by oxalate than in wheat leaves, which leads to differences in oxalate accumulating rates (Igamberdiev et al., 1988a). Oxalate exudation by roots may be very intensive and important for extraction of iron in calcareous soils and detoxification of aluminium in calcifuge soils (Ryan et al., 2001). Oxalate is decomposed to CO_2 by oxalate oxidase enzyme, which in turn generates hydrogen peroxide in vacuoles and cell walls and is involved in the hypersensitive response to the pathogen attack (Bolwell and Wojtaszek, 1997; Zhou et al., 2000). The balance between oxalate formation and oxalate oxidation can be regulated by abscisic acid, a phytohormone which increases the level of mRNA for oxalate oxidase (Hurkman and Tanaka, 1996). The apoplastic oxalate oxidase protein (known as germin), which also has Mn-superoxide dismutase activity, is involved in morphogenetic processes during seed germination and responses to different stresses (Woo et al., 2000).

Calcium oxalate formation and dissociation is an important tool for regulating the level of free calcium in plants. It has been shown that its formation in calcephylic plants is a precondition for their survival in calcareous soils (Tyler and Strom, 1995; Jones, 1998). Free calcium is a major signal in transduction pathways connected with morphogenesis, stomatal opening and stress response (De Silva et al., 1996; Evans et al., 2001).

Oxalate is a potent uncompetitive inhibitor of cytosolic NADPH-dependent hydroxypyruvate reductase (Kleczkowski et al., 1991, 1992; Igamberdiev and Kleczkowski, 2000). The free oxalate, will thus suppress the oxidation of cytosolic NADPH and the scavenging of hydroxypyruvate and glyoxylate leaked from peroxisomes increasing the inhibitory action of glyoxylate on photosynthesis. For human cells, it was shown that the targeting of a peroxisomal aminotransferase to mitochondria leads to increased calcium oxalate formation (Leiper et al., 1996).

Glycerate strongly affects the activities of chloroplastic sedoheptulose-1,7-bisphosphatase and fructose-1,6-bisphosphatase (Igamberdiev and Kleczkowski, 1997 and references therein). Since glycerate kinase is regulated by the redox level of the chloroplast, at a lower reduction level the accumulation of glycerate can restrict the rate of photosynthesis. Thus, photorespiratory intermediates can act as important messengers regulating photosynthesis and mitochondrial respiration.

β-Oxidation chains are also important in regulating essential developmental processes, such as the flower formation (Richmond and Bleecker, 1999). A defect of the tetrafunctional protein involved in β-oxidation alters fatty acid composition and reduces fertility. This may be connected with the role of some fatty acids as signals of floral development, via the synthesis of substances serving as plant growth regulators, e.g. indole-3-butyric acid (Ludwig-Müller, 2000). Peroxisomes themselves are targets for phytohormone action: peroxisomal enzymes that scavenge reactive oxygen species are down regulated prior to gibberellic acid-induced programmed cell death in the barley aleurone (Fath et al., 2001). The amount of NADH-hydroxypyruvate reductase is regulated by cytokinin (Wingler et al., 1998). Thus peroxisomes both produce messenger molecules and undergo regulation by phytohormones.

7. Evolution of peroxisomes: acquisition of specific enzymes coupled with flavin-dependent oxidation

Almost all peroxisomes contain flavin oxidases, which may possess a broad specificity, and catalase. These enzymes are absent from glycosomes—glycolytic organelles in parasitic protozoa, but their homology to peroxisomes is doubtful (Michels et al., 2000). In different organisms, a range of enzymes supplements this primary oxidative system. In many algae, peroxisomes also carry out the reduction of glyoxylate (and/or hydroxypyruvate) for the dissipation of excess energy during photosynthesis (Huang et al., 1983). Glyoxysome-like peroxisomes are also present in many algal species. They contain only isocitrate lyase and malate synthase from the enzymes of glyoxylate cycle. The same has also been observed in the protozoan *Tetrahymena* (Huang et al., 1983).

In the Euglenophyceae, glycolate excreted from the chloroplast can undergo limited oxidation in the mitochondria rather than the peroxisome, by glycolate dehydrogenase (Yokota, 1992). A part of the glyoxylate pool is aminated in the mitochondria forming glycine, which then leaks out into cytosol, the remainder of the glyoxylate returns to chloroplasts where it is converted to formate and CO_2 in a reaction with H_2O_2. Formate enters the cytosol where the tetrahydrofolate derivative interacts with glycine, with the resulting formation of serine. The latter is converted to hydroxypyruvate and glycerate in peroxisome-like particles (Fig. 6).

In heteroconts, including golden (Chrysophyceae), brown (Phaeophyceae) and yellow-green (Xanthophyceae) algae, peroxisomes usually contain H_2O_2-producing glycolate oxidase, catalase and a transaminase forming glycine (Gross et al., 1985). These reactions are coupled with the reactions of glyoxylate cycle (malate synthase and isocitrate lyase), as in greening cotyledons (see below), β-oxidation of fatty acids takes place in the mitochondria (Stabenau, 1992). In some representatives (Chrysophyceae), the oxidation of glycolate takes place in mitochondria and peroxisomes and the formation of glycine takes place in mitochondria, hydroxypyruvate reductase was not found in these algae (Stabenau, 1992).

Investigations of the peroxisomes isolated from diatoms (Bacillariophyceae), algae which are also included in the group of heteroconts, revealed very unusual properties that indicate their possible different origin. In neither mitochondria nor peroxisomes, could catalase or any H_2O_2-forming oxidase be demonstrated. The glycolate-oxidizing enzyme present in the peroxisomes is a dehydrogenase also capable of oxidizing L-lactate. The peroxisomes also contain the glyoxysomal markers isocitrate lyase and malate synthase, thus photorespiratory glyoxylate is converted into malate, as in other heteroconts. However, the enzymes of the fatty-acid β-oxidation pathway are located exclusively in the mitochondria. The mitochondria additionally possess glutamate-glyoxylate aminotransferase and a glycolate dehydrogenase, which differs from the peroxisomal glycolate dehydrogenase, since it preferentially utilizes D-lactate as an alternative substrate. Hydroxypyruvate reductase and glyoxylate carboligase were not found in the cells of either diatom (Fig. 7). By culturing the diatom *Nitzschia laevis*, it was demonstrated that decreasing the CO_2 concentration in the aeration mixture from 2 to 0.03% and increasing the irradiance from 40 to 250 μmol quanta m^{-2} s^{-1}, resulted in an increase of all peroxisomal enzyme activities. In addition, enzyme activities of the β-oxidation pathway were increased. However, the activities of mitochondrial glycolate dehydrogenase and the aminotransferase were not

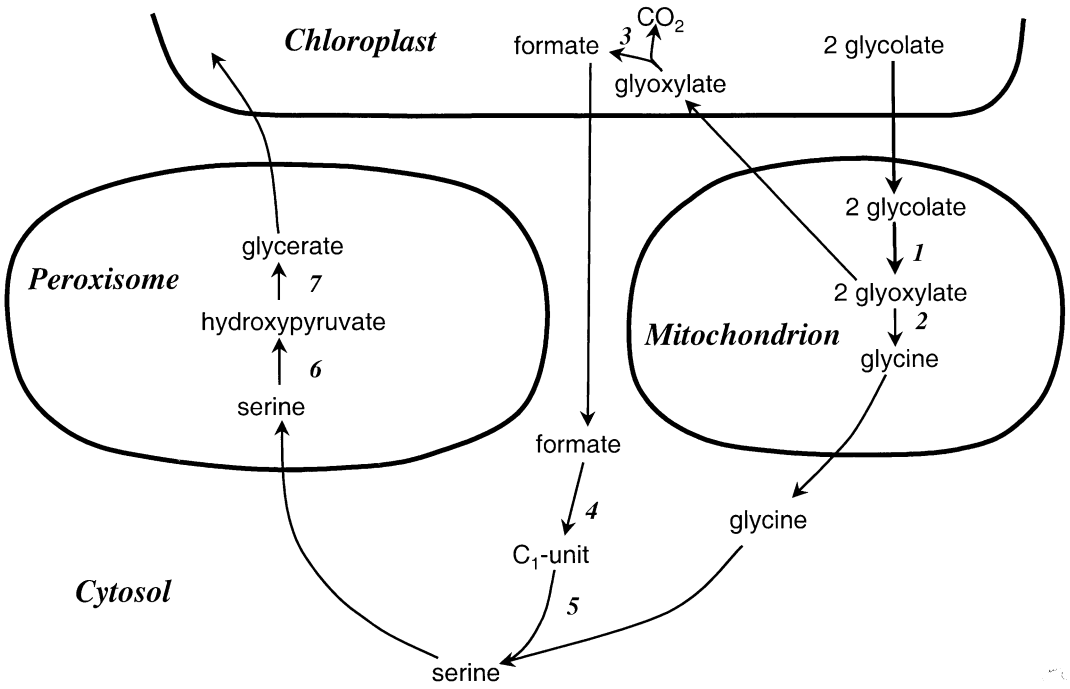

Fig. 6. Glycolate metabolism in *Euglena*. Glycolate formed in the chloroplast is oxidized in mitochondria by glycolate dehydrogenase (1) to glyoxylate. A part of the glyoxylate pool is transaminated to glycine (2). Another part enters the chloroplast where it is decarboxylated to formate in the non-enzymatic reaction with the hydrogen peroxide (3). Formate leaks to the cytosol where it forms tetrahydrofolate derivative (4), the latter interacts with the glycine molecule yielding serine (5). Serine in the peroxisome is transaminated to hydroxypyruvate (6), which is further reduced to glycerate (7). Glycerate enters the chloroplast where after phosphorylation it returns to the Calvin cycle.

altered under these conditions. It was postulated that there are two different pathways for the metabolism of glycolate in the diatoms localized in the mitochondria and the peroxisome-resembling organelles (Winkler and Stabenau, 1995). Such unusual organization in diatoms may be connected with the special CO_2 concentrating mechanism resembling that of C_4 plants but operating within one cell discovered in the marine diatom *Thalassiosira weissflogii* (Reinfelder et al., 2000). It strongly reduces photorespiratory rates in diatoms and causes greater enrichment by ^{13}C compared to other algal groups.

The red algae (Rhodophyceae) contain peroxisomes with glycolate oxidase activity. Some thermophilic species of red algae possess ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) with a high specificity for CO_2 and a very low oxygenase reaction (Uemura et al., 1997). A thermoacidophilic cyanidiophycean line of *Rhodophyta* exhibits in peroxisomes activities of catalase, glycolate oxidase and glutamate-glyoxylate aminotransferase. Other enzymes that attributed to peroxisomes in higher plants in this microalgal group are either in the cytosol (e.g. serine-glyoxylate aminotransferase and hydroxypyruvate reductase) or in the mitochondrion (e.g. fatty acid β-oxidation) (Seckbach et al., 1992).

In the primitive green algae (Prasinophyceae), peroxisomes are not involved in glycolate metabolism and contain enzymes of β-oxidation. The glycolate pathway is exclusively mitochondrial, and activities of the corresponding enzymes are low. All green algae (Chlorophyceae, Ulvophyceae), except Charophyceae, possess mitochondrial enzymes of glycolate metabolism (Frederick et al., 1973). In primitive algae, glycolate is metabolized by mitochondrial glycolate (D-lactate) dehydrogenase. This reaction is coupled with electron transport in mitochondria, and with the photosynthetic electron transport chain in cyanobacteria (Tolbert, 1992). The primitive unicellular green algae *Dunaliella salina* and *D. primolecta* possess only glycolate dehydrogenase. After separation of organelles by gradient centrifugation, glycolate dehydrogenase along with hydroxypyruvate reductase was found exclusively in the mitochondria. Thus the peroxisomes from *Dunaliella* are not of the leaf-type: because of their content of catalase, uricase and hydroxyacyl-CoA dehydrogenase, they appear to be of the same type as in *Eremosphaera* and other chlorophycean algae. No activity of glycolate dehydrogenase was found in the chloroplast fraction, when the 2,6-dichlorophenol-indophenol test was used (Stabenau et al., 1993).

In *Chlamydomonas reinhardtii*, the level of catalase activity was higher in cells grown in the dark than in the light (Kato et al., 1997). Subcellular fractionation on a sucrose density gradient revealed that catalase is localized in the mitochondria. Mitochondria purified on a

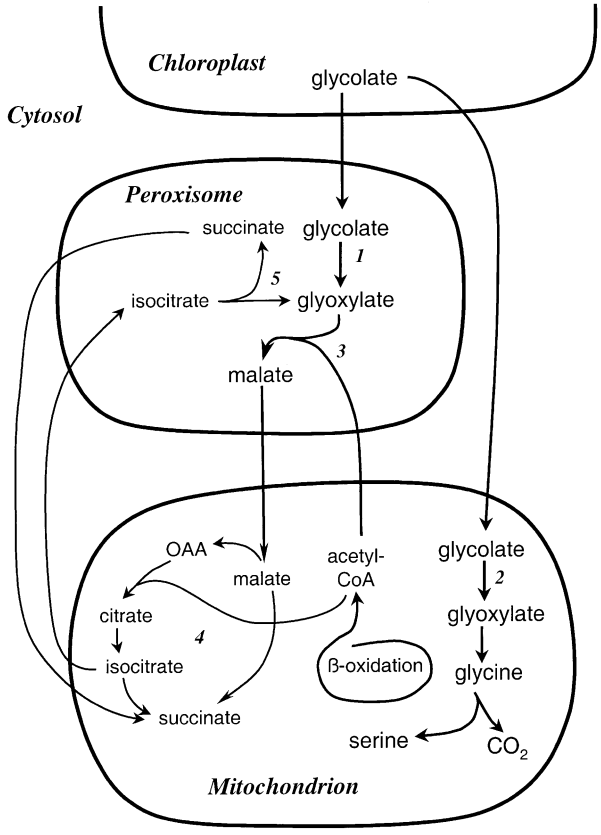

Fig. 7. Glycolate metabolism in diatoms. Glycolate formed in the chloroplast is oxidized either by glycolate (D-lactate) dehydrogenase (1) in mitochondria where the glyoxylate formed in transaminated to glycine or by glycolate (L-lactate) dehydrogenase (2) in peroxisomes, where the glyoxylate formed is condensed with acetyl-CoA in the malate synthase reaction (3). Acetyl-CoA is formed during the mitochondrial β-oxidation of fatty acids and is utilized both in the tricarboxylic acid cycle in mitochondria (4) or in malate synthase reaction in peroxisomes. Isocitrate formed in mitochondria is an additional source for glyoxylate in peroxisomes in isocitrate lyase reaction (5) whilst succinate formed in this reaction returns to mitochondria.

Percoll gradient contained three isoforms. The catalase of *C. reinhardtii* is unique among enzymes from photosynthetic organisms in that it is a dimer located in the mitochondria, instead of a tetramer located in microbodies. The activity of glycolate dehydrogenase in algae is low (less than 2 μmol h^{-1} mg^{-1} Chl) but CO_2-concentrating mechanism prevents the formation and excretion of large amounts of glycolate (Tolbert, 1982). The photorespiratory excretion of glycolate, glyoxylate and glycine may have been important in establishing symbiotic relationships between autotrophs and heterotrophs (Goldsworthy, 1969) and can play a role in the endosymbiosis between cyanobacteria and flagellatae and the exosymbiosis between algae and fungi.

It was shown that leaf-type peroxisomes are not present in the primitive unicellular prasinophycean line of algae, but in the multicellular algae *Mougeotia*, *Chara*, and *Nitella*, which are in the evolutionary line of Charophyceae, that led to higher plants (Kehlenbeck et al., 1995). The algal CO_2-concentrating mechanism and alkalization of the medium during photosynthesis were not lost when peroxisomes appeared in the members of the charophycean line of algae. Therefore, it is unlikely that lowering of the CO_2 concentration in the environment was a major factor in the evolutionary appearance of peroxisomes. Multicellular *Mougeotia*, an early member of the charophycean line of algae, has peroxisomes, but it excretes an excess of glycolate into the medium. The oxygenase activity of Rubisco and glycolate oxidase require about 200 to 400 μM O_2 for 0.5 V_{max}, i.e. near or above the present atmospheric level (21% ~ 250 μM at sea level). These high O_2 requiring steps in glycolate metabolism would have functioned faster with increasing atmospheric O_2, thus, it is possible that O_2 and not CO_2 concentration could have been the causative factor in the induction of peroxisomes (Kehlenbeck et al., 1995).

Structural, biochemical, and molecular evidence support the hypothesis that charophycean green algae are the closest relatives of the land plants (embryophytes). charophycean algae include the filamentous and unicellular *Zygnematales*, represented by the familiar *Spirogyra* and desmids; the relatively large and complex *Charales*, such as *Chara*; the less conspicuous, but well-studied Coleochaetae; and several other less well-known taxa. Ultrastructural studies of these green algae have revealed a variety of subcellular structures which are shared with land plants: cytoskeleton (including mitotic and meiotic division apparatus), peroxisomes, and cell wall features which are absent from most other algae (Graham and Kaneko, 1991).

It is clear from the previous consideration that glycolate metabolism has different intercompartmental localization in different groups of algae. Peroxisome-like particles fulfill different functions in different groups, and only in the Charophyceae do they possess all enzymes of the glycolate and glycerate pathway, as in higher plants. Mitochondrial metabolism of glycolate can be found very often in algae and a large part of the glycolate pool can be excreted into the external environment. The CO_2-concentrating mechanism via carbonic anhydrase prevents high rates of photorespiration in algae. It is interesting to note that the aquatic angiosperms also excrete glycolate into the external milieu, as was shown for aquatic macrophytes (Kolesnikov et al., 1985) and for the secondary simplified plant *Wolffia arrhiza* (L.) Hork. ex Wimmer (Igamberdiev and Zabrovskaya, 1994; Bykova et al., 1998).

8. Peroxisomes and the evolution of land plants

The important role that peroxisomes play in the metabolic pathways of the cell cannot be overemphasized.

Simpler organisms can oxidize glycolate to glyoxylate and convert the latter to glycerate without forming glycine, glyoxylate carboligase and tartronate semialdehyde dehydrogenase are the enzymes, which fulfill this role (Chang et al., 1993). The conversion of glyoxylate to glycine by an aminotransferase reaction led to the appearance of normal photorespiratory C_2 cycle. A high activity of glycine decarboxylase in photorespiring water plants could be harmful since the ammonia released in this reaction is very soluble and would readily diffuse into the surrounding environment. This possibly explains why the normal photorespiratory cycle is not common in algae and develops in connection with the origin of land plants. Consideration of the localization of photorespiratory enzymes in algae, shows that only in multicellular algae (Charophyceae) and higher plants are all enzymes of the glycolate and glycerate pathways enclosed within the peroxisomes. Only in these organisms are peroxisomes important for the metabolism of the whole flux of glycolate, and peroxisomal respiration can be considered as an unavoidable consequence of multicellular organization on land (Igamberdiev, 1989). Leaf cells grown in culture also contain little peroxisomal activity relative to the whole tissue (Tolbert, 1992).

Peroxisomes of lower plants reveal less specialization than that of angiosperms. Peroxisomes of ferns show the absence of temporal separation of autotrophic and heterotrophic metabolism (DeMaggio et al., 1980), since germinating spores contain chlorophyll and can carry out photorespiration as well as β-oxidation of fatty acids linked to the glyoxylate cycle. Thus, the link between glyoxylate formed in photorespiration and its utilization in the malate synthase reaction is possible. A similar link exists only during a short period of germination in greening cotyledons, still utilizing fatty acids and already photosynthesizing (Igamberdiev et al., 1988b). The operation of the glyoxylate cycle in germinating seeds is connected with transfer of the glyoxysome formation programme in germinating spores to the stage of germinating seeds. Glyoxysomes are present in germinating spores: with the appearance of seed plants this type of organelle developed in germinating seeds, although in germinating pollen it is also present. The autonomy of seed metabolism, which is a key factor in the appearance of flowering plants, is provided for by the acquisition of glyoxysomal enzymes. In senescing leaves, when the β–oxidation pathway and glyoxylate cycle enzymes (isocitrate lyase and malate synthase) are induced for the breakdown of membrane lipids and gluconeogenesis (Chen et al., 2000), a part of photorespiratory glyoxylate could also be channeled through the glyoxylate cycle.

Glyoxysomal metabolism in higher plant seeds is located mostly in the cotyledons, but in some cases it operates in the endosperm (e.g. castor bean). The presence of glyoxylate cycle enzymes in maturing seeds has been demonstrated (Baqui et al., 1977). Incomplete glyoxysomes appear during maturation of seeds and contain catalase and malate synthase. Isocitrate lyase and other enzymes of glyoxylate cycle appear later, only during germination (Frevert et al., 1980). The aconitase participating in the glyoxylate cycle is located in the cytosol, which prevents oxidation of its iron-sulfur clusters by hydrogen peroxide (Courtois-Verniquet and Douce, 1993; Cots and Widmer, 1999).

A special type of peroxisome is the glyoxysome in the scutella of monocotyledonous plants. The scutellum has to a certain extent similar functions to the cotyledon of dicots but reveals a homology to the third leaf of nymphealean seedlings (Burger, 1998). Besides a fat-storing function, the scutellum together with the aleurone layer has a "digestion" function participating in supplying low molecular weight organic acids for the hydrolysis of the endosperm (Drozdowicz and Jones, 1995). During the germination of cereal seeds, carbohydrates are provided by the endosperm, thus the role of glyoxysomes may be different from that in dicots. Incorporation of label from ^{14}C-acetate by the scutellum was observed mostly in amino acids, but not in carbohydrates (Igamberdiev and Rodionova, 1991). This shows that the operation of the glyoxylate cycle in the scutellum provides carbon skeletons for amino acid synthesis. In contrast to cotyledons of dicotyledonous plants, the mitochondria of maize scutellum exhibit some activity of the glycine decarboxylase complex, whose development during germination has a similar profile to the glyoxylate cycle enzymes (Igamberdiev et al., 1997). The explanation of this may be the outflow of glyoxylate from the glyoxylate cycle to glycine and serine synthesis. Another important feature of glyoxysomes in monocots is the ability to oxidize succinate to form malate. The system is membrane-bound, has low affinity for succinate, contains flavin and forms hydrogen peroxide (Igamberdiev et al., 1995). Hence, the function of glyoxysome-like organelles in the scutellum of monocots may be the interconnection of fatty acid degradation with amino acid synthesis and probably with the synthesis of organic acids, which cause an acidification of the endosperm.

Recent works on the mutants lacking key enzymes of the glyoxylate cycle revealed that the coupling of β-oxidation pathway with the glyoxylate cycle is not crucial for seed germination (Eastmond et al., 2000b; Eastmond and Graham, 2001). Moreover, the glyoxylate cycle is absent in the embryo of germinating barley being present only in aleurone cells (Holtman et al., 1994). In *A. thaliana*, photosynthesis can compensate for the absence of the glyoxylate cycle during postgerminative growth, and only when the light intensity or day length is decreased does seedling establishment become compromised. An additional anaplerotic source

of carbon for lipid breakdown and seedling establishment can be provided in the absence of the glyoxylate cycle by exogenous sucrose or photosynthesis (Eastmond et al., 2000b). In a range of tissues and plant species different interactions between β-oxidation and the glyoxylate cycle have been observed, which could be more efficient in large fat-storing seeds and even absent in tissues of monocot embryos. Coupling of glyoxylate cycle with photorespiratory glycolate cycle is also possible. This all shows great flexibility of peroxisomal metabolism in the formation of interconnections between different metabolic pathways.

Peroxisomes were also important for the establishment of different types of photosynthetic metabolism. Special modifications of photosynthetic metabolism in aquatic angiosperms strongly reduce the flux of glycolate. A C_4-cycle concentrating CO_2 in the submerged monocot *Hydrilla verticillata* is confined to chloroplasts indicating that Kranz anatomy is not obligatory for the C_4-type photosynthesis (Magnin et al., 1997). In the evolutionary transition from land to water in aquatic grasses, different modifications of C_4 metabolism have occurred (Keeley, 1998). Also, CO_2 concentrating mechanisms, including high levels of the carbonic anhydrase activity, have developed in a similar manner to algae, in *Elodea* leaves (Fagerberg et al., 1991), that also contain some structural features found in both C_3 and C_4 plants (Eighmy et al., 1991).

In some aquatic angiosperms, evolutionary reversal to the types of peroxisomal metabolism present in algae has also been observed. Even glycolate dehydrogenase activity was detected in some marine angiosperms along with an absence of glycolate oxidase (Tolbert, 1982). In fact, low activities of a light-dependent, glycolate dehydrogenase, which is inhibited by salicylhydroxamic acid has been demonstrated in chloroplasts, where it is associated with photosynthetic electron transport of many, if not all higher plants, (Goyal and Tolbert, 1996). It represents an efficient system of recycling of carbon from glycolate, while regenerating energy under extreme environmental conditions. ATP is generated by glycolate oxidation through the photosystem I, this promotes more cyclic phosphorylation (Goyal and Tolbert, 1996). However the absence of glycolate oxidase is very unusual in higher plants.

Excretion of glycolate into the surrounding medium has been shown in different aquatic angiosperms (Kolesnikov et al., 1985). The most simplified aquatic angiosperms are Lemnaceae, and especially the genus *Wolffia*, the absence of roots, a vascular system, and infrequent flowering, being possibly the result of evolutionary adaptation to photoheterotrophic growth conditions. Photorespiratory metabolism in *Wolffia* differs significantly from photosynthetic higher plants. Glycine conversion to serine in this plant proceeds at low rate. Glycolate oxidase activity in *Wolffia* was low, and glycolate dehydrogenase activity could not detected (Bykova et al., 1998). Thus, similarly to other higher plants, and in contrast to most algae, *Wolffia* possesses the common glycolate pathway. It includes glycolate oxidase and glycine decarboxylase, but activities of these enzymes are relatively low and glycine metabolism has lost the characteristic features of its preferential (to other mitochondrial substrates) oxidation. The reduction of the photorespiratory pathway is consistent with the observation that glycolate and glycine were excreted into the surrounding medium. It was shown that *Wolffia* had a lower capacity for the refixation of photorespiratory ammonium, compared to other higher plants including the plant of the same family, *Lemna gibba* L. (Monselise and Kost, 1993). Excretion of other compounds, especially phenolics, reflects a significant reduction of secondary metabolism characteristic for all Lemnaceae (Landolt and Kandeler, 1987).

The importance of photorespiratory peroxisomes for adaptation of plants to high O_2 and low CO_2 has been clearly shown using photorespiratory mutants, deficient in key enzymes of glycolate metabolism (Blackwell et al., 1990; Leegood et al., 1995; Wingler et al., 1997, 2000). Photorespiration protects plants against photoinhibition caused by different stresses including drought (Wingler et al., 1999b, 2000). Photorespiration decreases the redox and energy level in chloroplasts under limiting CO_2 concentrations and at high light intensity (Heber et al., 1996; Igamberdiev et al., 2001). The O_2/CO_2 ratio in the Earth's atmosphere increased with the appearance of land plants, which depleted CO_2 by weathering during the formation of soils (Berner, 1997) and reached the highest values (more than 1000) 300 million years ago, at the end of the Carboniferous period (Permo-Carboniferous boundary) (Fig. 8). From this period, photorespiration became a very important process, possibly regulating the CO_2 and O_2 content of the air (Tolbert et al., 1995). During the Mesozoic era, the O_2/CO_2 ratio was lower and then increased in the Cenozoic era with the spreading of angiosperms, reaching the highest values in the glacial periods, compared to that in Permo-Carboniferous (Fig. 8). Lower CO_2 concentrations corresponded to the higher rates of divergence and evolution of land plants and other organisms (Rothman, 2001). Also higher O_2 concentrations led to enhanced rates of divergence particularly connected with the formation of various secondary metabolites and with increased woodiness (Gottlieb and Borin, 1998, 2000). Due to high rates of anthropogenic release, the atmospheric CO_2 has increased and the relative rate of photorespiration decreased. The consequence of this process may be the loss of the selective advantages of C_4 plants, even in the areas where they are abundant. When the C_4 plant sorghum, was grown at 700 μl l^{-1} CO_2, the activity of phosphoenolpyruvate carboxylase

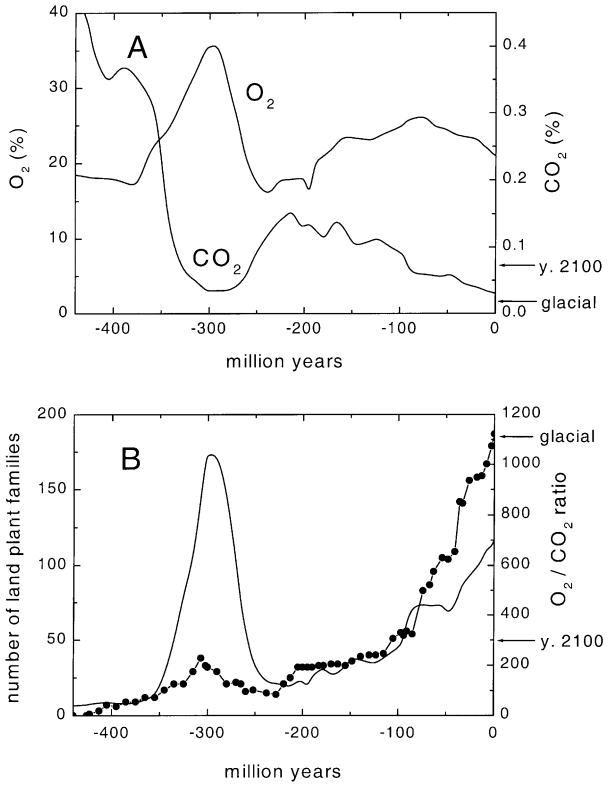

Fig. 8. Changes in atmosphere composition during the evolution of land plants. A—Changes in O_2 (Berner, 1999) and CO_2 (Berner, 1994) concentration. B—Changes in the number of land plant families (–●–●–) (Rothman, 2001) and in O_2 to CO_2 ratio (——) (derived from A). The glacial minimum of CO_2, the glacial maximum of O_2 to CO_2 ratio and the corresponding expected values in the year 2100 (Falkowski et al., 2000) are shown by the arrows.

was reduced and the bundle sheath cells become permeable to CO_2, thus approaching to the characteristics of a C_3 plant (Watling et al., 2000).

The importance of peroxisomal metabolism is also evident for C_4 and C_3–C_4-intermediate plants, that evolved in response to low CO_2 concentration in the air and which exhibit a reduced rate of photorespiration, due to the presence of a CO_2 concentrating mechanisms. In C_4 plants, the activities of the glycolate pathway enzymes are comparable to those in C_3 plants, but are confined to the bundle sheath cells, except glycerate kinase which is present in the mesophyll cells (Osmond and Harris, 1971; Usuda and Edwards, 1980). Maize appears to lack the GR-1 isoenzyme of glyoxylate reductase but activities of both the HPR-1 and HPR-2 isoenzymes of hydroxypyruvate reductase are high (Kleczkowski and Edwards, 1989). It seems evident that the presence of the photorespiratory pathway and glycine oxidation in bundle sheath cells serves to prevent CO_2 escaping from the environment of Rubisco and allows for immediate reassimilation (Lacuesta et al., 1997). Glycerate enters into mesophyll where it is used as a source of 3-phosphoglyceric acid for biosynthetic purposes. Glycolate may be produced in C_4 plants in the Mehler reaction via the oxidation of organic acids by superoxide radicals and hydrogen peroxide (Karpilov and Lyubimov, 1977; Gaudillerre et al., 1983) and then oxidized in the peroxisome.

Laisk and Edwards (1998) showed that when C_4 photosynthesis is limited by the supply of atmospheric CO_2 to the C_4 cycle, the C_3 cycle becomes limited by the regeneration of ribulose 1,5-bisphosphate (RuBP), which in turn limits RuBP oxygenase activity and photorespiration. The rate of excess electron transport over that consumed for CO_2 fixation in C_4 plants was very sensitive to the presence of O_2 in the gas phase, rapidly increasing between 0.01 and 0.1% O_2, and at 2% O_2 it was about 60% of that at 21% O_2. This shows the importance of the Mehler O_2 reduction reaction as an electron sink, compared with photorespiration in C_4 plants. Using C_4 plants deficient in C_3 and C_4 cycles it was shown that the investment in the C_3 and C_4 cycles must be balanced for maximum efficiency (Maroco et al., 1998a,b). A limitation in the C_4 cycle due to the deficiency in phosphoenolpyruvate carboxylase led to the appearance of C_3-like properties (higher photorespiration) while a limitation in the C_3 cycle due to a deficiency of Rubisco resulted in CO_2 leakage due to overcycling of the C_4 pathway (Maroco et al., 1998a,b).

The C_3–C_4 intermediate species have a reduced rate of photorespiration although they contain Rubisco in both the mesophyll and bundle sheath cells. However, the glycine decarboxylase complex and, to a lesser extent, serine hydroxymethyltransferase are confined to the latter tissue (Hylton et al., 1988). As a consequence, glycine formed in the mesophyll must move to the bundle sheath cells for decarboxylation to serine. The glycine pool in C_3–C_4 plants is much larger than in C_3 species, and a massive flux of this metabolite must exist between the two cell types to sustain high rates of photosynthesis. In C_3–C_4 intermediate species, a CO_2 concentrating mechanism results from an effective capture of glycine-derived CO_2 by bundle sheath chloroplasts, which tightly overlie mitochondria in these cells (Rawsthorne, 1992).

Evolution of land plants included mechanisms of heterotopy (Meyen, 1988), i.e. of the transfer of constituent features from one part of organism structure to another occurring together with heterochrony, the evolutionary change in developmental rate of timing leading to differential rates of metabolic processes (Li and Johnston, 2000). Loading of peroxisomes by corresponding enzymes is an example of such a heterotopy at the subcellular level. During the evolution of land plants peroxisomal heterotopy also played a key role. Thus, the evolution of cultivated plants was accompanied by an increase in the role of the peroxisomal compartment. In high-oil containing maize plants, isocitrate lyase activity

is higher, whereas malate synthase activity is similar to low-oil containing maize cultivars (Tsaftaris and Scandalios, 1983). The same tendency is observed for the evolution of cultivated soybean, which contains higher concentrations of lipids than the original ancestor and exhibits higher levels of isocitrate lyase activity in the cotyledons of germinating seeds and a higher activity of photorespiratory enzymes in the mature leaves (Igamberdiev and Lavlinsky, 1989). In beet cultivars with high rate of sugar accumulation, the capacity of photorespiratory flux and the activities of peroxisomal enzymes are higher than in low sugar cultivars (Igamberdiev, 1990).

9. Conclusions

The flux through the non-coupled processes in the plant cell is high and intensified under certain (e.g. stress) conditions. According to non-linear thermodynamics, the non-coupled processes, i.e. the processes characterized by a decrease of free energy, lead to the appearance of conditions for the emergence of pathways, not present before, which determines the generation of new structures. This is an example of order generation from chaos far from equilibrium (Prigogine, 1980). Metabolic organization can be viewed as a result of the evolutionary transformation of a few primary reactions into different forward and reverse routes with the subsequent 'unfolding' of the initial futile cycles (Igamberdiev, 1994, 1999). This corresponds to a transition from simple uncoupled processes to integrated strongly coupled reaction networks.

Peroxisomes fulfill an important role in the evolutionary formation of metabolic networks, via establishing interconnections between different metabolic compartments. In lower organisms, peroxisomes carry out terminal oxidation, in higher organisms, intermediate oxidation, which is an important stage of metabolism in multicellular organisms. In evolution they exhibit the phenomenon of intracellular heterotopy, i.e., of the transfer of proteins from one compartment to another. Peroxisomes are loaded with specific enzymes coupled to dissipative flavin dependent oxidation connected with catalase-mediated splitting of hydrogen peroxide and acquire specialization.

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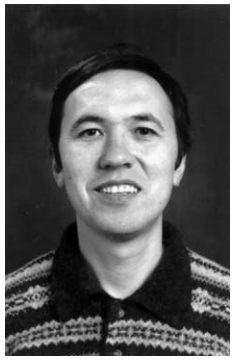

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