DISTINCT SUBCELLULAR LOCALIZATION OF NAD-LINKED AND FAD-LINKED GLYCEROL-3-PHOSPHATE DEHYDROGENASES IN *N*-ALKANE-GROWN *CANDIDA TROPICALIS*

Susumu KAWAMOTO, Takao YAMADA, Atsuo TANAKA and Saburo FUKUI
Laboratory of Industrial Biochemistry, Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Kyoto 606, Japan

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1. Introduction

Peroxisomes (microbodies) appear abundantly in alkane-grown cells of *Candida* yeasts [1,2]. In the isolated peroxisomes, several enzymes were detected, e.g., catalase, isocitrate lyase, malate synthase, fatty acid β-oxidation system, acyl-CoA synthetase and carnitine acetyltransferase [3–6]. We have suggested that peroxisomes in alkane-utilizing yeasts play essential roles in the metabolism of higher fatty acids derived from alkane substrate, i.e., fatty acid degradation and gluconeogenesis.

NADH is generated during fatty acid β-oxidation in peroxisomes [4], probably at the step of oxidation of 3-hydroxyacyl-CoA to 3-oxoacyl-CoA. Reoxidation of NADH must be necessary for continuous operation of fatty acid oxidation. Although the mode of NADH reoxidation in castor bean glyoxysomes (microbodies) has been investigated [7], it has not been solved so far.

We describe here the localization of NAD-linked glycerol-3-phosphate dehydrogenase (EC 1.1.1.8, *sn*-glycerol-3-phosphate:NAD oxidoreductase) (NAD-G3PDH) in the peroxisomes and FAD-linked glycerol-3-phosphate dehydrogenase (EC 1.1.99.5, *sn*-glycerol-3-phosphate: (acceptor) oxidoreductase) (FAD-G3PDH) in the mitochondria of alkane-grown yeast. Our results suggest that so-called 'glycerol-3-phosphate (G3P)/dihydroxyacetone phosphate (DHAP) shuttle' [8,9] may be involved in transport of reducing equivalents from peroxisomes to mitochondria, by analogy with its function between cytosol and mitochondria of animal tissues.

2. Materials and methods

2.1. *Cultivation of yeast and subcellular fractionation of cells*

Cultivation and subcellular fractionation of *Candida tropicalis* (Castellani) Berkhout strain pK 233 were carried out as in [4].

2.2. *Enzyme assay*

Catalase, cytochrome oxidase, NAD-linked glutamate dehydrogenase and protein were assayed as in [10,11]. Measurement of NADH-cytochrome *c* reductase was done as that of NADPH-cytochrome *c* reductase [3] by using NADH in place of NADPH. NADH-glyoxylate reductase and NADH-hydroxypyruvate reductase were assayed by the methods in [7] with slight modifications. The reaction mixture contained 33 mM potassium phosphate buffer (pH 7.2), 3.3 mM sodium azide, 0.25 mM NADH, substrate (1–20 mM sodium glyoxylate or 1–20 mM lithium hydroxypyruvate) and enzyme in 1.5 ml final volume. NAD reduction activity with an organic acid (1 mM) as substrate was measured as that of glyoxylate reductase or hydroxypyruvate reductase by using an organic acid in place of glyoxylate or hydroxypyruvate. NAD-linked glycerol-3-phosphate dehydrogenase (NAD-G3PDH) was assayed by the methods in [12] using the following two systems: NADH oxidation with dihydroxyacetone phosphate (DHAP) and NAD reduction with glycerol-3-phosphate (G3P). The reaction mixture for assay of NADH-linked DHAP reduction activity contained 37 mM potassium phosphate buffer (pH 7.2), 0.25 mM NADH, 3.3 mM

sodium azide, 0.5 mM DHAP and enzyme in 1.5 ml final volume. A decrease in A_{340} was followed spectrophotometrically. The DHAP solution was prepared from DHAP cyclohexylamine salt dimethyl ketal H_2O (Sigma, St Louis) according to Sigma directions. The reaction mixture for assay of NAD-linked G3P dehydrogenation activity was composed of 37 mM pyrophosphate buffer (pH 9.3), 3.3 mM NAD, 3.3 mM sodium azide, 33.3 mM DL-α-glycerophosphate disodium salt (Nakarai Chemicals, Kyoto) and enzyme in 1.5 ml final volume. An increase in A_{340} was measured spectrophotometrically. FAD-linked glycerol-3-phosphate dehydrogenase (FAD-G3PDH) was assayed by the phenazine methosulfate (PMS) method [13]. G3P-dependent oxygen consumption was measured by a Beckman fieldlab O_2 analyzer with a Clark-type O_2 electrode in 11 ml reaction mixture. The reaction mixture consisted of 47.3 mM potassium phosphate buffer (pH 7.6), 4.6 mM PMS, 1 mM sodium azide, 33 mM DL-α-glycerophosphate disodium salt and enzyme. The enzyme activity was expressed in nmol O_2/min.

All the reactions were carried out at 30°C.

3. Results and discussion

As in [4], reduction of NAD is involved in the fatty acid β-oxidation, which proceeds exclusively in the peroxisomes of the yeasts. NADH reoxidation system must be present in the peroxisomes for the successive operation of fatty acid β-oxidation.

Therefore, we investigated the subcellular localization of several NAD-linked enzymes in a typical alkane-utilizing yeast, *C. tropicalis*. The activities of NADH-cytochrome *c* reductase and NAD-linked glutamate dehydrogenase were not observed in the peroxisomes. Although NADH-glyoxylate reductase and NADH-hydroxypyruvate reductase were demonstrated in the glyoxysomes of castor bean [7], the activities of these enzymes in this yeast were too low to confirm their exact subcellular localization. Furthermore, any activities of NADH oxidation were not detected in this yeast when the TCA cycle intermediates except for oxalacetate were used individually as substrate. The activity of malate dehydrogenase was present in the cells, but not in the peroxisomes [3]. DHAP, however, was found to be effective in the oxidation of NADH by the cell-free extract of the yeast, indicating the presence of NAD-linked G3PDH in the yeast. This activity was significantly higher in the alkane-grown cells than the glucose-grown cells of the yeast (table 1).

As shown in table 2, most of FAD-G3PDH and NAD-G3PDH (both NADH-DHAP reduction activity and the reverse reaction, NAD-G3P dehydrogenation activity) were detected in the particulate fraction (P_2, 20 000 × *g* pellets). When P_2 fraction was subjected to sucrose density gradient centrifugation, FAD-G3PDH was detected mainly in fraction 2 (30–40% sucrose fraction), while NAD-G3PDH mostly in fraction 5 (42.5–50% sucrose fraction). When cytochrome oxidase and catalase were taken as each marker enzyme of mitochondria and of peroxisomes, FAD-G3PDH was concluded to be located in the mitochondria and NAD-G3PDH in the peroxisomes (fig.1).

Several shuttle systems for transfer of reducing equivalents, including glycerol-3-phosphate (G3P)/dihydroxyacetone phosphate (DHAP) shuttle (NAD-G3PDH and FAD-G3PDH), have been known in animal mitochondria [8,9]. By analogy with animal mitochondria, operation of G3P/DHAP shuttle was suggested in animal peroxisomes [12] and malate/aspartate shuttle (malate dehydrogenase and glutamate-oxalacetate transaminase) in plant peroxisomes [14]. Although glutamate-oxalacetate transaminase was detected in the mitochondria, peroxisomes and cytosol of alkane-grown *C. tropicalis* (unpublished results), malate dehydrogenase was not localized in the peroxisomes, but in the mitochondria [3]. Thus, malate/aspartate shuttle may not operate

Table 1
Activity of NAD-linked glycerol-3-phosphate dehydrogenase in *Candida tropicalis* cells grown on different carbon sources

Cell	Enzyme activity (nmol.min^{-1}.mg protein^{-1})
Alkane-grown	169
Glucose-grown	10.7
Glycerol-grown	16.5

Enzyme sources were the 3000 × *g* supernatant solutions of the protoplast homogenates. The enzyme activity was measured by the reduction of DHAP with NADH

Table 2
Subcellular localization of enzymes in alkane-grown *Candida tropicalis* cells

	Fraction		
	S_1	P_2	S_2
Protein (mg)	77.1	26.6	36.6
Cytochrome oxidase			
Relative activity (%)	100	96.4	0.5
Specific activity (nmol/min × mg protein)	85.9	240	0.9
Catalase			
Relative activity (%)	100	79.4	41.1
Specific activity (μmol/min × mg protein)	364	838	315
FAD-G3P dehydrogenase			
Relative activity (%)	100	98.8	nil
Specific activity (nmol/min × mg protein)	54.1	155	nil
NAD-G3P dehydrogenase (NAD-G3P dehydrogenation activity)			
Relative activity (%)	100	88.0	12.0
Specific activity (nmol/min × mg protein)	16.2	41.3	4.1
NAD-G3P dehydrogenase (NADH-DHAP reduction activity)			
Relative activity (%)	100	92.7	12.2
Specific activity (nmol/min × mg protein)	169	454	43.4

The experimental procedures were as in [3]. S_1, 3000 × *g* supernatant of the protoplast homogenate; P_2, 20 000 × *g* pellets of S_1; S_2, 20 000 × *g* supernatant of S_1. Activities in S_1 were expressed as 100%

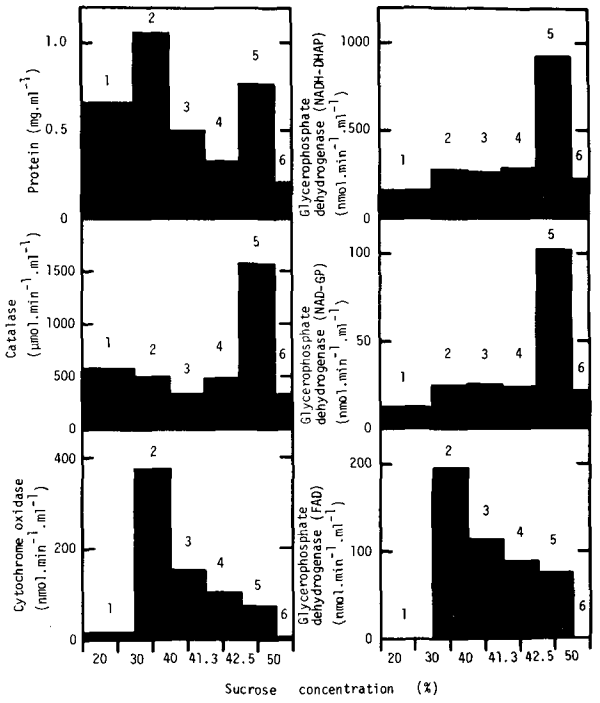

Fig.1. Particulate localization of enzymes in P_2. The experimental procedures were as in [3]. The volume of each fraction was as follows: 1, 3.75 ml; 2–5, 2.5 ml each; 6, 1.25 ml.

for reoxidation of NADH in the peroxisomes of the yeast.

The results obtained here suggest that G3P/DHAP shuttle may operate between the peroxisomes and mitochondria in alkane-utilizing *C. tropicalis* for the transfer of reducing equivalents generated during fatty acid oxidation. Another possibility is that effluent supply of DHAP may permit the reoxidation of NADH independent of the shuttle. However, this seems not to be plausible under the gluconeogenic conditions where the yeast is assimilating alkanes.

G3P and DHAP have been also known as the key metabolites in glycerol metabolism, glycolytic system and lipid metabolism in mammalian cells [9]. In *Trypanosoma*, not only NAD-G3PDH but also the other several glycolytic enzymes are located in microbody-like organelles ('glycosomes') [15,16]. In

mammalian cells, acyl-CoA-DHAP acyltransferase, which is the key enzyme in glycerolipid biosynthesis, was reported to be located in peroxisomes [17]. However, in the glycerol-grown or glucose-grown cells of *C. tropicalis*, the level of NAD-G3PDH activity was 1/10th–1/15th that of the alkane-grown cells (table 1). The results obtained strongly suggest that the high NAD-G3PDH activity of the alkane-grown cell must participate in the shuttle system rather than in glycerol metabolism or phospholipid metabolism.

Although we have no information whether peroxisomal membrane has free permeability to NADH to be oxidized directly at mitochondria, the shuttle system mentioned above appears to have an important role in the reoxidation of NADH formed in the peroxisomes of the yeast.

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