Distribution and Glycosylation of Rat Liver Cytochrome b_5*

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A number of cytoplasmic membranes contain cytochrome b_5. This enzyme is, however, most abundant in the endoplasmic reticulum. The cytochrome obtains electrons exclusively from NADH-cytochrome b_5 reductase and this electron transport chain is present in microsomes prepared from all organs. Cytochrome b_5 is present in the membrane in a ten-fold excess over its flavoprotein counterpart.[1] This NADH-oxidizing electron transport chain participates in microsomal fatty acid desaturation, but its main function is unknown. The orientation of cytochrome b_5 is characteristic: the hydrophilic portion at the aminoterminal end extends into the aqueous phase on the cytoplasmic side; while the C-terminal end, consisting of mainly hydrophobic amino acids, is inserted in the lipid bilayer.[2]

The distribution of cytochrome b_5 in the intracellular membranes of rat liver was studied by immunoprecipitation of the enzyme after *in vivo* labeling with [^{3}H]leucine (Table 1). For the preparation of antibodies, detergent-isolated enzyme which contains both the hydrophilic and the hydrophobic portions was used. Complete isolation of the antibody precipitate was achieved by protein A – Sepharose chromatography. Cytochrome b_5 is broadly distributed in the liver cell, with highest concentration in rough and smooth microsomes, but it is also present in Golgi and outer mitochondrial membranes. The enzyme also occurs in peroxisomes. In order to determine the site of synthesis of the enzyme, bound and free ribosomes were isolated and antibody precipitation was performed after release of the nascent peptides with puromycin. Nascent enzyme could be detected only on bound ribosomes.

Cytochrome b_5 is present in membranes not only as the holoenzyme, but also in apoenzyme form (Table 2). Since the apoenzyme can be associated with heme with a stoichiometry of one to one, heme incubation was used to determine the amount of apoenzyme present. The iron of the heme was exchanged for ^{59}Fe and after incubation of the isolated fractions immunoprecipitation, adsorption to protein A – Sepharose and extensive washing were performed. No difference in incorporation appears between intact and solubilized membranes, which indicates that the heme binding site is

Table 1. Distribution of cytochrome b_5 in intracellular membranes. Rats were injected with 9250 kBq/100 g body weight of [^{3}H]leucine into the portal vein 60 min before decapitation (exp. 1) or 3 min before decapitation (exp. 2). Fractions were isolated and the enzyme was precipitated with antibodies against cytochrome b_5.

Exp.	Fraction	Anti-cyt. b_5 precipitable cpm/mg protein
1	Rough microsomes	920
	Smooth microsomes	1075
	Golgi membranes	610
	Outer mitochondrial membranes	361
	Peroxisomes	340
	Soluble supernatant	139
2	Bound ribosomes	1775
	Free ribosomes	76

Table 2. Presence of apo-cytochrome b_5 in subcellular membranes. Control and trypsin-treated (50 μg/mg protein, 30 °C, 10 min) intact or deoxycholate-solubilized microsomes were incubated with heme containing ^{59}Fe. After solubilization, precipitation with anti-b_5 was performed.

Fractions	Heme incorporated pmol/mg protein
Rough microsomes	
Intact	0.027
Solubilized	0.024
Rough microsomes	
Trypsin-treated	0.009
Solubilized + trypsin-treated	0.005
Smooth microsomes	0.020
Golgi fraction	0.015
Outer mitochondrial membranes	0.006
Peroxisomes	0.002
Erythrocyte ghosts	0.001

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available on the cytoplasmic surface of the membrane. Trypsin treatment destroys the apoenzyme completely, while the same treatment does not attack the catalytic part of the holoenzyme. Consequently, if heme incubation is applied after the proteolytic treatment, no incorporation of labeled heme could be observed. The apoenzyme present in rough microsomes corresponds to about 5 % of the total cytochrome b_5 content of this membrane. A high concentration of apoenzyme was also found in smooth microsomes and in the Golgi fraction. The peroxisomes and probably also the outer mitochondrial membranes are devoid of the apoenzyme.

These results were controlled by experiments in which [^{14}C]-α-aminolevulinic acid was injected *in vivo*. In this manner the transfer of heme synthesized *in vivo* to the apoenzyme could be followed. Rapid incorporation both into rough and smooth microsomes occurred and no difference in incorporation time could be observed between these two fractions. Therefore, it appears that heme is not added to all of the apoenzyme in rough microsomes, but that the apoenzyme is also transported in the endoplasmic membrane system.

Cytochrome b_5 is a glycoprotein and can be labeled by injection of radioactive sugars into rats (Table 3). In previous experiments we have found that glucosamine, mannose and glucose are incorporated into the newly synthesized enzyme at the ribosomal level.[3] After labeling *in vivo* with [^{14}C]galactose, radioactivity was demonstrated by chromatographic separation both in the galactose and glucose of the immunoprecipitated enzyme. On the other hand, when labeling *in vivo* was continued for 24 h, the radioactivity in the glucose moiety disappeared, in contrast to the labeling of the galactose.

Table 3. Double labeling of cytochrome b_5 *in vivo*. Rats were injected intraportally with 9250 kBq [^{3}H]leucine and 1850 kBq [^{14}C]galactose per 100 g body weight. Cytochrome b_5 was precipitated with antisera from total microsomes, the sugars were removed by hydrolysis and separated by paper chromatography. The radioactivity in the protein and in the chromatography spots were measured in a Packard Oxidizer.

Time	Protein	Radioactivity Galactose cpm	Glucose
6 min	15370	1720	2710
60 min	3620	727	540
24 h	1630	320	0

These results indicate that cytochrome b_5 is synthesized on bound ribosomes and subsequently appears as an apoenzyme on the cytoplasmic surface of the microsomal membranes. Conversion to holoenzyme may take place in both microsomal subfractions. The enzyme is glycosylated while still attached to the ribosomes, but its oligosaccharide chain is modified during transport in the endoplasmic membrane system. In a manner similar to the secretory proteins[4] glucose is removed from the growing oligosaccharide chain, which may be necessary both for the conversion of apo- to holoenzyme and for further glycosylation, which is probably required for the transport to other cytoplasmic organelles.

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