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Role of a peroxisomal NADP-specific isocitrate dehydrogenase in the metabolism of the riboflavin overproducer *Ashbya gossypii*

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Abstract

During growth on soybean oil as carbon source a high activity of NADP-specific isocitrate dehydrogenase (ICDH) of up to 3 U/mg was found in cell-free extracts of the filamentous fungus *Ashbya gossypii*. Since this activity could mean a loss of carbon for riboflavin formation, the subcellular localization of this enzyme and its role in the metabolism were studied. While the NADP-specific ICDH, localized by density gradient centrifugation in peroxisomes, followed Michaelis–Menten-type kinetics for the substrate isocitrate, the mitochondrial NAD-specific isoenzyme exhibited an allosteric regulation by adenine nucleotides. Localization of enzymes involved in the substrate supply and the conversion of reaction products was investigated to explain the metabolic function of the peroxisomal ICDH. NADPH-oxidizing 2,4-dienoyl-CoA reductase was exclusively found in peroxisomes, while citrate synthase and α-ketoglutarate dehydrogenase complex (KGDH) were found only in mitochondria, and NAD-specific glutamate dehydrogenase was found in the cytosol. The data shown are consistent with the assumption that the NADP-specific ICDH of *A. gossypii* provides reducing equivalents for the peroxisomal metabolism, probably for the degradation of unsaturated fatty acids.

Keywords: *Ashbya gossypii*; Isocitrate dehydrogenase; Peroxisome

1. Introduction

Ashbya gossypii (Ashby and Novell) [1] is a filamentous hemiascomycete recently classified as belonging to the Saccharomycetaceae family [2]. The fungus isolated from cotton bolls and other crops is able to overproduce vitamin B_2 (riboflavin) [3] and improved strains are used for industrial riboflavin production. Besides glucose plant oils are suitable substrates because of their yield-enhancing effect [4]. An essential aspect for the metabolism of fatty acids, released in *A. gossypii* by an extracellular lipase [5], is the glyoxylate cycle. Schmidt et al. [6] found a correlation between the isocitrate lyase (ICL) specific activity, which represents one key enzyme of this pathway, and riboflavin formation. In contrast to *Saccharomyces cerevisiae*, where ICL is a cytosolic enzyme [7,8], the enzyme of *A. gossypii* [9], like that of several other fungi, e.g. *Candida tropicalis* [10], *Yarrowia lipolytica* [11] and *Aspergillus nidulans* [12], is localized in peroxisomes. Since an NADP-specific isoenzyme of isocitrate dehy-

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drogenase (ICDH) has also been found in this compartment of *S. cerevisiae* [13,14] and *C. tropicalis* [15], the question of localization of this enzyme activity in *A. gossypii* is raised. ICDHs, which catalyze the oxidative decarboxylation of isocitrate to form α-ketoglutarate, thereby reducing a dinucleotide cofactor, have been found in all compartments of eukaryotic cells. In *S. cerevisiae* there are four different isoenzymes. The mitochondrial NAD-specific ICDH has long been known to be an allosteric regulated enzyme of the tricarboxylic acid cycle [16]. The metabolic function of the second mitochondrial ICDH, an NADP-specific enzyme, is unclear since disruption of the corresponding gene had no effect in growth experiments [17]. It was recently clarified that the common function of the two other NADP-specific isoenzymes of *S. cerevisiae* is the supply of reducing equivalents. While in peroxisomes NADPH produced by ICDH is needed for the degradation of unsaturated fatty acids [13,14], the cytosolic isoenzyme provides NADPH for the reduction of endogenous or exogenous sources of hydrogen peroxide [18].

Since isocitrate is an important precursor of riboflavin biosynthesis for *A. gossypii* when growing on soybean oil, high activities of its degradative pathway, represented by ICDH, gained attention. The first objective was to localize the NADP-specific ICDH activity in the subcellular compartments of *A. gossypii*. Because of a co-localization with ICL in peroxisomes, its regulation and affinity for the substrate isocitrate was of interest. To explain the metabolic function of the peroxisomal ICDH the origin of the substrate and the fate of the reaction products were investigated by localization of the respective enzymes.

2. Materials and methods

2.1. Microorganism and cultivation

A. gossypii strain ATCC 10895 was grown in liquid medium consisting of 10 g/l yeast extract and 10 g/l soybean oil (YS) as main carbon source. Cultivation conditions have been described elsewhere [5].

2.2. Protoplast formation and membrane permeabilization

Mycelium grown for 48 h on YS medium was harvested by filtration and rinsed with 50 mM phosphate buffer (pH 7.0). Protoplasts were released from hyphae during incubation at 28°C in KEKS buffer (50 mM K-phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM KCl, 1 M sorbitol) with 2 mg/ml Lysing Enzyme (Sigma). Protoplasts were recovered by centrifugation (5 min, $1500 \times g$), washed once and resuspended in the same buffer containing the protease inhibitor Complete® (1 pill/50 ml; Roche). After microscopic enumeration the suspension was adjusted to 2×10^8 protoplasts per ml. One volume of protoplast suspension was added to an equal volume of KEKS buffer containing different concentrations of digitonin (Roche) thus giving final concentrations ranging from 0 ng to 15 ng digitonin per protoplast. Digitonin is known to form pore complexes with cholesterol so that membranes become permeable [19]. After membrane permeabilization, performed for 10 min at 30°C, the protoplasts were pelleted by centrifugation (2 min, $10{,}000 \times g$). The supernatants were removed and like the pelleted protoplasts, which were resuspended in 1 ml KEKS buffer containing Complete® and 0.1% (v/v) Triton X-100, they were further analyzed.

2.3. Cell extraction and subcellular fractionation

Cell-free extracts for enzyme activity measurements were prepared in 200 mM Tris/HCl buffer (pH 6.5) containing 1 mM of the protease inhibitor phenylmethane sulfonyl fluoride (PMSF) [20]. Cell organelles were isolated from protoplast homogenate [9] and separated either by Percoll density gradient centrifugation [9] or by sucrose density gradient centrifugation. Dis-

Table 1
Assays used for the analysis of in vitro enzyme activities

Enzyme	Reaction conditions	References
Catalase (EC 1.11.1.6)	50 mM phosphate (pH 7.0), 30 mM H_2O_2. 240 nm ($\varepsilon = 40.0\ mM^{-1}\ cm^{-1}$)	[39]
3-Ketoacyl-CoA thiolase (EC 2.3.1.16)	100 mM Tris/HCl (pH 8.3), 30 mM $MgCl_2$, 50 mM KCl, 0.1 mM CoA, 0.01 mM acetoacetyl-CoA. 303 nm ($\varepsilon = 16.9\ mM^{-1}\ cm^{-1}$)	[40] modified
Cytochrome *c* oxidase (EC 1.9.3.1)	50 mM phosphate (pH 7.2), 0.038 mM cytochrome *c* (reduced with sodium dithionite). 550 nm ($\varepsilon = 21.1\ mM^{-1}\ cm^{-1}$)	[41] modified
Fumarase (EC 4.2.1.2)	100 mM Tris/HCl (pH 7.5), 50 mM malate. 240 nm ($\varepsilon = 40.0\ mM^{-1}\ cm^{-1}$)	[42] modified
Hexokinase (EC 2.7.1.1)	50 mM Tris/HCl (pH 7.5), 30 mM $MgCl_2$, 2.5 mM NADP, 5 mM ATP, 0.35 U glucose-6-phosphate dehydrogenase (Roche), 50 mM glucose. 340 nm ($\varepsilon = 6.3\ mM^{-1}\ cm^{-1}$)	[43] modified
Malate dehydrogenase (EC 1.1.1.37)	100 mM phosphate (pH 7.5), 0.2 mM NADH,2 mM oxaloacetate. 340 nm ($\varepsilon = 6.3\ mM^{-1}\ cm^{-1}$)	[44] modified
Glutamate dehydrogenase (NAD^+) (EC 1.4.1.2)	100 mM Tris/HCl (pH 8.0), 150 mM NH_4Cl, 0.25 mM NADH, 20 mM α-ketoglutarate. 340 nm ($\varepsilon = 6.3\ mM^{-1}\ cm^{-1}$)	[45] modified
Citrate synthase (EC 4.1.3.7)	100 mM Tris/HCl (pH 8.0), 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.2 mM acetyl-CoA, 0.15 mM oxaloacetate (neutralized with $KHCO_3$). 412 nm ($\varepsilon = 13.6\ mM^{-1}\ cm^{-1}$)	[46] modified
Isocitrate dehydrogenase (NAD^+) (EC 1.1.1.41)	200 mM Tris/HCl (pH 8.0), 1 mM $MnCl_2$, 1.5 mM AMP, 1 mM NAD, 4 mM *threo*-DL-isocitrate. 340 nm ($\varepsilon = 6.3\ mM^{-1}\ cm^{-1}$) 37°C	[47] modified
Isocitrate dehydrogenase ($NADP^+$) (EC 1.1.1.42)	200 mM Tris/HCl (pH 8.0), 1 mM $MnCl_2$, 1 mM NADP, 4 mM *threo*-DL-isocitrate. 340 nm ($\varepsilon = 6.3\ mM^{-1}\ cm^{-1}$) 37°C	[47] modified
2,4-Dienoyl-CoA reductase (EC 1.3.1.34)	50 mM phosphate (pH 7.4), 0.1 mM NADPH, 0.1 mM 2,4-decadienoyl-CoA. 340 nm ($\varepsilon = 6.3\ mM^{-1}\ cm^{-1}$) 37°C	[48]
Δ^3,Δ^2-Enoyl-CoA isomerase (EC 5.3.3.8)	50 mM Tris/HCl (pH 9.0), 50 mM KCl, 50 μg/ml BSA, 2 U 3-hydroxyacyl-CoA dehydrogenase (Sigma), 1 U crotonase (Sigma), 0.1% (v/v) Triton X-100, NAD-regenerating system (1 mM NAD, 1 mM sodium pyruvate, 25 mM $MgCl_2$, 2 U lactate dehydrogenase (Roche)), 50 μM 3-*cis*-decanoyl-CoA. 303 nm ($\varepsilon = 13.9\ mM^{-1}\ cm^{-1}$)	[49,50] combined
α-Ketoglutarate dehydrogenase complex (EC 1.2.4.2; EC 2.3.1.61; EC 1.8.1.4)	100 mM TES/NaOH (pH 7.3), 0.9 mM thiamine PP_i, 10 mM α-ketoglutarate, 3 mM dithiothreitol, 10 mM $MgCl_2$, 2 mM NAD, 0.2 mM coenzyme A. 340 nm ($\varepsilon = 6.3\ mM^{-1}\ cm^{-1}$)	[51] modified

continuous sucrose gradients composed of 2.5 ml each of 50% (w/v), 45%, 40%, 35%, and 2.0 ml of 30% sucrose in 50 mM phosphate buffer (pH 7.2) were overlaid with 2 ml of the organelle suspension (20% sucrose in the same buffer). After centrifugation in a Ti50 rotor (Beckmann) for 4 h at 75,000 × *g* at 4°C, fractions of 1 ml were collected from the bottom of the tube and analyzed for enzyme activities. The highest activity of each enzyme within a gradient was set at 100%.

2.4. Enzyme assays

Enzyme activities were assayed spectrophotometrically as described in Table 1. The assays were performed, if not otherwise indicated, in a final test volume of 1 ml at 30°C. Protein concentrations were measured by the method of Bradford [21] with BSA as standard. One unit of enzyme activity was defined as the amount of enzyme required to form 1 μmol of product per minute. Specific activities are given as units per milligram protein. Kinetic data were calculated with the ENZFITTER program [22].

3. Results

3.1. NADP-specific ICDH subcellular localization

Isocitrate is a metabolite at the branch point of the glyoxylate and tricarboxylic acid cycle. Since the ICL branch is favored during growth on C_2-compounds with respect to growth and riboflavin formation, compartmentation of ICDH activities was investigated to estimate their impact on carbon flux. In cell-free extracts of *A. gossypii* mycelium grown on YS medium NAD- and NADP-specific ICDH activities of 0.2–0.5 and 2.5–3.0 U/mg, respectively, were measured. For subcellular localization of these enzyme activities, isolated organelles were fractionated by means of sucrose density gradient centrifugation. Enzyme activity measurements demonstrated the NADP-specific ICDH activity in accordance with the peroxisomal marker enzyme catalase and the β-oxidation enzyme 3-ketoacyl-CoA thiolase (Fig. 1). NAD-specific ICDH activity co-segregated with the mitochondrial marker enzyme cytochrome *c* oxidase shows a distinct separation from peroxisomes. Activity of the NAD-specific malate dehydrogenase (MDH) was detectable in both the mitochondrial and peroxisomal fraction. This gradient did not permit us to rule out an NADP-specific ICDH activity in the mitochondria, since both peroxisomal marker enzymes achieved rather small local maxima in the corresponding fractions as well. However, in frac-

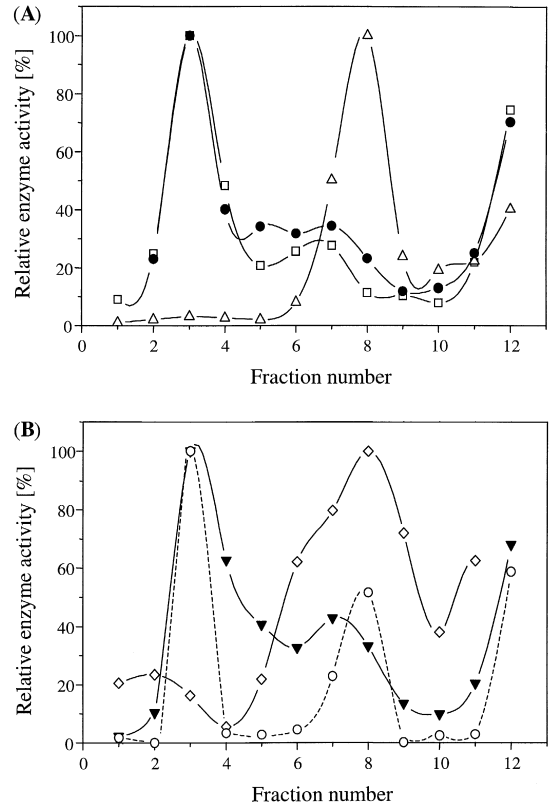

Fig. 1. Subcellular localization of ICDH isoenzymes in *A. gossypii* cells. (A) Catalase (□) and 3-ketoacyl-CoA thiolase (●), used as peroxisomal marker, cytochrome *c* oxidase (△), used as mitochondrial marker, and (B) NAD- (◇) or NADP-specific ICDH (▼) and NAD-specific malate dehydrogenase (○) were measured in fractions of a linear sucrose density gradient.

tions of a Percoll density gradient no NADP-specific ICDH activity was detectable in the mitochondrial fractions (Fig. 2).

3.2. Properties of NAD- and NADP-specific ICDH

In order to evaluate the significance of the detected peroxisomal NADP- and mitochondrial NAD-specific ICDH in the metabolism of *A. gossypii* both enzymes were kinetically characterized using the corresponding fraction of the density gradient or cell-free extracts. The peroxisomal ICDH revealed Michaelis–Menten-type kinetics with a K_m value for DL-isocitrate of 0.11 ± 0.01 mM (Table 2). The pH optimum was found to be pH 8.0 with the Tris/HCl buffer system. Enzyme activity was dependent on bivalent cations with 1 mM Mn^{2+} being twice as effective as 1 mM Mg^{2+} (data not shown). The addition of 3 mM ATP to the standard assay had a pronounced inhibitory effect (60% inhibition), while no effect was observed when AMP was added. NAD-specific ICDH exhibited a sigmoidal saturation curve characteristic of an allosteric enzyme. The $S_{0.5}$ value for DL-isocitrate was found to be 1.33 ± 0.07 mM without the addition of adenine nucleotides. Enzyme activity was enhanced significantly by AMP, but inhibited by ATP, leading to a lower or higher $S_{0.5}$ value, respectively,

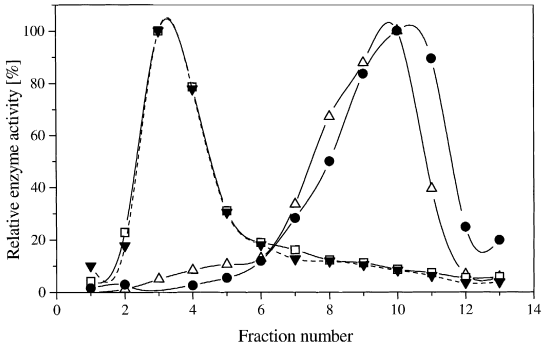

Fig. 2. Compartmentation of citrate synthase activity in cells of *A. gossypii*. Activities of the marker enzymes catalase (□) and cytochrome *c* oxidase (△), NADP-specific ICDH (▼) and of citrate synthase (●) were assayed in fractions of a Percoll density gradient.

Table 2
Kinetic parameters for isocitrate of the NAD- and NADP-specific ICDH of *A. gossypii*
Measurements were performed using cell-free extracts or isolated peroxisomes from the corresponding fractions of a sucrose density gradient.

Enzyme	Kinetics	$S_{0.5}$ (K_m) [mM]	V_{max} [U/mg]
NAD-ICDH	sigmoidal	$S_{0.5}$ 1.33 ± 0.07	0.23 ± 0.01
1.3 mM AMP		$S_{0.5}$ 0.32 ± 0.01	0.21 ± 0.01
1.3 mM ATP		$S_{0.5}$ 3.24 ± 0.06	0.21 ± 0.01
NADP-ICDH	Michaelis–Menten	K_m 0.11 ± 0.01	3.24 ± 0.10

without changing V_{max} (Table 2). Like the NADP-specific isoenzyme, NAD-specific ICDH exhibited a dependence on bivalent cations and maximal activity at pH 8.0.

3.3. Localization of isocitrate-supplying and α-ketoglutarate- or NADPH-converting enzymes

Furthermore, the metabolic function of the peroxisomal ICDH in *A. gossypii* was investigated by localizing enzymes involved in supplying the substrate isocitrate and converting the reaction products α-ketoglutarate and NADPH. For subcellular localization of corresponding enzymes, Percoll density gradient centrifugation was preferred because of the shorter processing time important for enzyme stability and better organelle separation without osmotic stress.

Citrate synthase catalyzes the condensation of acetyl-CoA, formed by the β-oxidation, with oxaloacetate into citrate, which is subsequently converted into isocitrate by an aconitase. Citrate synthase activity in *A. gossypii* was demonstrated to be localized only in mitochondria as indicated by the marker enzyme cytochrome *c* oxidase (Fig. 2).

In order to elucidate a potential metabolic role of the peroxisomal ICDH in glutamate biosynthesis the localization of the α-ketoglutarate dehydrogenase complex (KGDH) and the NAD-specific or NADP-specific glutamate dehydrogenase (GDH) was determined. KGDH activity was recovered exclusively in the mitochondrial fraction of a Percoll density gradient,

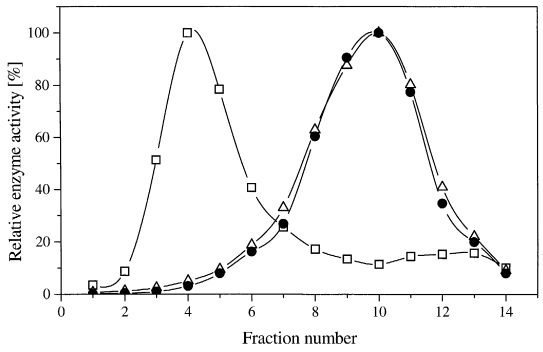

Fig. 3. Subcellular distribution of KGDH in *A. gossypii* cells. The peroxisomal marker catalase (□), mitochondrial NAD-specific ICDH (△) and KGDH (●) were measured in fractions of a Percoll density gradient.

identified by NAD-specific ICDH activity (Fig. 3). GDH activity was detectable only with NAD as cofactor in cell-free extracts of *A. gossypii*. Because this activity failed to be measurable in fractions of a density gradient, compartmentation of NAD-specific GDH was investigated by digitonin-conditioned membrane permeabilization. Due to the concentration-dependent effect of digitonin on membrane cholesterols, enzymes were dynamically released from treated protoplasts (Fig. 4). Enzyme activities, measured after centrifugation in supernatants and resuspended pellets, revealed that the cytosolic marker enzyme hexokinase was already released at low digitonin concentrations (80% at 3 ng digitonin/protoplast). Release of the peroxisomal marker enzyme catalase was achieved at higher concentrations (90% at 15 ng digitonin/protoplast). But even treatment with the highest concentrations of digitonin failed to release all the enzyme activity of the mitochondrial matrix enzyme fumarase (60% at 15 ng digitonin/protoplast). NAD-specific GDH showed a pattern of release comparable to that of hexokinase, suggesting an exclusively cytosolic localization of this enzyme in *A. gossypii*.

The function of the peroxisomal ICDH in *S. cerevisiae* is, as concluded from disruption of the encoding gene, to provide NADPH for the peroxisomal degradation of unsaturated fatty acids [13,14]. The reduction of double bonds of unsaturated fatty acids requires auxiliary enzymes in peroxisomes, an NADPH-oxidizing 2,4-dienoyl-CoA reductase and a $\Delta^2\Delta^3$-enoyl-CoA isomerase. To find out whether these enzymes are present in peroxisomes of *A. gossypii* fractions of a Percoll density gradient were assayed for both enzymes. Activity of the NADPH-consuming 2,4-dienoyl-CoA reductase as well as of the $\Delta^2\Delta^3$-enoyl-CoA isomerase cosegregated with catalase activity (Fig. 5), indicating a peroxisomal localization of these enzymes also in *A. gossypii*.

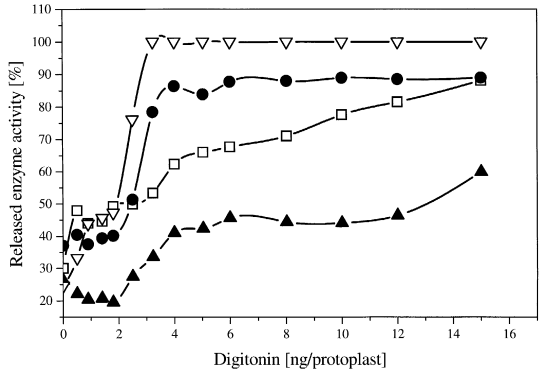

Fig. 4. Release of enzymes from *A. gossypii* protoplasts treated with different concentrations of digitonin. Fumarase (▲), catalase (□), hexokinase (●), and NAD-specific glutamate dehydrogenase (▽) activities are expressed as % released activity to total activity.

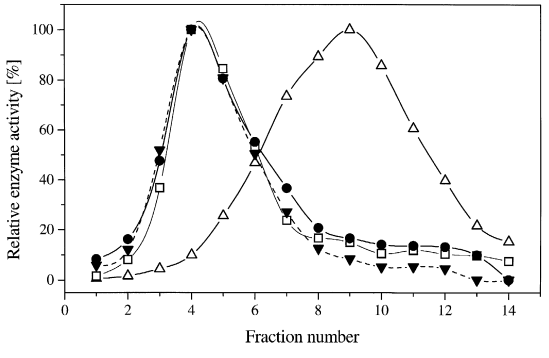

Fig. 5. Investigation of *A. gossypii* peroxisomes on auxiliary enzymes for the degradation of unsaturated fatty acids. Fractions of a Percoll density gradient were assayed for activity of the peroxisomal marker catalase (□), the mitochondrial enzyme NAD-specific ICDH (△), and 2,4-dienoyl-CoA reductase (●) and $\Delta^2\Delta^3$-enoyl-CoA isomerase (▼).

4. Discussion

Cell fractionation methods were used to investigate localization of the NADP-specific ICDH in the fungus *A. gossypii* grown on triglycerides, and subsequently its metabolic function. Based on the results obtained hitherto, a detailed model of compartmental pathways and intercompartmental metabolite exchange in *A. gossypii* is drawn (Fig. 6).

Density gradient centrifugation indicated that, like *S. cerevisiae* [13,14] and *C. tropicalis* [15], *A. gossypii* exhibited an NADP-specific ICDH within peroxisomes. Peroxisomes are the sole compartments associated with the β-oxidation pathway for the degradation of fatty acids in yeasts [23]. This was confirmed in *A. gossypii* by measurement of 3-ketoacyl-CoA thiolase activity within the peroxisomal fraction. The NADH generated during β-oxidation could be reduced as described for *S. cerevisiae* [24] by an MDH isoenzyme, since NAD-specific MDH activity was measured in peroxisomes as well as in mitochondria of *A. gossypii*. Compartmentation of the glyoxylate cycle enzyme ICL in peroxisomes of *A. gossypii* was shown recently by both density gradient centrifugation and immunogold labeling [9]. So both ICL and ICDH are localized in peroxisomes of *A. gossypii* as in *C. tropicalis* [25] and in glyoxysomes of plants [26]. Co-localization of these enzymes seems detrimental, since compartmentation is supposed to prevent the partition of isocitrate between nonhydrolytic cleavage and oxidative

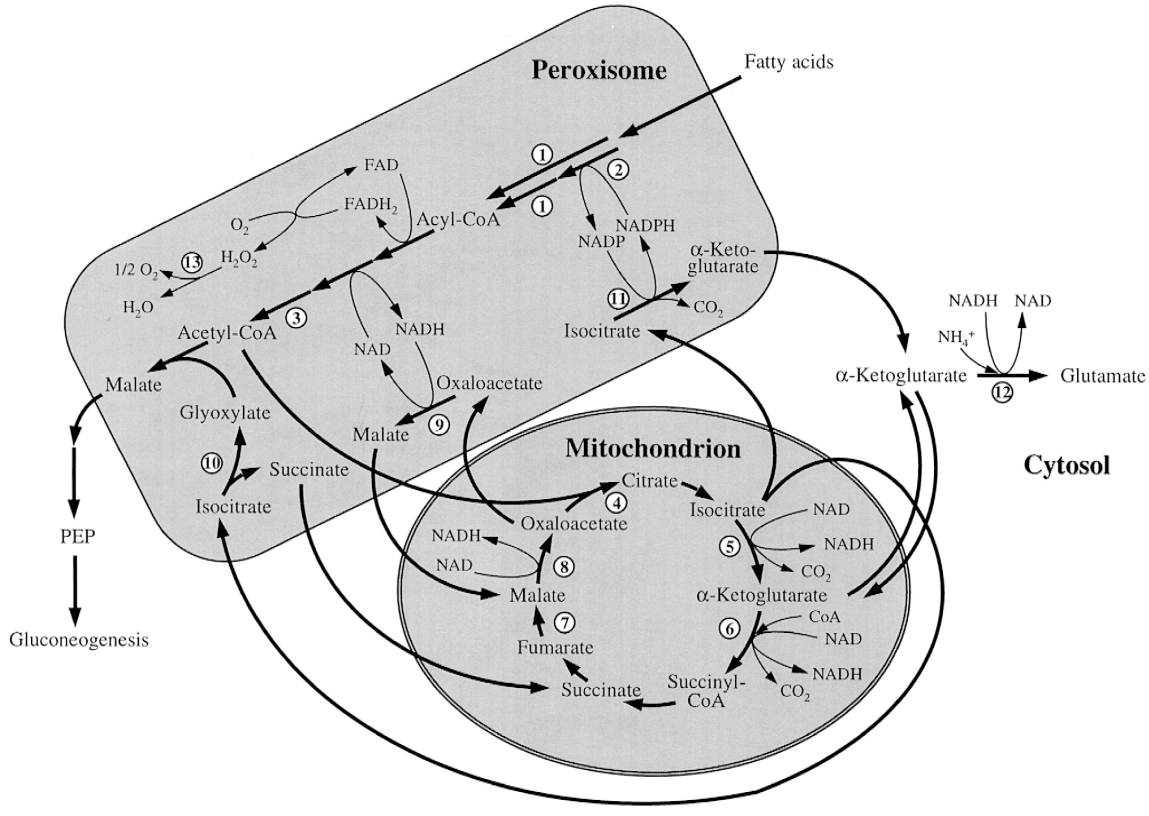

Fig. 6. Proposed model of compartmental pathways and intercompartmental metabolite exchange in *A. gossypii* during cultivation on fatty acids. Enzymes: (1) $\Delta^2\Delta^3$-enoyl-CoA isomerase, (2) 2,4-dienoyl-CoA reductase, (3) 3-ketoacyl-CoA thiolase, (4) citrate synthase, (5) NAD-specific isocitrate dehydrogenase, (6) KGDH, (7) fumarase, (8) mitochondrial or (9) peroxisomal NAD-specific malate dehydrogenase, (10) isocitrate lyase, (11) NADP-specific isocitrate dehydrogenase, (12) NAD-specific glutamate dehydrogenase, (13) catalase.

decarboxylation. With respect to carbon flux towards biomass and riboflavin in *A. gossypii* the peroxisomal ICDH would induce a net loss of carbon, whereas the ICL directs the carbon into gluconeogenesis with a minimum loss. Since affinity for the common substrate was about fivefold higher, peroxisomal NADP-specific ICDH ($K_m = 0.11$ mM) represented a potential competitor for ICL ($K_m = 0.55$ mM [6]). For the peroxisomal NADP-specific ICDH of *C. tropicalis* [15] similar kinetic data are reported with respect to K_m (0.11 mM for DL-isocitrate), which is 10-fold higher than the ICL K_m value of that yeast, and kinetic characteristics, although it was not affected by ATP as the *A. gossypii* enzyme was. Further investigations are needed to estimate whether the in vitro inhibition of the peroxisomal ICDH by ATP is of physiological significance. In *Escherichia coli* the activity of the NADP-specific ICDH is regulated by reversible phosphorylation, allowing effective competition by ICL [27,28]. A similar regulation in peroxisomes of *A. gossypii* seems improbable, since phosphorylation has not been described for either eukaryotic ICDH or for any peroxisomal enzyme.

The NAD-specific ICDH of *A. gossypii* was found in mitochondria like that of all fungi investigated up to now. Its allosteric characteristics and sensitivity to adenine nucleotides are common features for that enzyme participating in the tricarboxylic acid cycle [16,29].

As in *C. tropicalis*, *C. lipolytica*, *C. utilis*, *Hansenula polymorpha*, *A. oryzae* and *A. nidulans* [25,30–32], citrate synthase activity was solely found in mitochondria of *A. gossypii*. In contrast, *S. cerevisiae*, which is more closely related to *A. gossypii*, possesses in addition to two mitochondrial citrate synthase enzymes a third isoenzyme in peroxisomes [33]. However, the peroxisomal enzyme was shown to be involved together with the acetylcarnitine shuttle in the transport of acetyl residues out of this compartment [24].

Furthermore, the function of the α-ketoglutarate provided by the peroxisomal ICDH and the fate of the reduced NADP generated in this reaction was investigated. Activity of the KGDH of *A. gossypii* was strictly localized in mitochondria as described for *Neurospora crassa* and *S. cerevisiae* [34,35]. Activity of the α-ketoglutarate-converting NAD-specific GDH was exclusively found to belong to a cytosolic enzyme by digitonin-conditioned permeabilization of *A. gossypii* protoplasts. This is in agreement with NAD-specific GDH of *S. cerevisiae* [36] and with NAD- and NADP-specific GDH of *C. tropicalis*, *N. crassa*, *A. oryzae* and *A. nidulans* [31,32,37,38]. Thus an involvement of the peroxisomal ICDH of *A. gossypii* in glutamate biosynthesis seems unlikely.

On the other hand, taken together all the results obtained suggested that the NADPH formed is needed as a reduction equivalent in peroxisomes. For *S. cerevisiae* it was shown recently that peroxisomal NADP-specific ICDH is a putative constituent of a redox-regenerating system essential for the degradation of unsaturated fatty acids within this compartment [13,14]. Analogous to *S. cerevisiae*, both auxiliary enzymes of the degradation pathway, the $\Delta^2\Delta^3$-enoyl-CoA isomerase as well as the NADPH-oxidizing 2,4-dienoyl-CoA reductase, were determined in peroxisomes of *A. gossypii*. Final evidence that NADP-specific ICDH of *A. gossypii* regenerates the NADPH needed for the peroxisomal degradation of unsaturated fatty acids will be given by the disruption of the corresponding gene and the investigation of the phenotype obtained on fatty acids. Additionally, by this disruption, the hypothesis of the competition of ICL and ICDH for the common substrate in peroxisomes will be examined. If in *A. gossypii* the net loss of carbon initiated by peroxisomal ICDH is prevented, ICL should redirect more carbon into gluconeogenesis thus resulting in an improved biomass and/or riboflavin formation. Moreover, disruption of the peroxisomal NADP-specific ICDH encoding gene will elucidate whether *A. gossypii* possesses additional isoenzymes as described for other fungi. However, comparison of the activ-

ity ratio catalase:NADP-specific ICDH of cell-free extracts with isolated peroxisomes suggested a minor role of e.g. a cytosolic isoenzyme.

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