BIOSYNTHESIS OF DOLICHOL PHOSPHATE BY SUBCELLULAR FRACTIONS FROM LIVER

Gustavo R. DALEO, H. Esteban HOPP, Pedro A. ROMERO and Rafael PONT LEZICA
Departamento de Biología, Fundación Bariloche, Casilla de Correo 138, 8400 San Carlos de Bariloche, Argentina

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1. Introduction

Since the first report of Behrens and Leloir in 1970 [1] suggesting that dolichyl phosphate glucose could serve as glucosyl donor for the synthesis of glucose-containing proteins, a large amount of work has been done in order to establish the central role of dolichyl phosphate in the biosynthesis of eukaryotic glycoproteins [2]. Nevertheless, it is suprising that very little attention has been given to the metabolism of dolichol. This is a generic name for long-chain polyprenols which are composed of an isoprenoid chain of 80 to 110 carbon atoms. Most of the double bonds are *cis*, and only two isoprene residues near the ω end are *trans*, the α-isoprene unit being saturated.

It is generally accepted that the biosynthesis of dolichyl phosphate follows the standard reaction pattern of head-to-tail condensations of allylic prenyl pyrophosphates with successive isopentenyl pyrophosphates units. These reactions are catalyzed by enzymes usually known as prenyl transferases. A *cis* prenyl transferase has been partially purified from *Lactobacillus plantarum*, giving undecaprenyl pyrophosphate as final product [3].

In vivo synthesis of dolichol has been achieved in rat liver from [4 *S*-^{3}H]-mevalonate [4]. Most of the radioactive dolichol was found in mitochondrial fraction. These results are in agreement with previous work [5,6] indicating that free dolichol was found in higher concentrations in mitochondria. On the other hand, an indirect estimate of the levels of dolichyl phosphate in subcellular fractions gives the nuclear and the Golgi fractions as being the richest [7]. It seems that neither the actual concentration of free dolichol nor the stimulation of glucolipid formation indicates necessarily the subcellular fraction where synthesis of dolichol occurs.

Recently the in vitro synthesis of dolichyl phosphate by a plant system has been described [8]. This provides a useful method for the study of dolichyl phosphate biosynthesis. We report here in the direct evidence of the synthesis of dolichyl phosphate by subcellular fractions of rabbit and chicken liver.

Abbreviations: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; Dol-P, dolichyl phosphate

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2. Materials and methods

RS-[2-^{3}H]Mevalonic acid (MVA) lactone (382 mCi/mmol) and Δ^3-[1-^{14}C]isopentenyl pyrophosphate (61 mCi/mmol) were obtained from Amersham/Searle Corporation. Dolichol and dolichyl phosphate were purchased from Sigma Chemical Co. Ficaprenyl phosphate was synthesized as described [9]. Dolichyl monophosphate-[^{14}C]-glucose was a gift from R. Staneloni. All other chemicals were analytical grade.

2.1. *Subcellular fractionation and assay for marker enzymes*

Rabbit and chicken livers were perfused with 10 mM Hepes, pH 7.5, in 0.25 M sucrose immediately after

the animals were killed, and subjected to subcellular fractionation as described by Fleischer and Kervina [10]. The fractions isolated were: nuclei, plasma membrane, mitochondria, Golgi, total microsomes and supernatant. The purified mitochondria were subfractioned into the outer and inner membranes and the matrix, after swelling in 10 mM potassium phosphate buffer, pH 7.4, at 0°C as described [11].

Marker enzymes were used for the determination of purity of subcellular fractions. Glucose-6-phosphate for total microsomes, 5'-nucleotidase for plasma membrane, and thyamine pyrophosphatase for the Golgi were assayed as described [12]. The inner mitochondrial membrane was associated to cytochrome *c* oxidase activity and measured according to Simon [13]. NADH-cytochrome *c* reductase was assayed as described [14] for outer mitochondrial membrane and microsomes marker. To evaluate the enzymatic activity associated with mitochondrial matrix, malate dehydrogenase was measured [15].

2.2. *Assay for Dol-P biosynthesis*

When MVA was used as precursor, assays were performed for 75 min at 30°C with the following mixture: 2.5 μmoll Tris–HCl, pH 7.5, 0.5 μmol ATP, 0.5 μmol $MgCl_2$, 2.5 nmol *RS*-[2-^{3}H]MVA (382 mCi/mmol) and protein from different subcellular fractions. When [1-^{14}C]IPP was used as Dol-P precursor, the assays as well as extraction and fractionation of the products were made as described [8], independently of the radioactive precursor used. The acid resistant material eluting from the Sephadex LH-20 column with Dol-P marker, was considered as the biosynthesized dolichyl-P. This assumption was confirmed by submitting the radioactive material to enzymatic and alkaline hydrolysis, followed by reverse-phase TLC, and comparison with authentic dolichol as described [8]. The ability of the biosynthesized Dol-P to form sugar derivatives was also confirmed with microsomal enzyme and UDP-[^{3}H]glucose or UDP-[^{14}C]glucose, as reported previously [8].

Column chromatography on Sephadex LH-20 (1 × 20 cm) was performed as described [8] and eluted with 0.1 M ammonium acetate buffer in 99% methanol [16].

Phosphate was determined by the Taussky and Shorr method [17] and proteins by the Lowry method [18].

3. Results and discussion

In vitro incorporation of radioactivity from [1-^{14}C] IPP or [2-^{3}H]MVA into dolichyl phosphate was investigated in membranes separated by centrifugation on discontinuous sucrose-density gradients. Table 1 shows the results and the purity of different fractions measured by the specific activity of marker enzymes. The biosynthesized Dol-P from Golgi, mitochondria and the outer mitochondrial membrane fractions were submitted to phosphatase or alkaline hydrolysis and the resulting free alcohols were chromatographed on reversephase TLC in order to confirm their chain lengths.

The mitochondria showed the highest specific activity for the direct synthesis of Dol-P from IPP or MVA. This result is in agreement with an earlier suggestion of Martin and Thorne [4] based on the specific activity of free dolichol synthesized in vivo from mevalonate. The determination of free dolichol in different subcellular fractions from pig liver [5,6] are also coincident with our results. The indirect estimate of Dol-P levels by extraction of subcellular fractions and measuring their effectiveness in stimulating the formation of glucolipid after incubation with microsomal enzymes [7], gave some different results. The Golgi apparatus and the nuclear fraction seem to be the richest ones. Our results show that the Golgi system can synthesize Dol-P. This activity does not seem to be derived from contaminating material from mitochondria. On the other hand, experiments with rabbit or chicken liver consistently showed that the Golgi had about half the specific activity of mitochondria.

We did not find significant synthesis of Dol-P in the nuclear fraction. Nevertheless, free dolichol [4] and a substance that mimics the effect of Dol-P in the synthesis of glucolipids from UDP-glc [7] were found in this fraction. This negative result may reflect only enzyme inactivation during the isolation procedure, and not the inability of the nuclear fraction to synthesize Dol-P.

Once established that the mitochondria are the principal site of Dol-P synthesis, it was important to study the Dol-P biosynthesis at submitochondrial level. The inner and outer membranes and the matrix were isolated from purified mitochondria. Table 2 shows the incorporation into Dol-P as well as the marker enzymes.

Table 1
Specific activity of marker enzymes and synthesis of dolichyl phosphate by different subcellular fractions from rabbit and chicken liver

Fractions	Glucose-6-Pase (Units/mg)		5'-Nucleotidase (Units/mg)		Thyamine pyrophosphatase (Units/mg)		NAD-Cyt *c* reductase (Units/mg)		Dolichyl-P synthesis (pmol/75 min/mg)	
	Rabbit	Chicken	Rabbit	Chicken	Rabbit	Chicken	Rabbit	Chicken	Rabbit	Chicken
Total mitochondria	7	3	8	4	6	26	36	–	7.50	3.78
Golgi apparatus	60	ND	21	4	190	150	6	–	3.55	1.46
Total microsomes	450	240	9	5	1	40	230	–	1.80	0.08
Nuclear fraction	88	41	ND	21	13	12	ND	–	1.25	0.04
Plasma membrane	50	40	320	980	20	5	ND	–	0.15	–

Fractions were separated by differential and discontinuous sucrose-gradient centrifugation. Enzymes were assayed on 0.1 ml samples, Dol-P biosynthesis was made on 0.03 ml samples from IPP for rabbit and MVA for chicken. For glucose-6-phosphatase, 5'-nucleotidase and thyamine pyrophosphatase one unit was the hydrolysis of 1 nmol/min of phosphorus: for NADH-cyt *c* reductase, the reduction of 1 nmol/min of cytochrome *c*. ND: non-detected

Table 2
Specific activities of rabbit liver submitochondrial fractions

Fractions	Dolichyl-P synthesis		NADH-Cyt *c* reductase		Cytochrome *c* oxidase		Malate dehydrogenase	
	Spec.activity (pmol/75 min/mg)	RSA	Spec.activity (nmol/min/mg)	RSA	Spec.activity (nmol/min/mg)	RSA	Spec.activity (nmol/min/mg)	RSA
Total mitochondria	7.5	–	44.8	–	20.80	–	46.0	–
Outer membrane	10.0	1.33	54.0	1.20	1.56	0.07	11.0	0.24
Inner membrane	1.0	0.13	5.6	0.12	11.23	0.54	22.0	0.47
Matrix	4.5	0.60	ND	–	1.17	0.05	45.5	0.99

Purified mitochondria from rabbit liver were subfractioned as previously described and incubated with IPP as precursor. ND: non-detected; RSA: relative specific activity (spec. act. in each fraction/spec. act. in total mitochondria).

It seems clear that the outer membrane is responsible for Dol-P biosynthesis. The relative specific activities for Dol-P synthesis and NADH-cytochrome *c* reductase showed a similar degree of purification, indicating that both activities were together in the outer membrane. A recent report [19] demonstrated that intravenously injected dolichol was rapidly concentrated in the outer membrane of rat liver mitochondria.

The discovery that Dol-P synthesis occurs in mitochondria is of considerable interest, since another polyprenol is also synthesized in this organelle: the side chain of ubiquinone [20]. Another important point is that this is an all *trans* polyprenol and the enzymes involved in its synthesis from IPP were located in the inner membrane of mitochondria. Our results show that the outer mitochondrial membrane has the necessary enzymes for the synthesis of Dol-P from IPP, which contain *cis* and *trans* double bonds. This means that the following enzymes must be present: IPP isomerase, a minimum of one *trans*-prenyltransferase, and one *cis*-prenyltransferase necessary for the formation of both types of double bonds. Another enzyme that must be present is the one responsible for the saturation of the α-isoprene unit.

The fact that most of the enzymes required for ubiquinone synthesis are in the inner mitochondrial membrane [20] is in agreement with the current ideas that mitochondria in eukaryotes arise from a symbiotic incorporation of an anaerobic prokaryote. On the other hand, the fact that Dol-P is synthesized by the outer mitochondrial membrane, whose enzymes are codified by the nuclear genoma, is in agreement with the different type of polyprenyl phosphates involved in glycan synthesis, undecaprenyl phosphate for prokaryotes and dolichyl phosphate for eukaryotes [21].

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