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Estimation of dolichol and cholesterol synthesis in microsomes and peroxisomes isolated from rat liver

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Abstract The participation of peroxisomal and microsomal fractions from rat liver in dolichol and cholesterol synthesis was investigated using marker enzymes. Recovery was 8% for peroxisomes and 33% for microsomes, with virtually no cross-contamination between these fractions. Using these data, it was calculated that the peroxisomal branch-point enzyme activities for dolichol and cholesterol biosynthesis, i.e. *cis*-prenyltransferase and squalene synthase, were 25% and 12%, respectively, of the total homogenate activity. Treatment with mevinolin increased the peroxisomal contribution in the case of both enzymes, to levels almost equal to that of their microsomal counterparts. These results indicate that peroxisomes play a role in the biosynthesis of isoprenoid lipids and that the extent of this participation is increased extensively when peroxisomes are induced by various treatments.

Key words: Cholesterol synthesis; *cis*-Prenyltransferase; Dolichol synthesis; Microsome; Peroxisome; Squalene synthase

1. Introduction

The initial reactions of the mevalonate pathway yield farnesyl pyrophosphate (FPP), which is utilized for the synthesis of dolichol and cholesterol. FPP is also the substrate for *trans*-prenyltransferase, which forms the side-chain of ubiquinone [1]. FPP and its condensation product, geranylgeranyl pyrophosphate (GGPP), also serve as substrates for protein isoprenylation.

Originally, the initial portion of the pathway was thought to be localized to the cytoplasm and the terminal reactions in dolichol and cholesterol synthesis were considered to occur in the microsomes. However, investigations in recent years have demonstrated the presence of various biosynthetic steps leading to mevalonate pathway lipids in several subcellar locations. In the presence of [^{3}H]mevalonate and cytosol, not only microsomes, but also peroxisomes, from rat liver can synthesize dolichol [2]. The properties of the peroxisomal *cis*-prenyltransferase, the first commited enzyme of dolichol synthesis, were investigated and found to differ from those of the corresponding microsomal activity [3].

Peroxisomes are also able to synthesize cholesterol in the presence of [^{3}H]mevalonate and cytosol [4,5]. Peroxisomal squalene synthase exhibits regulation by mevinolin and by various inducers distinct from that of the microsomal enzyme [3,6]. Several other enzymes involved in cholesterol synthesis could also be detected in peroxisomes [7].

Interest in this organelle has increased during recent years, because of its extensive involvement in lipid metabolism. β-Oxidation of long-chain and very-long-chain fatty acids, plasmalogen synthesis and involvment in bile acid production are among the peroxisomal functions which have been studied in great detail [8]. Peroxisomes exhibit an unusual capacity for adaptation: upon treatment of rodents with hypolipidemic drugs, other chemicals and in certain dietary conditions, the number of hepatic peroxisomes is increased several-fold, with parallel and extensive induction of specific enzyme activities [9]. On the other hand, there are an increasing number of reports on diseases where some or most peroxisomal enzymes are absent with serious consequences for cellular life [10,11].

In order to increase understanding of the importance of peroxisomal participation in isoprenoid metabolism, it is important to quantitate the percentage of total hepatic dolichol and cholesterol synthesis which occurs in this organelle. This question is of considerable interest, since even a relatively low peroxisomal activity is often increased several-fold by physiological or pharmacological stimulation or under various pathological conditions.

2. Materials and methods

Male Sprague–Dawley rats (180 g) were used without starvation. Mevinolin treated rats were fed chow mixed with mevinolin (500 mg/kg food) for 21 days. The livers were homogenized in a medium containing sucrose, EDTA and ethanol, and used for subfractionation as described earlier [7]. Peroxisomes were isolated from the light mitochondrial fraction by centrifugation on a preformed linear Nycodenz gradient. After centrifugation in a vertical rotor, the peroxisomal fraction, banding at a density of about 1.23 g/ml, was collected. The microsomal fraction was prepared by centrifugation of the $10{,}000 \times g$ supernatant, at $105{,}000 \times g$ for 120 min.

NADPH-cytochrome *c* reductase (NADPH-cyt. *c* red.), urate oxidase and catalase activities were measured as described earlier [12–14]. *cis*-Prenyltransferase activity in the subfractions was assayed by using [^{3}H]isopentenyl pyrophosphate and unlabeled FPP as substrates [15]. In the case of microsomes, the incubation medium contained 100 mM KF and 1.0% Triton X-100 and, for peroxisomes, 5 mM KF and no detergent. After incubation, the lipids were extracted with butanol and dephosphorylated enzymatically [16]. The polyprenols were quantitated by reversed-phase HPLC, with direct elution through a radioactivity flow-detector.

For determination of squalene synthase activity, the incubation medium contained [^{3}H]FPP as the only substrate [3]. The products were extracted with chloroform/methanol (2:1), and analyzed using reversed-phase HPLC. Protein was measured according to Lowry, with bovine serum albumin as the standard [17].

3. Results and discussion

Contamination of isolated fractions by various membranes occurs when liver homogenate is fractionated. The presence of

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Table 1
Specific activity of marker enzymes in total liver homogenate, microsomes and peroxisomes

Enzymes	Homogenate	Microsomes	Peroxisomes
NADP-cyt. *c* red.[a]	23 ± 2.9	110 ± 1.0	2.8 ± 0.4
Urate oxidase[b]	9.0 ± 1.0	3.5 ± 0.4	290 ± 33
Catalase[c]	410 ± 50	62 ± 7.0	4500 ± 410

The marker enzyme activities were determined in microsomes and peroxisomes and were used to estimate cross-contamination. For microsomes, the membrane enzyme NADPH-cytochrome *c* reductase was employed. In the case of peroxisomes, two enzymes were selected – urate oxidase, which is firmly associated with the vesicles, and catalase, which is easily released. The values are the mean ± S.D. of six experiments. [a]nmol cyt. *c* reduced/min/mg protein; [b]nmol urate oxidized/min/mg protein; [c]μmol H_2O_2 decomposed/min/mg protein.

microsomal vesicles in the peroxisomal fraction was estimated by determining the activity of NADPH-cytochrome c reductase, an endoplasmic reticulum (ER) membrane enzyme (Table 1). Using Nycodenz as the density medium and a vertical rotor for separation, microsomal contamination of peroxisomes was negligible.

Peroxisomal contamination of microsomes was estimated by measuring urate oxidase and catalase, enzymes specifically associated with peroxisomes. The peroxisomal contamination in microsomes was only 1–2%. Thus, there is virtually no cross-contamination between these two preparations and the fractions isolated are therefore very suitable for enzyme distribution studies, at least from the point of view of purity.

In any fractionation studies, high purity has a price, which is low recovery. In order to estimate organelle recovery from the homogenate, the marker enzymes, were assayed both in the homogenate and the isolated fractions (Table 2). The results with respect to NADPH-cytochrome *c* reductase activity indicated that the yield of the ER fragments in the microsomes was 33% of the total.

The yield of peroxisomes was estimated by measuring two enzymes known to show different behaviour. Urate oxidase is present as a crystalloid structure and is consequently not released, even from broken peroxisomes [18]. On the other hand, catalase is easily released from peroxisomes as are certain other enzymes present in the peroxisomal lumen. The recoveries of peroxisomes was quite poor, in agreement with previous studies [19]. The yield calculated on the basis of urate oxidase distribution, was about 8%, while recovery calculated using catalase was about 3%. Available experiments indicate that the enzymes involved both in dolichol and cholesterol synthesis are relatively firmly associated with the inner peroxisomal compartment and, for this reason, we calculated our data using the value for recovery obtained with urate oxidase.

With our isolation procedure, the protein content of the fraction consisting of microsomal vesicles (washed to remove adsorbed cytosolic proteins) was about 15 mg/g liver (Table 3). Thus, the total microsomal content of liver homogenate calculated on the basis of a 33% recovery was 45 mg protein/g liver. The Nycodenz gradient procedure provides very pure peroxisomes but the amount was only 0.5 mg protein/g liver. This value requires substantial correction for the low recovery rate so that 6 mg peroxisomal protein was actually present per g liver under normal conditions.

The specific activity of the branch-point enzyme of dolichol synthesis, *cis*-prenyltransferase, was found to be 2.5-fold higher in peroxisomes than in microsomes (Table 4). Considering the relative recoveries, one may conclude that 75% of the total activity in the homogenate is localized in microsomes, while 25% of this activity originates from peroxisomes.

Upon mevinolin treatment, the specific activity doubled in peroxisomes and remained unchanged in microsomes. Consequently, about 43% of the total activity in the homogenate after this treatment was present in peroxisomes.

The specific activity of squalene synthase was about the same in microsomes and peroxisomes (Table 5). It is known that during homogenization and fractionation, the microsomal enzyme may be subject to proteolysis, resulting in the release of an enzymatically active fragment into the cytosol [20]. With the mild homogenization procedure employed here such protein hydrolysis was limited and only about 2% of the total squalene synthase activity was recovered in the cytosol. Quantitative calculation of the distribution of this branch-point enzyme in cholesterol biosynthesis revealed that 86% of the total activity was present in microsomes and 12% in peroxisomes.

Mevinolin treatment increased the specific activity of squalene synthase in microsomes by 60% while in peroxisomes the increase was 12-fold. For this reason, 46% of the total activity in the homogenate was found to be peroxisomal. The cytosolic contribution was less than 1%. *cis*-Prenyltransferase and squalene synthase are considered to be the terminal regulatory enzymes of dolichol and cholesterol biosynthesis [21]. If these enzymes are rate-limiting in these biosynthetic processes, one can conclude that about 25% of dolichol and 12% of cholesterol synthesis in rat liver in vitro takes place in peroxisomes. This calculation is, however, only approximative since we do not know to what extent in vitro measurements represent in vivo activities. This question will require detailed investigation in the future. Even a few percent synthetic activity can make up a substantial proportion since upon peroxisomal proliferation this contribution is increased several-fold. β-Oxidation of fatty acids occurs predominantly in mitochondria, and the corresponding peroxisomal process plays a minor role from a quantitative point of view. Upon induction, however,

Table 2
Distribution of marker enzymes in isolated microsomes and peroxisomes

Enzyme	Homogenate	Microsomes		Peroxisomes	
	Total activity	Total acitivity	Recovery %	Total activity	Recovery %
NADPH-cyt. *c* red.[a]	4.7	1.6	33	0.0014	–
Urate oxidase[b]	1800	52	–	140	7.9
Catalase[c]	84000	920	–	2300	2.7

The marker enzyme activities were measured in homogenate, microsomes and peroxisomes. The specific activities were used to calculate the amount of microsomes and peroxisomes in the homogenate in order to obtain the yield. The values are the mean of six experiments.[a]μmol cyt *c* reduced/min/g liver; [b]nmol urate oxidized/min/g liver; [c]μmol H_2O_2 decomposed/min/g liver.

Table 3
Total protein content of microsomes and peroxisomes in liver homogenate

	Homogenate	Microsomes	Peroxisomes
Control			
Protein content in isolated fractions[a]	203 ± 17	14.9 ± 1.6	0.5 ± 0.06
Calculated total protein content[a]	203 ± 20	45 ± 5.3	6.1 ± 0.5
Mevinolin			
Protein content in isolated fractions[a]	211 ± 15	15.7 ± 1.8	0.5 ± 0.07
Calculated total protein content[a]	211 ± 23	46.2 ± 6	6.0 ± 0.8

The protein content in the homogenate, alkaline-washed microsomes and peroxisomes was determined after fractionation. Using the values obtained in Table 2, the total microsomal and peroxisomal protein content in the homogenate was calculated. The values are the mean ± S.D. of six experiments. [a]mg/g liver.

peroxisomal fatty acid β-oxidation can be increased 30–50 times [9].

Analysis of the organisation of biosynthetic sequences requires isolation of the individual enzymes involved in order to compare them chemically and functionally. In the case of fatty acid β-oxidation it has been established that the peroxisomal enzymes involved are different from the mitochondrial ones [22]. This may also be the case for the branch-point enzymes investigated here. The regulation of both *cis*-prenyltransferase and squalene synthase in microsomes and peroxisomes occurs independently. This became clear when these enzyme activities were assayed after various dietary treatments, administration of inducers of peroxisomes and microsomes, drugs and other chemicals [6]. Obviously, the localization of these biosynthetic pathways in the two organelles raises not only quantitative considerations, but the lipids synthesized at these two locations may fulfil different functions. One suggestion is that the lipids produced in the endoplasmic reticulum participate in lipoprotein production and membrane biogenesis, whereas those synthesized in peroxisomes may serve as substrates for further metabolism and also be transported to the bile, where they are constitutive components.

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Table 4
Distribution of *cis*-prenyltransferase in microsomes and peroxisomes

Fraction	Specific activity (pmol/mg prot./h)	Total activity (nmol/g liver/h)	% of total activity in homogenate
Control			
Microsomes	270 ± 30	12 ± 1.5	75
Peroxisomes	680 ± 76	4.1 ± 0.58	25
Mevinolin			
Microsomes	260 ± 30	12 ± 1.9	57
Peroxisomes	1480 ± 160	9 ± 1.3	43

cis-Prenyltransferase activity was determined in the various fractions and the values given in Table 2, used to estimate the percentage distribution of the activities in the homogenate. The values are the mean ± S.D. of four experiments.

Table 5
Distribution of squalene synthase activity in microsomes and peroxisomes

Fraction	Specific activity (pmol/mg prot./h)	Total activity (nmol/g liver/h)	% of total activity in homogenate
Control			
Microsomes	430 ± 48	19.3 ± 2.2	86
Peroxisomes	418 ± 43	2.6 ± 0.29	12
Cytosol	8.0 ± 1.1	0.5 ± 0.2	2
Mevinolin			
Microsomes	716 ± 75	33 ± 3.8	54
Peroxisomes	4730 ± 520	28 ± 3.4	46
Cytosol	16 ± 2.1	0.4 ± 0.2	–

The squalene synthase activity was measured in microsomes, peroxisomes and cytosol. The data in Table 2 were used to calculate the percentage distribution of the activities in the homogenate. The values are the mean ± S.D. of four experiments.

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