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Review

Malate dehydrogenase isoenzymes: Cellular locations and role in the flow of metabolites between the cytoplasm and cell organelles

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Abbreviations: CS, citrate synthase; IL, isocitrate lyase; LDH, lactate dehydrogenase; gMDH, mMDH, glyoxysomal-, mitochondrial-malate dehydrogenase; MS, malate synthase; PEP, phospho*enol*pyruvate; PTS, peroxisomal targeting signal; SHMT, serine hydroxymethyltransferase; TCA, tricarboxylic acid cycle.

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Malate dehydrogenases belong to the most active enzymes in glyoxysomes, mitochondria, peroxisomes, chloroplasts and the cytosol. In this review, the properties and the role of the isoenzymes in different compartments of the cell are compared, with emphasis on molecular biological aspects. Structure and function of malate dehydrogenase isoenzymes from plants, mammalian cells and ascomycetes (yeast, *Neurospora*) are considered. Significant information on evolutionary aspects and characterisation of functional domains of the enzymes emanates from bacterial malate and lactate dehydrogenases modified by protein engineering. The review endeavours to give up-to-date information on the biogenesis and intracellular targeting of malate dehydrogenase isoenzymes as well as enzymes cooperating with them in the flow of metabolites of a given pathway and organelle.

I. Introduction

Plant tissue contains multiple molecular forms of malate dehydrogenases (L-malate-NAD-oxidoreductase, MDH, EC 1.1.1.37). The physiological significance of multiple forms of MDH lies in their participation in different metabolic pathways. Differences in function correspond to differences in subcellular locations: MDH is found in microbodies such as glyoxysomes and peroxisomes, in mitochondria, in chloroplasts and in the cytosol. Malate dehydrogenase strongly disfavours oxalacetate as a product. Whether malate or oxalacetate is formed depends on physiological parameters such as the NAD(P) redox state and tissue function. All MDHs are NAD-dependent except the chloroplast-type, which requires NADP as cofactor. The different isoenzymes are encoded in genes of the nucleus and syntnesised on cytoplasmic ribosomes.

The different cell compartments are working in close cooperation. Thus, the metabolic pathways within the microbody are catabolic leading to end-products like glycine or a C_4 acid to be used for synthetic processes elsewhere in the cell. This finds its morphological expression in glyoxysomes being tightly wedged between lipid bodies and mitochondria or peroxisomes being located in intimate contact with chloroplasts and mitochondria. Malate dehydrogenase isoenzymes can serve as a model system for studying protein sorting to different cell compartments and for comparison of relevant functional epitopes.

Malate dehydrogenase isoenzymes in plants and other organisms are reviewed for their significance in a given pathway, their primary structures and kinetic properties and their targeting into the appropriate organelle.

II. The malate dehydrogenase in glyoxysomes

II-A. β-Oxidation of fatty acids and the glyoxylate cycle

Glyoxysomes belong together with leaf peroxisomes to the family of microbodies. They lack DNA [1], are surrounded by a single membrane and share with peroxisomes of animals and fungi at least two biochemical capabilities: O_2-processing (based on the conversion of H_2O_2 by catalase) and fatty acid oxidation. Glyoxysomes have been isolated from seedling tissues metabolising fatty acids such as the endosperm of *Ricinus communis* [2] and the cotyledons of Cucurbitaceae [3]. They are found in the aleurone [4–6] and scutella [7] of cereal seedlings and in the cotyledons of the Jojoba bean (*Simmondsia* spec.) in which wax is the major storage material [8]. The metabolic pathways of glyoxysomes in relation to oleosomes and mitochondria are summarised in Fig. 1. Glyoxysomes convert the long-chain fatty acids [9] to their CoA esters (enzyme 2). Their membranes contain an alkaline lipase that hydrolyses triacylglycerols [10–12]. They possess a complete set of enzymes for the β-oxidation of fatty acids and the enzymes of the glyoxylate cycle [9,13–16] and therefore play a major role in the conversion of fat to sucrose in fatty seedling tissue. It has generally been accepted that in plants fatty acid β-oxidation is restricted to microbodies [17–19], although a contribution of mitochondria cannot be entirely ruled out [20].

The first enzyme of the β-oxidation spiral is a H_2O_2 forming acyl-CoA oxidase (enzyme 3); in rat liver [21] and *Candida tropicalis* [22,23] it is synthesised without a cleavable signal peptide. The second and third reactions are catalyzed by a single bifunctional protein possessing 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities (enzymes 4 and 5) both in cucumber [24] and rat liver [25]. In *Candida tropicalis* a larger trifunctional protein catalyzes these reactions as well as β-hydroxyacyl-CoA epimerisation [26]. More recent data proved the peroxisomal bifunctional protein from rat liver to be a trifunctional enzyme with 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and Δ^3,Δ^3-enoyl-CoA isomerase activities [27]. The cucumber bifunctional protein is synthesised in its final size [28], as is the case for the trifunctional enzyme from yeast [29] and the rat liver enzyme [30,31]. The biogenesis of the fourth enzyme in the β-oxidation spiral, the 3-ketoacyl-CoA thiolase (enzyme 6) has been investigated only in rat liver. Unlike other β-oxidation enzymes the peroxisomal thiolase is synthesised as a higher molecular weight precursor [30] with a presequence of 26 amino acids at the amino terminus [32]. It is remarkable that the rat mitochondrial 3-ketoacyl-CoA thiolase is synthesised without a transient pre-sequence [33].

The β-oxidation of fatty acids ends up with the production of acetyl-CoA. The enzymes of the glyoxylate cycle catalyze the conversion of two molecules of

acetyl-CoA to succinate and comprise citrate synthase (gCS), aconitase, isocitrate lyase (IL), malate synthase (MS), and malate dehydrogenase (enzymes 9–13).

II-B. Characterisation of glyoxosomal malate dehydrogenase and in vivo synthesis

It is established that in fatty cotyledons [34–39] endosperm tissue [2,40–42] and scutella [43] there is a glyoxysomal malate dehydrogenase (gMDH), which differs in its electrophoretic behaviour from the mitochondrial and cytosolic forms. It is a homodimer with M_r 67000 [44] and has a subunit molecular mass of 33 kDa [45]. It tends to form aggregates at higher concentrations [40,43,44] and exhibits an isoelectric point at pH 8.92, which is considerably more basic than the isoelectric point of pH 5.39 of mitochondrial MDH [44]. The same differences are found for the citrate synthase isoenzyme pair with isoelectric points of pH 9.1 for the glyoxysomal and of pH 5.9 for the mitochondrial enzyme [46]. A physiological explanation for this difference is not known. The microbody form of MDH is considerably less heat stable than the other isoenzymes [42,44]. Antibodies raised against gMDH distinguish the microbody MDH from the other isoenzymes by their capacity for selective inhibition and precipitation [38,42,47]. The kinetic properties, however, are not greatly different from the mitochondrial isoenzyme (cf. subsection III-C) [38,42,47]. The data for gMDH ($3.1 \cdot 10^{-10}$ M) are: V_{max} (μmol/min) = 6.06 for malate oxidation; V_{max} (μmol/min) = 19.57 for oxalacetate reduction; K_m(OAA) = 0.18 mM; K_m(NADH) = 0.13 mM; K_m(malate) = 7.18 mM; K_m(NAD) = 0.46 mM [48]. The glyoxylate cycle is substrate-inducible and so is the biosynthesis of glyoxysomes. As a consequence, the time-course of gMDH appearance and decline during plant development differs strikingly from that of mMDH. Glyoxysomal MDH is absent in dry seeds of

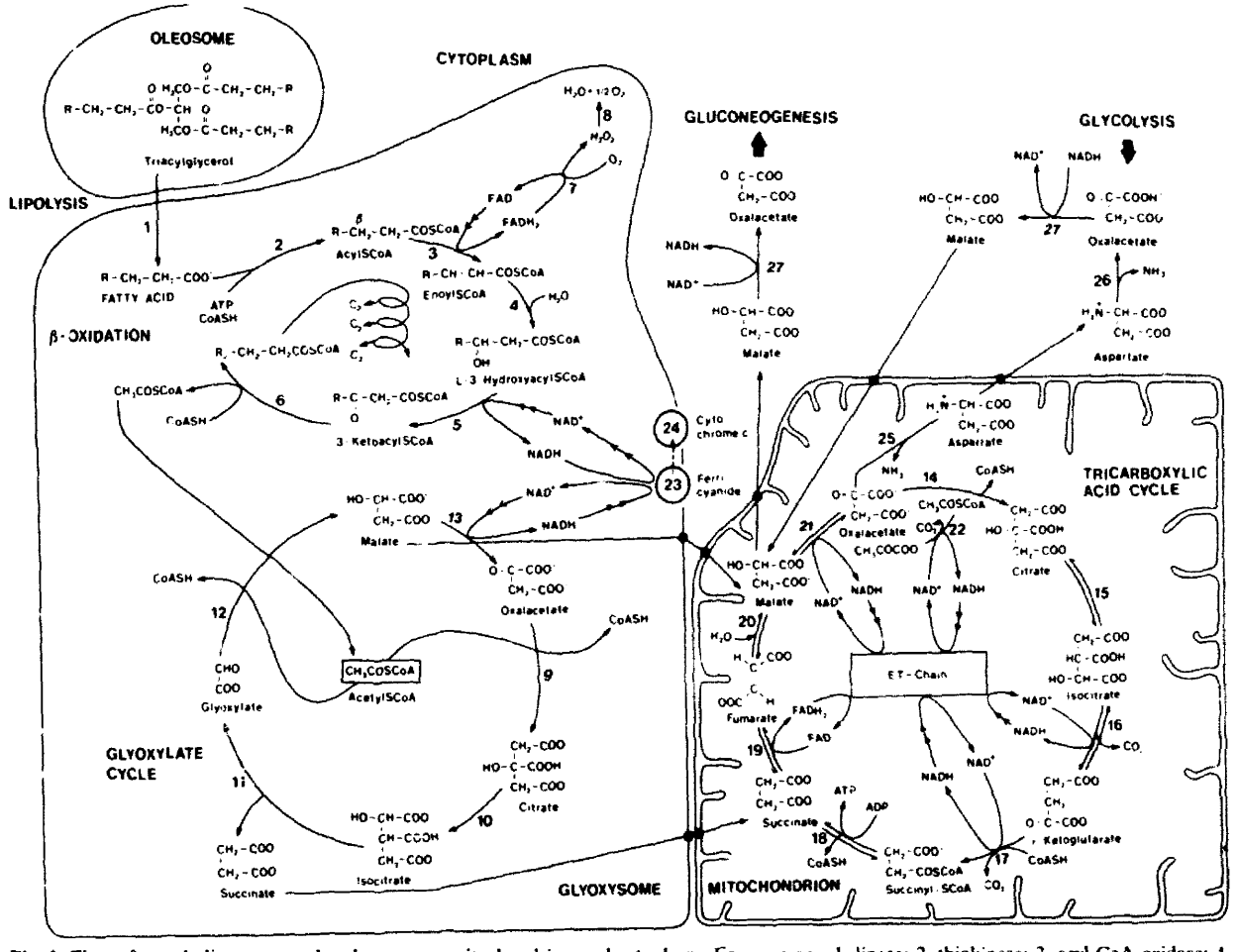


Fig. 1. Flow of metabolites among the glyoxysome, mitochondrion and cytoplasm. Enzymes are: 1, lipase; 2, thiokinase; 3, acyl-CoA oxidase; 4, enoylhydratase; 5, hydroxyacyl dehydrogenase; 6, ketoacyl thiolase; 7, flavin oxidase; 8, catalase; 9, citrate synthase; 10, aconitase; 11, isocitrate lyase; 12, malate synthase; 13, *malate dehydrogenase g*; 14, citrate synthase; 15, aconitase; 16, isocitrate dehydrogenase; 17, α-ketoglutarate dehydrogenase; 18, succinate thiokinase; 19, succinate dehydrogenase; 20, fumarase; 21, *malate dehydrogenase m*; 22, pyruvate dehydrogenase; 23, ferricyanide reductase; 24, cytochrome b_5; 25, aspartate aminotransferase m; 26, aspartate aminotransferase c; 27, *malate dehydrogenase c*.

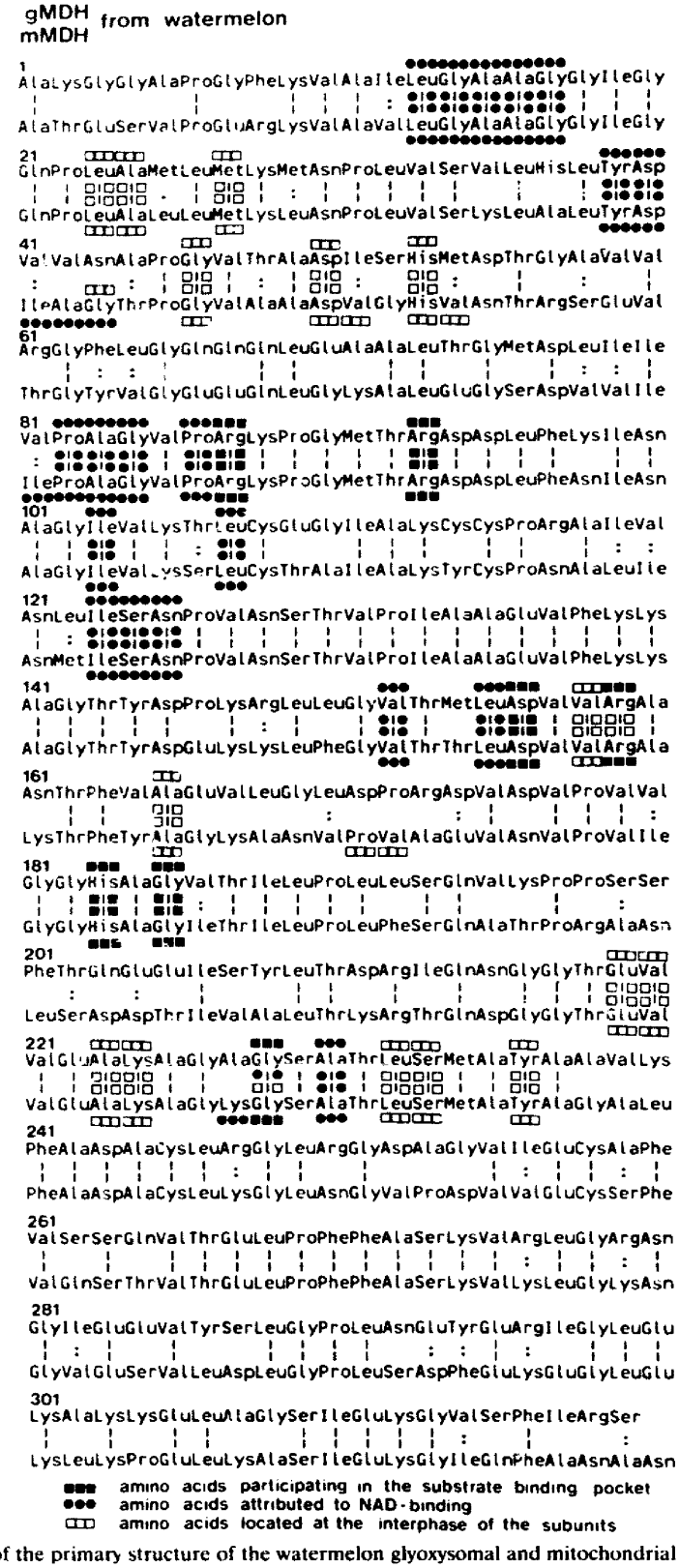


Fig. 2. Comparison of the primary structure of the watermelon glyoxysomal and mitochondrial malate dehydrogenase

watermelon. After a lag phase of 1.5 days, a large increase in amount and activity is observed, which reaches a peak at day 4 and then declines. On the other hand, mMDH is already present in dry seeds in substantial amounts, its activity increases 3-fold, and then remains at a constant level. During development of cotyledons both isoenzymes are synthesised de novo on cytoplasmic ribosomes [37,47,48].

II-C. In vitro synthesis of pre-glyoxysomal malate dehydrogenase and import into glyoxysomes

The transport of glyoxysomal MDH from the site of synthesis in the cytoplasm to the site of function in the organelle involves the translocation of the protein across the single membrane of the organelle. Glyoxysomal MDH from watermelon, with a mature subunit M_r of 33000 is exceptional, since it is synthesised as a larger precursor. In the wheat germ cell free protein synthesising system a protein with M_r 38000 [45,50] is made and in the reticulocyte system the product has a still higher apparent molecular mass $M_r = 41000$ [51]. Peroxisomal thiolase and sterol carrier protein 2 from rat liver [32,5Ja] are the only other microbody proteins known to be synthesised as higher molecular mass precursors. In vivo pulse chase experiments showed that the larger protein (pre-gMDH) is a precursor to the native enzyme, compatible with a posttranslational transport of gMDH [51]. Indeed, the cell free translation product is imported posttranslationally into glyoxysomes in vitro. However, glyoxysomes from watermelon cotyledons and castor bean endosperm behaved differently. When cell-free translation products from watermelon mRNA were incubated with watermelon glyoxysomes, the precursor of gMDH (pre-gMDH) was inefficiently processed, as revealed by immunoprecipitation with monospecific antibodies. Addition of a proteinase, cleaving at specific peptide bonds (trypsin, chymotrypsin) or unspecifically (proteinase K, pronase E, papain), to the mixture yielded a quantitative conversion of the precursor subunit to the 33000 M_r subunit which suggests proteinase sensitive substances inhibitory to import in this homologous in vitro organelle incorporation system [52]. Incubation of watermelon cell-free translation products with glyoxysomes from castor bean resulted in efficient processing and sequestering of pre-gMDH to the proteinase-resistant subunit form [53].

II-D. Sequence of cDNA clones and primary structure of the glyoxysomal malate dehydrogenase precursor protein

In principle, different isoenzymes can be derived from unrelated genes, from genes in the same family sharing a common ancestral gene or by alternative RNA splicing and posttranslational modification from a single gene. Comparison of glyoxysomal and mitochondrial MDH from watermelon cotyledons by means of N-terminal sequence analysis, serological methods and peptide patterns revealed a hign degree of similarity but excluded the possibility that both isoenzymes are encoded in a single gene [54]. Sequence analysis of a full-length cDNA clone for the gMDH precursor protein established the presence of an N-terminal transit peptide of 37 amino acids. Comparison of the primary structures in the mature part of the gMDH with mMDH in the higher plant (Fig. 2) as well as with mammalian and yeast mMDH demonstrates the prominent conservation of the NAD binding sites and the residues involved in catalysis [55]. Glyoxysomal MDH is very similar to mitochondrial and to cytoplasmic MDHs and lactate dehydrogenases (LDH). Genes encoding gMDH, mMDH, cytoplasmic MDH and LDH have apparently evolved from a common ancestral gene, as these enzymes constitute a family with very similar catalytic mechanism [56]. Especially amino acids Arg-87, Gly-185 and Gly-228, which are decisive for selecting malate as substrate, are rigorously conserved in MDH enzymes, while in LDH enzymes an equally strong conservation is observed for Gln, Asp and Thr in the same positions [57]. Furthermore, Asp-40, which is involved in NADH-binding, is highly conserved in the NADH-dependent 2-hydroxy acid dehydrogenases. Replacement of this aspartate by a serine shifts the coenzyme specificity towards NADPH [58].

The 37-residues-long N-terminal transit peptide poses the question if it is a glyoxysomal targeting signal. Glyoxysomal MDH and rat peroxisomal thiolase are the only microbody enzymes known to be synthesised with an amino-terminal transit peptide [32,55]. Microbody import is primarily studied with enzymes of β-oxidation located in the peroxisomes of liver and kidney of mammals and of yeast grown on alkanes [59]. It is analyzed in trypanosomes, a protozoan parasite, where glycosomes harbor glycolytic enzymes, which in other organisms are located exclusively in the cytosol [60] and with luciferase, an enzyme located in the peroxisomes of fireflies [61]. In plants, import studies concern glyoxysomes and peroxisomes [62]. So far three types of topogenic signal for microbody targeting are discussed:

(i) The positively charged domains and C-terminal extensions of the glycosomal proteins [63]. The primary structure of the C-terminus of gMDH is very similar to that of mitochondrial [55,64] and cytoplasmic MDHs [56]. No C-terminal extension is indicated and a comparison of the primary structures of the mature subunits of gMDH and mMDH does not identify obvious positively charged domains in gMDH. In both cases an average p*I* of 6.1 is found. If positively charged domains exist in the mature enzyme they have to arise by a special folding of the peptide chain or by post-trans-

lational modifications which give rise to the distinctly higher isoelectric point than the cytosolic and mitochondrial isoenzymes [44,65].

(ii) The peroxisomal targeting signal (PTS), a conserved tripeptide (Ser-Lys-Leu = SKL) first described for the firefly luciferase and shown to be necessary and sufficient to direct luciferase or reporter proteins into microbodies. The PTS permits Ser, Ala or Cys in the first position, Lys, Arg or His in the middle, and Leu in third position. Class I proteins contain the PTS at the C-terminus, and class II proteins contain an analogous sequence in at least one internal location [66]. The ubiquity of PTS suggests that the mechanism of protein translocation into microbodies with this signal has been conserved among plants, mammals, insects and yeast [67]. Glyoxysomal MDH does not contain this tripeptide in its mature part, but an Ala-His-Leu sequence is found in the transit peptide [55].

(iii) A highly suggestive targeting signal emerges from a comparison of gMDH with the rat peroxisomal 3-ketoacyl thiolase, which contains at the N-terminus a peptide extension of 26 residues:

gMDH NH_2-MQPIPDVNQRIARISAHLHPPKSQMEESSALRRANCR
pthiolase NH_2 MHRLQVVLGHL-AGRS--ESSSALQAAPC-

Both transit peptides have a net positive charge, they lack a long stretch of hydrophobic residues but they contain a glutamate and a cluster of serine residues at about the same distance from the cleavage site. Furthermore, 3-ketoacyl thiolase also lacks the PTS within its mature part and contains the tripeptide Gly-His-Leu in a position corresponding to the Ala-His-Leu tripeptide in the pre-sequence of gMDH. The mutation experiments of Gould and coworkers [66] did not analyze a Gly in the first position and therefore Gly-His-Leu may well be a targeting signal [55].

II-E. Molecular aspects of other glyoxysomal enzymes

The other enzymes of the glyoxylate cycle are citrate synthase, aconitase, isocitrate lyase and malate synthase [2]. Citrate synthase (CS) as well as aconitase exists as glyoxysomal and mitochondrial isoenzymes.

Citrate synthase isoenzymes were studied in corn scutella [68] in cucumber cotyledons [69], castor bean endosperm [42,46,70,71] and yeast [72,73]. Both native citrate synthases are dimers with a dimeric molecular mass of 95 kDa. Many properties are similar, including pH-dependence and temperature-sensitivity. The most pronounced differences were the isoelectric points of pH 9.1 for the glyoxysomal and pH 5.9 for the mitochondrial citrate synthase [46]. In *Saccharomyces cerevisiae* the peroxisomal and mitochondrial CS are encoded by two nuclear genes, CIT2 and CIT1, respectively. The gene for mCS encodes a typical targeting peptide for mitochondrial transport. The CIT2 product lacks this amino terminal leader and contains Ser-Lys-Leu (SKL) as a carboxy terminal leader peptide. Despite the similarity of the two isoenzymes in their amino-acid sequence (75% identity), antibodies raised against the two isoenzymes do not crossreact [72,74].

Isocitrate lyase and malate synthase are the key enzymes of the glyoxylate cycle. Isocitrate lyase (IL) exhibits a molecular mass of 255 kDa and is composed of four apparently identical subunits with an M_r of 64000. An isoelectric point of 5.9 was determined [75]. IL is synthesised with its final subunit size in vivo and in vitro in castor bean endosperm [76], cucumber [50,77] and also in *Neurospora crassa* [78]. The cDNA deduced amino acid sequence is known from rapeseed [79], castor bean [80], cotton [81] and *E.coli* [82]. The conserved tripeptide peroxisomal targeting signal was not found at the C-terminus.

Malate synthase has been purified from several oil seed species: from cucumber [50,69], castor bean [83,84], corn [85] and cottonseed [86]. Comparison of malate synthase labeled in vivo and in vitro revealed no detectable differences in subunit molecular mass [50,87–89] but the precursor form does not oligomerise and aggregate as is the case with the organellar protein. The 5 S monomer with a molecular mass between 57 kDa and 64 kDa is amphipatic and has a strong tendency to bind lipid or to oligomerise to a 20 S octamer [50,69,83,85,90,91] or dodecamer [86], giving estimated molecular masses of about 550 kDa or 730 kDa, respectively. A full-length cDNA clone for malate synthase was isolated from *Brassica napus*. The deduced polypeptide consists of 561 amino acids with a molecular mass of 63,700 daltons [92]. For cucumber a full-length cDNA clone and a genomic clone have been analyzed. Three introns could be identified and the deduced peptide sequence of 568 amino acids yields a molecular weight of 64961 [93]. Both cDNA-derived peptide chains contain the conserved tripeptide peroxisomal targeting signal at the C-terminus and in an internal location.

The five enzymes of the glyoxylate cycle catalyze the conversion of two molecules acetyl-CoA to succinate, which is transported into the mitochondria. Further consequences of the fatty acid degradation in the glyoxysomes are the formation of one $FADH_2$ by acyl-CoA oxidase and of two $NADH_2$ by hydroxyacyl dehydrogenase and malate dehydrogenase, respectively. $FADH_2$ is reoxidised by a flavin oxidase, resulting in the formation of H_2O_2, which is dismutated by catalase. Both enzymes associated with O_2 supply are unique for microbodies.

Catalase is a tetrameric haem protein [94] with known sequence in cotton seed [95], mammals [96–99] and yeast [100]. The C-terminus regions of the cDNA-derived peptide chains do not terminate with the conserved tripeptide peroxisomal targeting signal. The

subunit molecular masses are 55–65 kDa. In rat liver the extra-peroxisomal apomonomeric precursor and the subunit of mature peroxisomal catalase are indistinguishable by SDS-PAGE [101] or by one- and two-dimensional peptide mapping [102]. Catalase is imported into peroxisomes without detectable modification of its primary structure. Binding of the prosthetic haem group as well as oligomerisation occur within the peroxisomes [21]. A cytosolic factor(s) stimulates the import. Also in the yeast species *Candida tropicalis* and *Hansenula polymorpha* catalase mRNA yields a cell free translation product that comigrates in SDS-gels with the subunit of the mature enzyme [22,103,104].

Catalase biogenesis in pumpkins has unusual features [105,106]. The purified glyoxysomal catalase was found to consist of four identical subunits (55 kDa), whereas the leaf peroxisomal catalase contains two different forms of monomeric subunits (55 and 59 kDa). Catalase is synthesised in vivo and in vitro with an M_r of 59000, and imported into glyoxysomes or leaf peroxisomes posttranslationally. Inside the organelle it may be processed proteolytically to M_r 55000, bind haem and aggregate to an active tetrameric enzyme with a native M_r of 230000. Alternatively, it can escape proteolysis and assemble into a tetramer with little or no enzymatic activity: in etiolated (dark-grown) cotyledons, which have typical glyoxysomes, most of the catalase is processed to the active form. After greening of cotyledons, 60% of the catalase remains unprocessed within peroxisomes. The two types of tetramer are antigenically indistinguishable and copurify. Since the peroxisomal catalase consists of active and inactive forms, it displays only 40% of the specific activity measured with glyoxysomal catalase. Size differences between cell-free product and mature enzyme have also been observed in cucumber [?^.50].

II-F. Fate of $NADH_2$ made in glyoxysomes

The fate of $NADH_2$ synthesised within the glyoxysomes has not been fully clarified. Glyoxysomes do not contain an electron transport system linking $NADH_2$ to O_2 [15,107]. Two possibilities are discussed:

(i) Oxidation of $NADH_2$ in glyoxysomes by a malate-aspartate shuttle [108] would involve transport of malate from the glyoxysomes to mitochondria, oxidation of malate to oxalacetate, and transamination to aspartate, which returns to the glyoxysomes. Aspartate would combine with α-ketoglutarate to yield oxalacetate and glutamate. Glutamate : oxalacetate aminotransferase and malate dehydrogenase are present in both glyoxysomes and mitochondria, and are highly active [41]. This shuttle would also imply that malate in the glyoxysomes is not converted to oxalacetate, as previously assumed [2]. Instead, it is suggested that the MDH in the glyoxysomes functions in the reductive direction to consume $NADH_2$ and generate malate. The formation of oxalacetate from malate is a highly unfavorable reaction especially in the presence of $NADH_2$. The proposed shuttle mechanism would require transport of malate, aspartate, glutamate and α-ketoglutarate through the glyoxysomal and mitochondrial membranes at appropriate rates. Such movements have yet to be demonstrated. On the other hand, transport of succinate from glyoxysomes to mitochondria has been generally accepted. Transport between mitochondria or chloroplasts and the cytosol is controlled by specific shuttles and membrane-bound translocases. So far no membrane bound translocases have been reported for microbodies. The microbody is certainly a compartment for enzymes, but it is not yet known whether also the substrates are compartmentalised, which would require controls by membrane transport systems.

(ii) The alternative possibility is a coupling of the β-oxidation and glyoxylate cycles to NADH : cytochrome *c* and ferricyanide reductases in glyoxysomes, which may allow β-oxidation and the glyoxylate cycle to be partially uncoupled from mitochondrial oxidative phosphorylation [109]. Isolated glyoxysomal membranes contain both enzyme activities for oxidation of $NADH_2$ [110,111]. The malate dehydrogenase reaction alone does not favour malate oxidation. However, NAD-dependent malate oxidation was observed, when acetyl-CoA was available to condense with oxalacetate to form citrate, and thus relieve product inhibition. Alternatively, the addition of glutamate provided for the conversion of the oxalacetate to aspartate via the glutamate : oxalacetate aminotransferase [41]. Oxidation of malate also occurred if either cytochrome *c* or ferricyanide was added. $NADH_2$ oxidation by the malate aspartate shuttle would link β-oxidation to higher levels of ATP-generation. Thus, the balance of electron flow through the two routes would depend on the demand for ATP as fatty acid is oxidised and converted to sucrose in castor bean endosperm [109].

In conclusion, glyoxysomal malate dehydrogenase is – together with 3-ketoacyl thiolase – the only microbody enzyme known to be synthesised as a higher molecular mass precursor; gMDH is – together with catalase – the most active enzyme within the glyoxysomes and plays an important role in the glyoxylate cycle as well as in a putative malate/aspartate shuttle. The strong sequence similarity between glyoxysomal and mitochondrial malate dehydrogenase [55] supports an earlier hypothesis, that microbodies with their "archaic" O_2-processing also derive from an endosymbiont, but would have preceded mitochondria in the ancestral organism and would have lost their outer membrane [112].

III. The malate dehydrogenase in mitochondria

III-A. Multiple functions of mitochondrial malate dehydrogenase

Higher plant mitochondria resemble in many aspects those of mammals and yeast. For example, the sequence of electron carriers that mediates the flow of electrons from $NADH_2$ and succinate to O_2 via cytochrome oxidase (i.e., the cyanide-sensitive electron pathway), the phosphorylation system (ATPase complex) and the tricarboxylic acid cycle are functioning in a similar way.

However, there are distinct differences between animal and plant mitochondria, which may reflect the autotrophic metabolism of the latter: For instance, fatty acid oxidation is either very low [20,113,114] or not detectable (the bulk of fatty acid oxidation in the plant cell being confined to microbodies [17,115]). Oxalacetate has been found to traverse the inner membrane of plant mitochondria. The size and complexity of mitochondrial DNA is different [116].

Mitochondrial malate dehydrogenase participates in three different pathways: (1) In the tricarboxylic acid cycle (Fig. 1). (2) in the conversion of glycine to serine by providing reducing equivalents (Fig. 3) and (3) in the supply of CO_2 for fixation in bundle sheath chloroplasts (Fig. 4). The latter two pathways signify cooperation of the mitochondrion with the peroxisome in photorespiration and with the chloroplast in the concentration mechanism of CO_2 for C_4 photosynthesis, respectively.

In the tricarboxylic acid cycle mitochondrial MDH provides together with isocitrate dehydrogenase and α-ketoglutarate dehydrogenase $NADH_2$ to be oxidised in the respiratory chain, while succinate dehydrogenase yields $FADH_2$. Plant mitochondria preferentially oxidise $NADH_2$ produced in the matrix space as a result of substrate oxidation. Since $NADH_2$ produced during glycolysis cannot enter the mitochondrion, reducing equivalents are shuttled into the organelle by malate and pyruvate. However, plant mitochondria show a great flexibility by using a concerted action of malate dehydrogenase, NAD-dependent malic enzyme, and pyruvate dehydrogenase to provide citrate in the anaplerotic function of the TCA cycle. Matrix $NADH_2$ produced by the dehydrogenases and by malic enzyme can be oxidised equally well by the respiratory chain or by the malate dehydrogenase working in the reverse direction from oxalacetate to malate. Carbon input into the TCA cycle could occur in the form of cytosolic oxalacetate and malate (the latter produced by the successive operation of phospho*enol*pyruvate carboxylase and malate dehydrogenase in the cytosol). Pyruvate

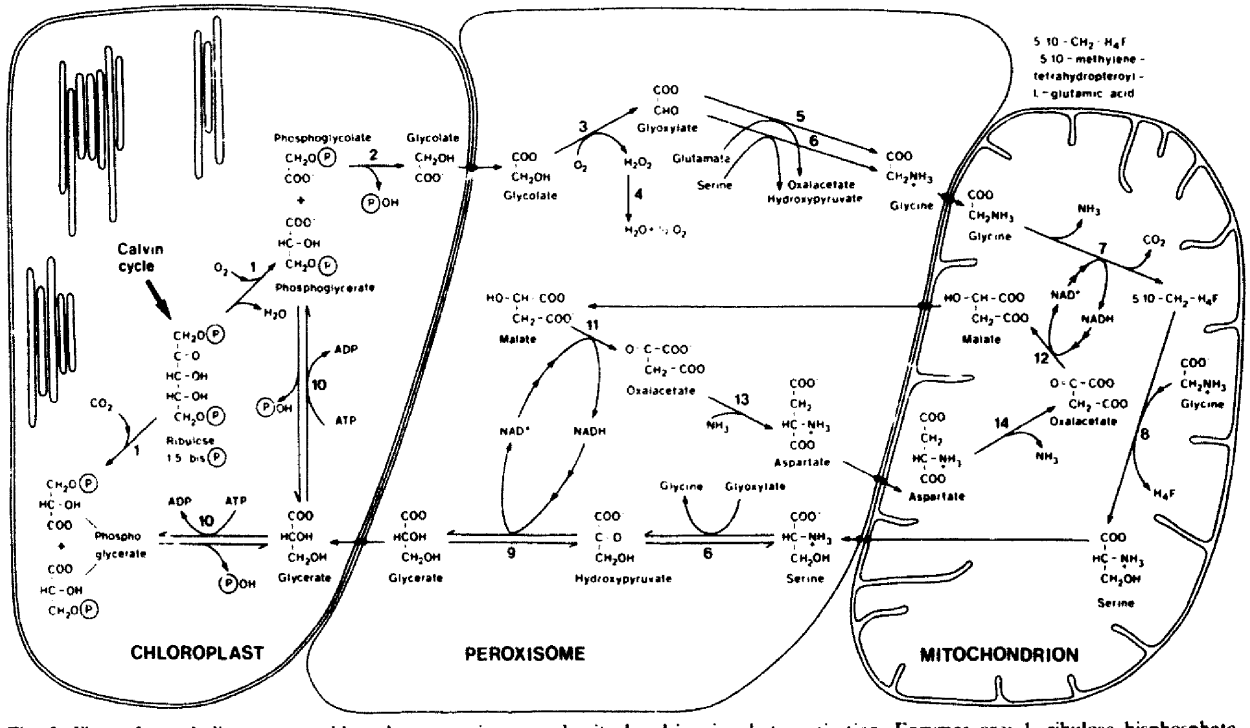


Fig. 3. Flow of metabolites among chloroplast, peroxisome and mitochondrion in photorespiration. Enzymes are: 1, ribulose bisphosphate carboxylase/oxygenase; 2, glycolate phosphatase; 3, glycolate oxidase; 4, catalase; 5, glutamate:glyoxylate aminotransferase; 6, serine:glyoxylate aminotransferase; 7, glycine cleavage system; 8, serine hydroxymethyltransferase (SHMT); 9, hydroxypyruvate reductase; 10, glycerate kinase; 11, malate dehydrogenase p; 12, malate dehydrogenase m; 13, aspartate aminotransferase p; 14, aspartate aminotransferase m.

can be provided either by the action of pyruvate kinase in the cytosol or by operation of malic enzyme in the matrix, utilising malate generated either in the matrix or the cytosol [116]. In fat-degrading tissue glyoxysomes deliver succinate as carbon input. In mammalian cells the inner membrane seems impermeable to oxalacetate under normal physiological conditions [117]. In marked contrast, oxalacetate has been found to traverse the inner membrane rapidly in all plant mitochondria isolated so far [118]. In plant mitochondria malate transport is sensitive to 2-*N*-butylmalonate, an inhibitor of the dicarboxylate carrier, while that of oxalacetate apparently is not [119]. Conversely, phthalonate [120] has little effect on malate transport, but severely restricts oxalacetate transport [121,122]. These results strongly suggest that malate efflux and oxalacetate influx occur on separate carriers [119,123]. Carbon input into the TCA cycle in the form of cytosolic oxalacetate would interrupt the continuity of the TCA cycle.

In mammalian and yeast cells mitochondrial and cytosolic malate dehydrogenases are components of the malate/aspartate shuttle and represent an important mechanism for exchange of substrates and reducing equivalents between metabolic pathways separated by the mitochondrial membrane. A considerable fraction of the total $NADH_2$ synthesised by the eucaryotic cell is manufactured in the cytoplasm during glycolysis, and the reducing equivalents required for respiration or for a variety of metabolic processes [124] are transported from the cytoplasm to the mitochondria across the mitochondrial membrane by the malate/aspartate shuttle, as intact mitochondria are impermeable to $NADH_2$ [125,125a,126]. There is significantly less information available about this shuttle system in plant mitochondria.

III-B. Photorespiration

On the other hand, a malate/aspartate shuttle plays a predominant role in the exchange of reducing equivalents between mitochondria and peroxisomes during photorespiration (Fig. 3). Glycine is oxidised in the mitochondrial matrix space by the glycine cleavage system to produce CO_2, NH_3, $NADH_2$, and 5,10-methylenetetrahydropteroyl-L-glutamic acid (5,10-CH_2-H_4F). The latter compound reacts with a second molecule of glycine to form serine in a reaction catalyzed by serine hydroxymethyltransferase (SHMT). Oxalacetate formed by transamination from aspartate is reduced by malate dehydrogenase, which allows the regeneration of NAD for glycine oxidation and thus bypasses the respiratory chain. The malate returns to the peroxisome [127]. Its conversion to oxalacetate by the peroxisomal malate dehydrogenase generates the $NADH_2$ necessary for the reduction of β-hydroxypyruvate [123]. Oxalacetate is shuttled back via aspartate into mitochondria.

At the same time, a malate/oxalacetate shuttle operates to transfer redox equivalents from the mitochondrial matrix to the cytosol. This shuttle functions mainly

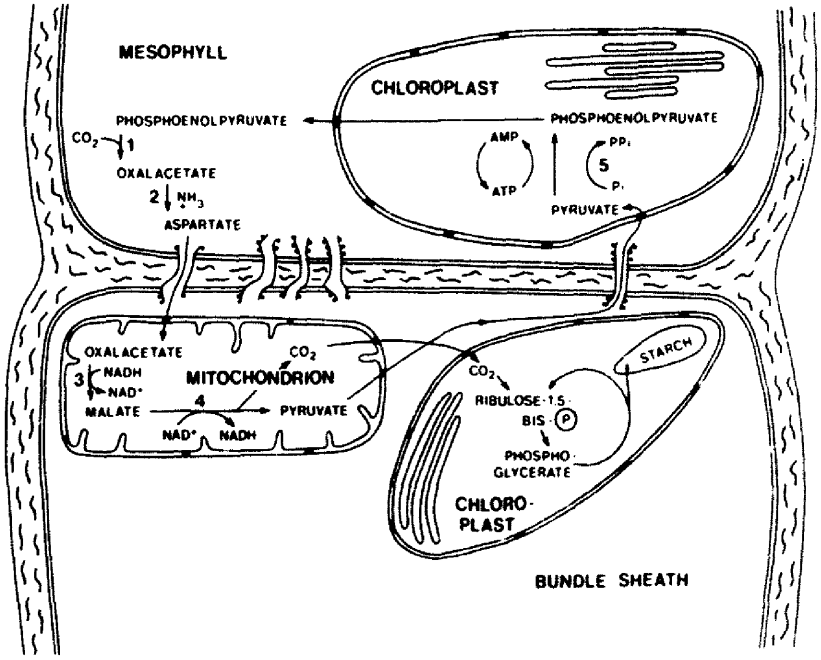


Fig. 4. Flow of metabolites among organelles in and between mesophyll and bundle sheath cells in C_4 pathway of photosynthesis as found in *Amaranthus*. Enzymes are: 1, phospho*enol*pyruvate carboxylase; 2, oxalacetate:aspartate aminotransferase; 3, NAD-malate dehydrogenase; 4, malic enzyme; 5, pyruvate, P_i dikinase.

in the export of malate from mitochondria, whereas the import of malate as respiratory substrate may proceed by the classical malate-phosphate antiport [128]. Potentially similar rates of $NADH_2$ export from pea leaf mitochondria in the malate/aspartate and the malate/oxalacetate shuttle are measured under photorespiratory conditions in vivo, but it is concluded that a significant amount of respiratory chain activity would also be required in the light to ensure the complete reoxidation of this $NADH_2$ [129].

In C_4 plants photorespiration is minimal because in the bundle sheath cells CO_2 is concentrated by decarboxylation of the C_4-acids malate or aspartate. This C_4 pathway of photosynthesis is always associated with Kranz anatomy [130,131]: CO_2 is first assimilated in the mesophyll cells into the C_4 carboxylic acids. These are then transported through the symplast to the sheath cells which surround the vascular bundles and appear like a wreath (Kranz) in leaf cross-sections. Here the acids are decarboxylated by one of three reactions [132,133]. Either a NAD-malic enzyme in the mitochondria converts malate to pyruvate and the formed CO_2 is used by the bundle sheath chloroplast in the Calvin cycle (Fig. 4) or the NADP-malic enzyme carries out the reaction in the chloroplast itself (Fig. 5). In a third group of plants the CO_2 is generated in the cytosol by a phospho*enol*pyruvate carboxykinase.

In those plant species (e.g., *Amaranthus retroflexus* or *Portulaca oleracea*) in which CO_2 is produced in the mitochondria by decarboxylation of malate, pyruvate is recycled into the mesophyll cells chloroplast. It is converted into phospho*enol*pyruvate by the pyruvate, P_i-dikinase and transferred into the cytoplasm. CO_2 assimilation by the phospho*enol*pyruvate carboxylase gives oxaloacetate, which is converted into aspartate by an aminotransferase. Aspartate is shuttled into the bundle sheath cell, where it is converted into oxalacetate and by the mitochondrial NAD-malate dehydrogenase into malate (Fig. 4).

III-C. Characterisation of mitochondrial malate dehydrogenase and in vivo synthesis

Mitochondrial malate dehydrogenase from cotyledons of germinating watermelon seedlings could be separated from the cytosolic and glyoxysomal isoenzymes by affinity chromatography on 5'-AMP-Sepharose [39]. It is a homodimer with an M_r (by gel filtration) of 74000 [44]. The subunits comigrate in SDS-polyacrylamide gel electrophoresis with a molecular mass of 38 kDa [51]. The development of mitochondrial and glyoxysomal MDH during seed germination was determined by means of radial immunodiffusion assays [47,134] as well as by fluorescence immunohistochemical localisation of the two isoenzymes with specific antibodies [49]. Cotyledons of ungerminated seeds were found to contain mMDH. During the first four days of germination the enzyme activity increased 3-fold finally contributing 16% of the total MDH activity extracted from cotyledon tissue. Mitochonurial MDH is synthesised de novo during the first 4 days of germination, as could be shown by density labeling in vivo

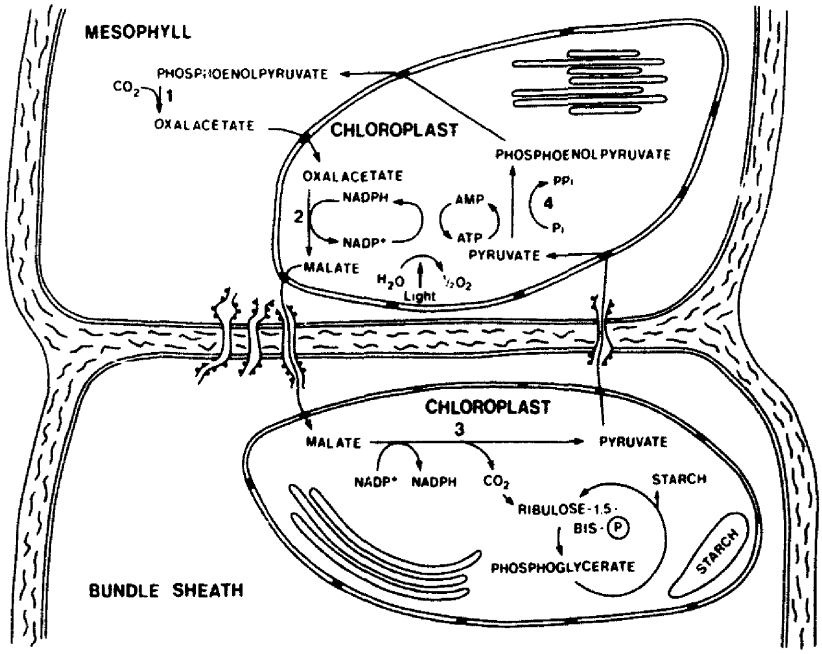


Fig. 5. Flow of metabolites among organelles in and between mesophyll and bundle sheath cells in C_4 pathway of photosynthesis as found in *Zea mays*. Enzymes are: 1, phospho*enol*pyruvate carboxylase; 2, NADP-malate dehydrogenase; 3, malic enzyme; 4, pyruvate, P_i dikinase.

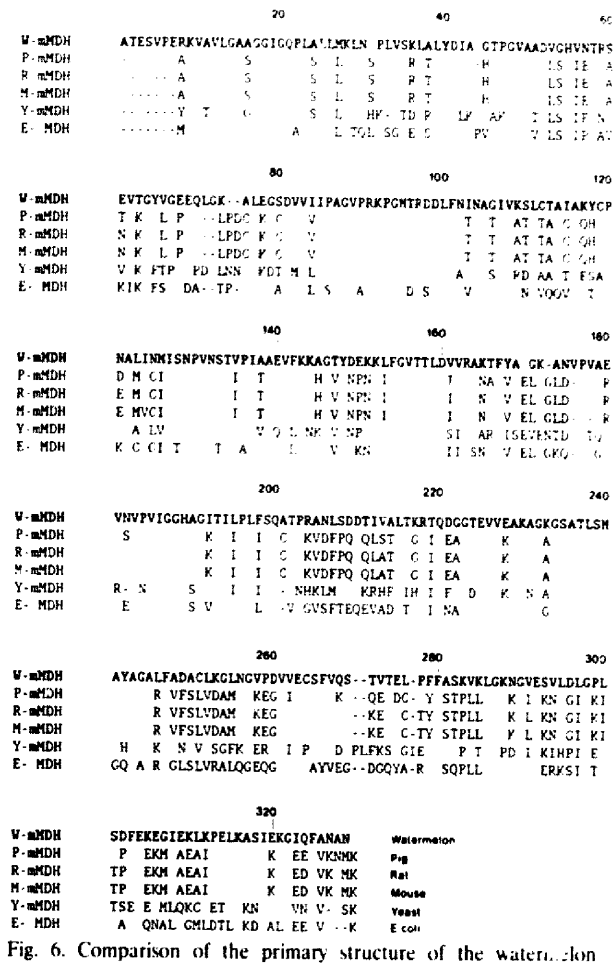
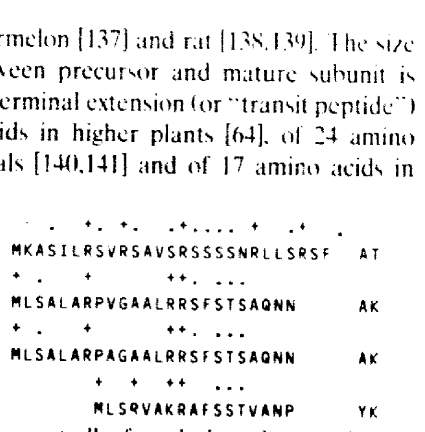


Fig. 6. Comparison of the primary structure of the watermelon mitochondrial malate dehydrogenase with the corresponding enzymes from pig [55], rat [140], mouse [152] and yeast [142]. Also given is the *E. coli* MDH amino-acid sequence [153]. Only amino-acid residues which differ from those found in the higher plant enzyme are written for the five sequences, an empty space signifying identity. Gaps introduced in the sequences for maximal alignment are indicated by a hyphen.

with D_2O and by labeling of cotyledons in vivo with [^{14}C]amino acids [134]. Synthesis occurs on cytoplasmic ribosomes. The kinetic parameters of mMDH are very similar to those of gMDH (cf. section II-B). For mMDH ($2.0 \cdot 10^{-9}$ M) they amount to V_{max}(μmol/min) = 5.54 for malate oxidation; V_{max}(μmol/min) = 18.70 for oxalacetate reduction; K_m(oxalacetate) = 0.15 mM; K_m($NADH_2$) = 0.11 mM; K_m (malate) = 3.69 mM; K_m (NAD) = 0.46 mM [148].

III-D. In vitro synthesis and import of malate dehydrogenase precursor into mitochondria

Mitochondrial MDH, like most mitochondrial enzymes, is synthesised as a higher molecular mass precursor in plants [51] and mammals [135,136]. In vitro import of pre-mMDH into mitochondria has been reported for watermelon [137] and rat [138,139]. The size differences between precursor and mature subunit is due to an NH_2-terminal extension (or "transit peptide") of 27 amino acids in higher plants [64], of 24 amino acids in mammals [140,141] and of 17 amino acids in yeast [142].



It has been repeatedly found that the spacing of arginines and the hydroxylated residues are of significance for the mitochondrial import of a specific protein. In this respect, the similarity of the spacing between the arginines in the targeting sequences of watermelon, rat, mouse and yeast is remarkable, as is the heaping of serine and threonine residues C-terminal to the conserved arginine in position 15 (position 8 for yeast mMDH) [64]. However, the fine tuning of mMDH-import is different in plants as compared to mammals and yeast.

A survey of amino-terminal proteolytic cleavage sites in mitochondrial precursor proteins revealed frequently an arginine at position -2 of the mature amino terminus, suggesting a one step cleavage, or at position -10, suggesting that some targeting peptides may be cleaved twice in succession by two different matrix proteinases [143]. For two-step processing, an arginine at -10, a hydrophobic residue at -8 (mainly phenylalanine) and a serine, threonine or glycine at -5 of the mature amino terminus is highly conserved [144]. For rat mMDH a two-step cleavage is established [145]. Protease I, which cleaves the precursor to the intermediate form, is insensitive to chelation by EDTA and to inactivation with *N*-ethylmaleimide. Protease II, which cleaves the intermediate form to the mature subunit, is inhibited by 5 mM EDTA and is inactivated by treatment with *N*-ethylmaleimide [146]. In rat the mMDH intermediate precursor is formed by cleavage between the arginine at -10 and the serine at -9 [145]. The ornithine transcarbamylase intermediate results from cleavage between an asparagine at -9 and a phenylalanine at -8 [147]. For the import of rat precursor mMDH the leucine in position -12 plays a crucial role in transit peptide binding to the mitochondrial surface [148,149], while the positive charge is important for membrane translocation, but does not affect binding [150]. In comparison with the 24-amino-acid leader in mammalian mMDH three amino acids, Arg-Ser-Phe, are inserted between the leader peptide and the mature amino terminus in the watermelon precursor mMDH. Furthermore, the mature form is distinguished from other mMDHs by an amino termi-

nal extension of 7 amino acids, which also may be involved in translocation. A two-step cleavage for watermelon mMDH in a manner comparable to rat mMDH is not very likely, because the distance between the relevant arginine at −13 and the mature NH_2-terminus seems too long. The valine at −15 could replace the leucine, but a hydrophobic residue like phenylalanine at −11 is missing. The arginine at −3 does not fulfill the expectations for a one-step cleavage, which needs an arginine at −2. Surprisingly, the motif leu-ser-arg-ser-phe (position −5 to −1) in watermelon pre-mMDH resembles very much the motif around the intermediate cleavage site in rat pre-mMDH leu-arg-arg-ser-phe (position −12 to −8), but its function remains to be analyzed [64]. Yeast pre-mMDH contains the same motif for a two-step cleavage as the rat mMDH in its leader peptide, but the presequence is dispensable for cellular localisation [151].

A genomic DNA fragment containing the mouse mitochondrial malate dehydrogenase was isolated. The gene is 12000 bp long and contains 9 exons separated by 8 introns of various sizes. In the 5′ flanking region neither a TATA box nor a CAAT box could be found. Instead, there are 6 copies of the GGGCGG or CCGCCC sequences which are a potential binding site for the transcription factor Sp1 [152].

III-E. Comparison of the mature mitochondrial malate dehydrogenase from the higher plant with that of other organisms: evolutionary aspects

Comparison of watermelon mMDH with mammalian, yeast and *E.coli* MDH [153] gives 55–60% overall identity of residues (Fig. 6). On the other hand, the amino-acid sequences of mouse mMDH and cytosolic MDH (cMDH) show only about 23% overall identity [154]. Surprisingly, comparisons of the amino-acid sequences among the eucaryotic and bacterial MDHs revealed that the similarity between the mitochondrial MDHs from plants, yeast, *Escherichia coli* and the thermophilic bacterium *Thermus flavus* [155] exceeds the *intra*species sequence similarity between mitochondrial and cytoplasmic MDH.

The amino-acid residues involved in catalysis, nucleotide binding, and helices forming the subunit interface are highly conserved in all MDHs (cf. Fig. 2). Mitochondrial MDHs, and *E.coli* MDH as well as glyoxysomal MDH from watermelon [55] derived from a common prototype encoded in an ancestral gene [156]. Close relationship is also found with other 2-hydroxy dehydrogenases such as lactate dehydrogenase [56]. Clarke et al. [157] constructed a catalytically active malate dehydrogenase by substituting only three amino acids in the active site of lactate dehydrogenase. Thus, the domains involved in the catalysis, substrate binding, and cofactor attachment are the most strongly conserved ones. Additional domains conserved specifically in the eucaryotic organelle-bound MDHs may reflect e.g., common regulatory functions or other complex formation demanded by interactions in the tricarboxylic acid cycle and the related glyoxylate cycle. In both cases a physical interaction with another enzyme such as citrate synthase could be necessary for direct transfer of oxalacetate between the two enzymes and for efficient functioning of the pathway [158]. This evolutionary aspect is illustrated by yeast mitochondrial and peroxisomal citrate synthase (CS) which are closely related but differ at the amino and carboxyl termini. Mitochondrial CS is synthesised with an N-terminal leader peptide whereas the peroxisomal CS contains the targeting signal SKL in the carboxy terminal tripeptide [72,74]. The mitochondrial and *E. coli* citrate synthases share identical residues in only 20% of the aligned amino-acid sequences [159].

Mitochondrial and cytosolic fumarases highlight a different mode of evolutionary conservation as these isoenzymes are encoded by a single gene in yeast [160] and rat liver [161]. The gene is transcribed from multiple start sites in the same reading frame, some of which are located inside the coding sequence. The major transcript presumed to code for mitochondrial fumarase encodes an additional sequence of 41 amino-acid residues on the NH_2 terminus. The shorter translation products lacking the amino terminal leader peptide are proposed to remain in the cytoplasm.

IV. The malate dehydrogenase in peroxisomes

Photorespiration is an example for the tight cooperation of metabolic pathways located in different cell compartments (Fig. 3). It is initiated by the oxygenase activity of the bifunctional enzyme ribulose-bisphosphate-carboxylase/oxygenase (Rubisco) [162]. Metabolism of glycolate carbon occurs sequentially in three organelles, the peroxisomes, the mitochondria, and the chloroplasts [163]. In the peroxisome glycolate is oxidised to glyoxylate and then transaminated to glycine with either glutamate or serine [164]. Glycine then exits the peroxisome and is oxidised to ammonia, CO_2 and serine in the mitochondrion [165]. Serine is converted to glycerate by serine : glyoxylate aminotransferase and hydroxypyruvate reductase [166,167] in the peroxisome, and in this form photorespiratory carbon returns to the chloroplast. Glycerate is phosphorylated to phosphoglycerate by the glycerate kinase and can reenter the photosynthetic cycle [168]. Based on glycolate and glyoxylate feeding experiments with peroxisomes and cells, it has been suggested that glyoxylate oxidation to H_2O_2 and CO_2 may make a significant contribution to photorespiratory CO_2 evolution [169–172].

The advantage of the photorespiratory cycle appears to be two-fold. When CO_2 in the plant canopy becomes limited in supply – which is frequent at midday – Rubisco functions as an oxygenase and thereby protects the photosynthetic machinery from damage as it continues to operate in the sunlight. It does so by using energy for respiration. At the same time this leads to the production of CO_2 and regeneration of the substrate to be used in the fixation of CO_2.

A malate/oxalacetate/aspartate shuttle has been proposed for plant microbodies similar to the one for mitochondria [127,129,173]. Leaf peroxisomes as well as seed glyoxysomes contain a large amount of activity of an isoenzyme of NAD : malate dehydrogenase [174,175]. Besides catalase the malate dehydrogenase with an activity amounting to about 50 μmol/min per mg protein is the most active enzyme in plant microbodies. The oxidised component of the leaf peroxisomal malate shuttle is proposed to be aspartate rather than oxalacetate, as aspartate is the substrate for aspartate aminotransferases functioning in three different pathways of leaf peroxisomes [164].

Glyoxysomes in fat-degrading tissue and the leaf peroxisomes are closely related microbodies in higher plants. In greening watermelon or cucumber cotyledons the microbodies undergo a functional transition from glyoxysomal to peroxisomal metabolism. Double-label immunoelectron microscopy with isocitrate lyase and hydroxypyruvate reductase [176] or isocitrate lyase and serine : glyoxylate aminotransferase [177] revealed both sets of enzymes to be present at the same time. They were either present in two distinct populations of microbodies with different functions or in a single population of microbodies with a dual function. These results support the one-population hypothesis first proposed by Trelease et al. [3], which implies that the microbodies persist during the transition and that only their enzymic content changes. An alternative two-population hypothesis had previously suggested that glyoxysomes are degraded and new peroxisomes are created during greening of cotyledons [178].

It is therefore not surprising that enzymes present in both glyoxysomes and peroxisomes are identical. Peroxisomal and glyoxysomal malate dehydrogenases are serologically indistinguishable, and have the same isoelectric points and function as dimers [42,179]. Likewise, catalases from pumpkin glyoxysomes [105,106] and cucumber or lentil peroxisomes [180] crossreact serologically and have identical subunit characteristics.

Glycolate oxidase has been purified from crude extracts of greening cucumber cotyledons. The enzyme exhibited an M_r of 180000 or 700000 and is accordingly a tetramer, or a 16-mer made of identical subunits of 43 kDa [181]. The subunits are synthesised on cytosolic ribosomes without a cleavable presequence [180,182].

For NADH-dependent hydroxypyruvate reductase from cucumber a full-length cDNA clone has been isolated [183]. The open reading frame encodes a polypeptide with 382 amino-acid residues corresponding to a calculated molecular mass of 41.7 kDa. The amino-acid sequence of hydroxypyruvate reductase has 26% identical and 50% similar residues to the amino-acid sequence of the *E. coli* enzyme phosphoglycerate dehydrogenase, which catalyzes a similar reaction. The "conserved tripeptide" microbody targeting signal cannot be found. Dot blot analysis indicated that a single gene is likely to be present per haploid genome.

V. The malate dehydrogenase in chloroplasts

Besides the NAD-dependent isoforms of malate dehydrogenase located in microbodies, mitochondria and the cytoplasma in higher plants a NADP-dependent form of the enzyme is found in the chloroplasts. The chloroplastic MDH is essential for both the universal C_3 photosynthesis (Calvin cycle) and the more spezialised C_4 cycles, which allow plants to limit the loss of assimilates by photorespiration. In C_3 plants, NADP-MDH is an essential component of the malate/oxalacetate shuttle, which balances reducing equivalents between the chloroplast and the cytosol [184]. In C_4 plants, NADP-MDH activity is 10-fold higher and acts to convert oxalacetate to malate in chloroplasts of mesophyll cells for transport to bundle sheath cells [185]. During C_4 photosynthesis, atmospheric CO_2 is fixed by carboxylation of phospho*enol*pyruvate (PEP) in mesophyll cells (by PEP carboxylase), giving C_4 dicarboxylic acids which are decarboxylated in bundle sheath cells by one of three different decarboxylases: NADP-malic enzyme, NAD-malic enzyme or PEP carboxykinase. Only in the group of C_4 species utilising NADP-malic enzyme (*Zea mays, Saccharum officinarum, Sorghum bicolor, Digitaria sanguinalis*) does NADP-MDH have a major role (Fig. 5). When malate is transported to bundle sheath cells and decarboxylated via NADP-malic enzyme, it acts as a carrier of reducing power as well as CO_2. The $NADPH_2$ formed is directly utilised for phosphoglycerate reduction.

Studies of kinetics with the maize enzyme [186] showed linear Lineweaver-Burk plots for all four substrates and K_m values of 18 μM for oxalacetate, 50μM for $NADPH_2$, 24 mM for malate, and 45 μM for NADP. Pea leaf NADP-MDH can be interconverted between monomer (M_r 40000), dimer, and tetramer by varying pH and ionic strength [187]. The tetramer represents an inactive and the dimer the active form of the enzyme [188]. The chloroplast NADP-MDH in both C_3- and C_4-plants differs from the NAD-dependent forms of the enzyme in that the former is regulated indirectly by light via the thiore-

doxin-ferredoxin system [189]. In order to be enzymatically active, disulfide bridges on the protein must be reduced by thioredoxin, which receives electrons from ferredoxin and the electron transport system of photosynthesis [186,190,191]. Messenger RNA levels of NADP-MDH in maize increase upon greening of etiolated tissue, as shown by in vitro translation and immunoprecipitation [192]. In sorghum, mRNA levels were shown to be light regulated [193]. A cDNA clone for maize NADP-malate dehydrogenase has been isolated [194]. The encoded amino-acid sequence predicts that NADP-MDH is synthesised as a preprotein of 432 amino acids (46865 Da) and processed into a mature protein of 375 amino acids (40934 Da) with removal of a 57-amino-acid-long transit peptide (5931 Da). Despite the lack of sequence similarities to other chloroplast transit peptides the extra sequence shows the common features [195]: it is rich in the hydroxylated amino acids serine and threonine (14%), it is also rich in small hydrophobic amino acids such as alanine and valine (28%); it shows a net positive charge (8 arginines, 1 lysine), and is generally deficient in acid amino acids (2 aspartates). In the mature part of the protein, the locations of four cysteine residues are identical in the pea [188] and maize proteins. These cysteins, two near the N-terminus and possibly two near the C-terminus, are the sites of regulation by thioredoxin via cysteine crosslinking [187,196]. The maize enzyme is similar to other MDHs in regions related to enzymatic function. Especially Arg-124, Arg-130, Asp-194, Arg-197 and His-222 (Maize NADP-MDH numbering), which belong to the active site pocket and are responsible for the catalytic mechanism [57] are highly conserved between glyoxysomal [55] and mitochondrial NAD-MDHs [64] from higher plants as well as between other mitochondrial NAD-MDHs [140–142], cytosolic NAD-MDHs [197] and lactate dehydrogenases [56]. Using site-directed mutagenesis, the NAD-dependent lactate dehydrogenase from *Bacillus stearothermophilus* has been specifically altered at a single residue to shift the coenzyme specificity towards $NADPH_2$ [58]. The single change is at position 53 (LDH numbering; corresponding to position 41 in the watermelon mMDH numbering) where the aspartate, which is conserved in all NAD-MDHs and LDHs, has been replaced by a serine. This substitution reduces steric hindrance on binding of the extra phosphate group of $NADPH_2$ and removes the negative charge of the aspartate group. NADP-MDH from maize contains a glycine at this position (72 for NADP-MDH numbering).

The similarity of the C_3 and C_4 forms of NADP-MDH suggests that genes for C_4 enzymes may have been recruited from existing genes encoding C_3 enzymes.

VI. Malate dehydrogenases in the cytoplasm

VI-A. Characterisation of cytoplasmic malate dehydrogenases and in vivo synthesis

Dark-grown watermelon seedlings possess a simple pattern of MDH isoenzymes. After separation of crude extracts from cotyledons by polyacrylamide gel electrophoresis and MDH-specific staining, four MDH isoenzymes are recognised during the first period of germination. After two days, a fifth isoenzyme becomes visible [34,35]. By isolation and fractionation of the cell organelles it was shown that the glyoxysomes contain isoenzyme V (gMDH) and the mitochondria isoenzyme III (mMDH), whereas isoenzyme I, II and IV belong to the cytosol [36]. In contrast to the cotyledons, the embryo axis contains only the cytosolic isoenzyme I and the mitochondrial isoenzyme III.

In a developmental study of NAD-malate dehydrogenase isoenzymes in the cotyledon of cucumber seedlings grown in darkness and in light [37] it was learned that cMDH I and mMDH (isoenzyme III) occur in substantial quantity already in cotyledons of dry seeds while cMDH II and IV and gMDH (isoenzyme V) are present in very low amount. During germination in darkness, the activities of the five isoenzymes increase at different rates, but they all peak together at day 3 and then decline gradually at a similar rate. Light applied at day 3 selectively eliminated the two cytosolic isoenzymes II and IV but did not affect the subsequent developmental patterns of the third cytosolic isoenzyme I, the mMDH and the gMDH. Such a selective elimination of these two cytosolic isoenzymes, and their absence in roots as well as green leaves, indicates that cMDH II and IV participate in the mobilisation of fat reserves during germination. Root tissue does not contain detectable amount of microbody MDH [198]. It seems that mMDH and cMDH I are the two isoenzymes for the basic cellular metabolism. Since both of them, unlike the other three, occurred in substantial amount in the cotyledon of dry seeds and increased at a low rate during the first three days of germination, they might be under a control mechanism of gene expression different from that for the other isoenzymes [37].

Biosynthesis of malate dehydrogenase isoenzymes was also studied in cotyledons of watermelon. Whereas the glyoxysomal and mitochondrial isoenzymes are synthesised as higher molecular weight precursors, the in vitro translation of the cytosolic MDH I yields a product which has the same molecular mass as the subunit of the native isoenzyme (39.5 kDa) [51]. In cotyledons of germinating watermelon seeds immunofluorescence microscopy detects cMDH I only in confined regions of the lower epidermis. The cMDH I was associated with

distinct groups of epidermal cells which probably are meristemoids giving rise to the stomata. The most intensive fluorescence was observed in 1-day-old cotyledons, and it decreased as germination progressed [49].

VI-B. Genomic clones of cytoplasmic malate dehydrogenase and protein structure

A cDNA and a genomic clone for cytoplasmic MDH has been isolated from mouse [154,197]. An amino-acid sequence has been determined for the porcine cMDH and a three dimensional structure proposed from X-ray crystallographic studies [56]. The mouse cDNA encodes 334 amino-acid residues, and the deduced amino-acid sequence of the cytoplasmic enzyme shows about 23% overall identity with the mMDH [141]. In spite of this low similarity, the residues responsible for catalysis, NADH-binding and subunit interaction are highly conserved among all NADH-dependent MDHs analyzed so far [56]. Surprisingly, the sequence identity between mouse cMDH and *Thermus flavus* MDH is 52% and that between mouse mMDH and *Escherichia coli* MDH amounts to 58%, thus exceeding the *intra* species homologies between cMDH and mMDH from the mouse. The mouse cytosolic malate dehydrogenase gene is interrupted by 8 introns. A comparison with the mouse mitochondrial malate dehydrogenase gene revealed that the position of the introns has been conserved considerably along the two genes, which suggests that a common ancestral gene for cMDH and mMDH was broken up by introns before its duplication and the divergent evolution of the two genes. The 5′ end of the gene lacks the TATA and CAAT boxes characteristic of eucaryotic promotors, but contains GC-rich sequences, one putative binding site for a cellular transcription factor, Sp1, and at least two major transcription-initiation sites. In these characteristics it resembles the mouse mMDH gene [152]. Nucleotide sequence comparisons of the promoter regions of the mouse cMDH gene and of the other three mouse genes coding for isoenzymes participating in the malate/aspartate shuttle (i.e. mMDH, cytosolic and mitochondrial aspartate aminotransferases) revealed highly conserved domains in the promoters. The primary function of cytoplasmic malate dehydrogenase in watermelon cotyledons (presumably cMDH I) may well be its participation in the shuttle of reducing equivalents between the cytosol and different organelles, especially the mitochondrion, as found in mammalian systems. It can be speculated that the functions of cytoplasmic MDH II and IV in watermelon cotyledons, which disappear after fat mobilisation and greening, include involvement in gluconeogenesis (Fig. 1, enzyme 27). It would be interesting if the cell affords an additional and probably independently regulated isoenzyme especially for this purpose.

VI-C. Crassulacean acid metabolism

Succulent plants with Crassulacean acid metabolism store water in cells containing besides chloroplasts large vacuoles. During night they fix CO_2 with cytoplasmic phospho*enol*pyruvate carboxylase and cMDH into malate which is stored in the vacuoles. In daytime malate is transported back into the cytoplasm and cleaved by malic enzyme to liberate CO_2 for use in the Calvin cycle [199]. Phospho*enol*pyruvate carboxylase and ribulose-bisphosphate carboxylase discriminate differently between the stable isotopes ^{13}C and ^{12}C. This is used to determine from the relative amounts of ^{13}C and ^{12}C in the photosynthetic products how much CO_2 originates from re-use of pre-fixed CO_2 and how much is additionally made from external CO_2 in the Calvin cycle [199]. Large variations are encountered in different species, tissues and environmental conditions such as water stress. Light or dark activation of phospho*enol*pyruvate carboxylase consists in phosphorylation of a single serine in the N-terminal domain and leads to increased catalytic activity and decreased feedback inhibition by malate. Regulation takes place by daily de novo synthesis and breakdown of the protein-serine kinase while the phosphatase 2A, the carboxylase itself and associated enzymes such as pMDH or pyruvate, Pi dikinase display only long-term turnover [200]. In the C_4 plants maize, sorghum and *Portulaca* the phosphokinase is synthesised in the light and degraded in the dark. In the Crassulacean plant *Bryophyllum fedtschenkoi* the protein kinase appears in an endogenous circadian rhythm 5 h after the onset of darkness and disappears 2 h before daybreak, at which time the phospho*enol*pyruvate carboxylase is dephosphorylated by phosphatase 2A [201]. Clearly, the enzyme during daytime should be sensitive to inhibition by malate mobilised from the vacuole to avoid refixation of the CO_2 generated by malic enzyme. It is expected that cMDH shows long-term turnover.

It will be of interest to assign the exact roles of the different cytoplasmic and organelle-bound malate dehydrogenase isoenzymes to the steps within the different photosynthetic pathways. This will be a pre-requisite to determine the function of their non-homologous domains in enzyme complex formation and regulation of activity.

Malate dehydrogenases in plants play a crucial role in several pathways and – at a closer look – turn out to be a very fascinating family of isoenzymes.

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