Vol. 84, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1978 Pages 572-580

GLYCOSYLATION OF RAT LIVER CYTOCHROME b_5 ON THE RIBOSOMAL LEVEL

Åke Elhammer, Gustav Dallner and Tsuneo Omura

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, Department of Pathology at Huddinge Hospital, Karolinska Institutet, Stockholm, Sweden and Department of Biology, Faculty of Science, Kyushu University, Fukuoka, Japan

Received July 31,1978

SUMMARY: The distribution and site of synthesis of cytochrome b_5 was studied by antibody precipitation of the enzyme labeled *in vivo*. The enzyme is present in rough and smooth microsomes, Golgi and outer mitochondrial membranes. The cytochrome is synthesized only on bound ribosomes, where glucosamine and galactose moieties are also added. The enzyme seems to be devoid of mannose and sialic acid residues.

INTRODUCTION

Cytochrome b_5 is present in rough and smooth microsomes, Golgi and outer mitochondrial membranes of rat liver. This enzyme is especially suited for studies of membrane biogenesis since it is an integral protein composed of two parts: a hydrophilic catalytically active portion situated on the cytoplasmic surface of the membrane and a hydrophobic portion required for binding to the membrane (1,2). Methods are available for the purification of two different forms of cytochrome b_5, detergent-b_5 which is the whole intact enzyme and trypsin-b_5 consisting of the hydrophilic portion only (3,4). In this paper the site of synthesis of cytochrome b_5 and its insertion into membranes was investigated and the possibility that cytochrome b_5 is a glycoprotein was studied.

MATERIALS AND METHODS

Male rats starved for 20 h were used. D-[1-^{3}H]Glucosamine (2000 mCi/mmol), D-[1-^{3}H]galactose (5000 mCi/mmol) and D-[1-^{3}H]mannose (2700 mCi/mmol) were purchased from the Radiochemical Centre, Amersham. NaCl solution was added to the labels to give a final concentration of 0.9 % and injections were made into the portal vein under phenobarbital anesthesia.

Rough and smooth microsomes, Golgi membranes and outer mitochondrial membranes were prepared as described previously (5-7). The purity of subcellular fractions was estimated by measuring various marker enzymes. The major contaminants in rough microsomes were 5 % lysosomes and 1 % mitochond-

0006-291X/78/0843-0572$01.00/0

ria, in smooth microsomes 11 % lysosomes and 6 % plasma membranes, in Golgi vesicles 4 % microsomes and 7 % plasma membranes, and in the outer mitochondria membrane fraction 4 % microsomes and 6 % lysosomes. Detailed discussion of such contamination is presented in recent methodological reviews (8,9). All membrane fractions were washed (by recentrifugation) with 0.15 M Tris-HCl buffer, pH 8.0. Particle-free supernatant was obtained by prolonged centrifugation (10). Free ribosomes and membrane-free bound ribosomes were prepared according to Blobel and Potter (11). Nascent proteins were released from ribosomes with puromycin (12). Trypsin-b_5 was prepared following the procedure of Omura and Takesue (3). Detergent-b_5 was isolated according to Ozols (4) with minor modifications. For preparation of antiserum against cytochrome b_5, detergent-b_5 was subjected to glutaraldehyde polymerization (13) and then injected into rabbits (1 mg every third week for 12 weeks). Soluble cytochrome b_5 from various preparations was incubated with anti-b_5 serum, adsorbed onto a Protein-A Sepharose column in the presence of buffer of high ionic strength, and, after extensive washing, eluted with an acidic buffer. After dialysis and lyophilization the residue was dissolved in 2 % SDS[1] and radioactivity was measured after addition of scintillator. All values were corrected for unspecific precipitation by using antibodies against egg albumin and chicken serum. Protein was determined as described earlier (14).

RESULTS AND DISCUSSION

The purity of the cytochrome b_5 used for the production of antisera was determined using SDS-acrylamide gel electrophoresis (Fig. 1). Only one major peak appeared on the gel in the molecular weight region of 17,000, indicating a high degree of purity in our detergent isolated cytochrome b_5 preparation.

Using previously reported methods (16) the purity and specificity of anti b_5-antibodies were tested by the Ouchterlony procedure. The antibodies formed single precipitation lines with the purified enzyme and the microsomal preparation and the precipitation bands fused, indicating immunological identity. The specificity was further tested by precipitation of [^{3}H]-leucine labeled cytochrome b_5 from solubilized rough microsomes with antibodies, followed by dissociation of the complex and by gel electrophoresis (Fig. 2). The two major protein bands are that of the large and small subunits of the antibody. The major radioactive peak was associated with the small protein peak of cytochrome b_5 in the 17,000 MW region. The procedure used failed to completely dissociate the antigen-antibody complex and consequently, part of the radioactivity remained associated with the large subunit.

[1] Abbreviation: SDS, sodium dodecyl sulphate.

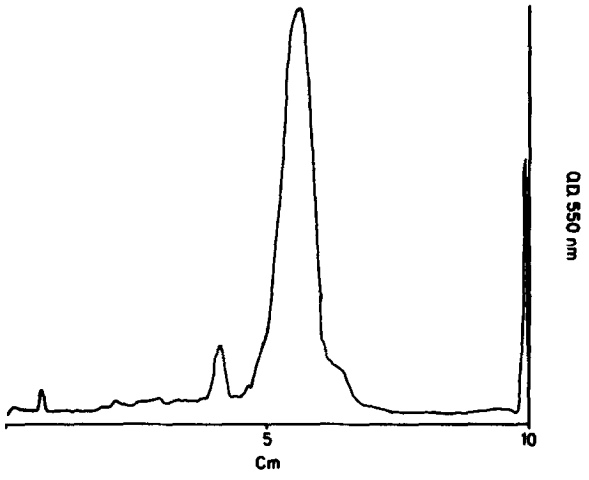

Fig. 1. SDS-acrylamide gel electrophoresis of the detergent isolated cytochrome b_5.
Gel electrophoresis was performed according to Weber and Osborn (15).

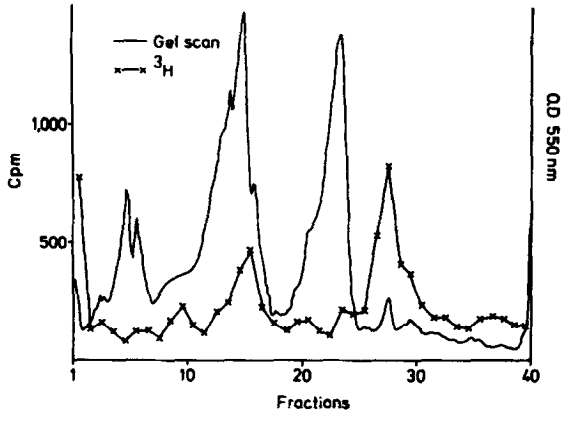

Fig. 2. SDS-acrylamide gel electrophoresis of the cytochrome b_5-anti cytochrome b_5 complex.
Rats were injected in the portal vein with 250 µCi [^{3}H]leucine/100 g body weight 10 min before decapitation. The samples were boiled 4 min in the presence of 2 % SDS; 8 M urea and 5 % β-mercaptoethanol. Gel electrophoresis was performed according to Weber and Osborn (15) and the 2 mm thick sliced gels were incinerated in a Packard Oxidizer for measurement of radioactivity.

Antiserum against microsomal cytochrome b_5 precipitates b_5 from solubilized rough and smooth microsomes, Golgi membranes and also outer mitochondrial membranes (Table 1). Thus, the cytochrome b_5 in microsomes and in

Table 1. DISTRIBUTION OF ANTIBODY-PRECIPITABLE CYTOCHROME b_5 IN INTRACELLULAR MEMBRANES

In exp. 1 rats were injected with 250 µCi/100 g body weight of [^{3}H]leucine into the portal vein 60 min before decapitation. Membranes (2 mg protein/ml) were dissolved in 1.5 % Triton X-100 and 0.1 % deoxycholate before anti-b_5 precipitation was carried out. The amount of radioactivity injected in exp. 2 was 500 µCi/100 g body weight and decapitation was performed after 3 min. In this case anti-b_5 precipitation was performed on the nascent proteins released by puromycin in the absence of detergents (12).

Exp.	Fraction	Total cpm/ mg protein	Anti-b_5 precipitable cpm/mg protein
1	Rough microsomes	23,800	920
	Smooth microsomes	32,800	1,075
	Golgi membranes	20,300	611
	Outer mitochondrial membranes		361
	Soluble supernatant	12,000	139
2	Bound ribosomes		1,775
	Free ribosomes		76

the outer mitochondrial membrane cross-react immunologically. Some cytochrome b_5 was also present in the particle-free supernatant; the exact origin of this b_5 is not clear. It may either represent newly synthesized apoenzyme in the process of being transported to the mitochondria or it may be microsomal enzyme that has been released during the preparation procedure. Table 1 also demonstrates that newly synthesized cytochrome b_5 is not present on free ribosomes but appears exclusively on bound ribosomes.

Isolated trypsin b_5 is relatively homogenous in terms of electrophoretic mobility (Fig. 3) and also in terms of spectrophotometric and fluorometric properties. When cytochrome b_5 was purified from rats labeled *in vivo* with [^{3}H]glucosamine and subjected to SDS electrophoresis, a peak of radioactivity coincident with the protein peak was seen.

In order to investigate the possible glycosylation of cytochrome b_5 during its biosynthesis, labeled glucosamine, mannose and galactose were injected into the animals 4 min before decapitation and the enzyme was pre-

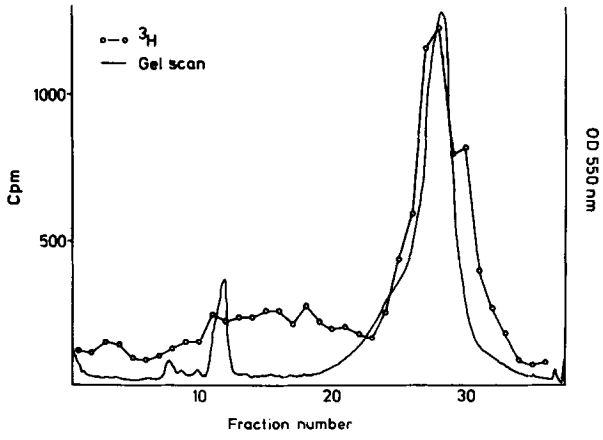

Fig. 3. SDS-acrylamide gel electrophoresis of the isolated trypsin-b_5. The rats were injected in the portal vein with 100 µCi [^{3}H]glucosamine/100 g body weight 30 min before decapitation. Gel electrophoresis was performed according to Weber and Osborn (13) and the gel was sliced into 2 mm slices. Radioactivity was determined using an Oxidizer (Packard).

cipitated both after release from ribosomes and from rough microsomal membranes (Table 2). After the short pulse with glucosamine, radioactivity appeared both on the nascent protein and on the enzyme in the rough microsomes, constituting 1.4 and 1.1 percent of the total radioactivity in these fractions, respectively. No mannose label could be detected in cytochrome b_5 after immunoprecipitation. On the other hand, the other neutral sugar galactose is clearly associated with b_5 both at the ribosomal and at the membrane level, where this protein contains 3.6 and 5.2 percent of the total radioactivity, respectively. The fourth sugar located in microsomes, i.e., sialic acid, appears to be absent from the cytochrome. After 60 min *in vivo* labeling, when a large part of the radioactive glucosamine has been converted to sialic acid (17), neuraminidase treatment did not remove any radioactivity from nascent cytochrome b_5 or from b_5 in rough microsomal membranes. To exclude the possibility that the injected sugars are metabolized to amino acids, cytochrome b_5 labeled *in vivo* with [^{3}H]galactose and solubilized from rough microsomes was precipitated with antibodies and the complex was subjected to acid hydrolysis to liberate the sugar component

Table 2. INCORPORATION OF [^{3}H]LABELED SUGARS INTO ANTIBODY-PRECIPITABLE CYTOCHROME $\underline{b}_5$

Rats were injected with 500 µCi of the appropriate sugar label/100 g body weight 4 min and 60 min before decapitation in exp. 1 and 2, respectively. Nascent proteins released by puromycin were precipitated directly, while rough membranes (2 mg protein/ml) were dissolved in 0.55 % deoxycholate before precipitation with anti-$\underline{b}_5$. In exp. 2 the nascent proteins and dissolved rough membranes were treated with 25 µg neuraminidase (Type VI, Sigma) per mg protein at 37°C for 15 min. After dialysis precipitation with anti-$\underline{b}_5$ was performed as is described in Materials and Methods.

Exp.		Nascent proteins from isolated 'bound' ribosomes[a]	Rough membranes[b]
		cpm/mg protein	
1	[^{3}H]Glucosamine	82	303
	[^{3}H]Mannose	8	16
	[^{3}H]Galactose	981	7,105
2	[^{3}H]Glucosamine	1,100	3,700
	[^{3}H]Glucosamine followed by neuraminidase treatment	1,140	3,850

[a] anti-$\underline{b}_5$ precipitable cpm/mg isolated nascent protein.

[b] anti-$\underline{b}_5$ precipitable cpm/mg rough membrane protein.

(17). After ion exchange chromatography practically all the radioactivity was recovered in the neutral sugar eluate (Table 3). On thin layer chromatography the radioactivity was associated with the galactose spot. Such experiments were also performed after labeling *in vivo* with [^{3}H]glucosamine and the results were similar.

The insertion of cytochrome $\underline{b}_5$ into rough microsomal membranes from which the ribosomes had been removed was studied by trypsin treatment after short pulse labeling (Fig. 4A). It has been established that the newly synthesized apo-enzyme, which contains a large amount of [^{3}H]leucine, is rapidly fragmented by trypsin treatment (20). For this reason only the

Table 3. THE RECOVERY OF [^{3}H]GALACTOSE INJECTED IN VIVO IN ANTIBODY-PRECIPITABLE CYTOCHROME b_5

Rats were injected with 250 µCi [^{3}H]galactose/100 g body weight 10 min before decapitation. Cytochrome b_5 was precipitated from solubilized rough microsomes with antisera, hydrolyzed in 1 M HCl (100°C, 8 hours) (17) and the supernatant was analyzed after filtration. The filtered supernatant was chromatographed on a Dowex 50 W-x 8 column to retain amino acids and amino sugars (18). The eluate was analyzed on thin layer chromatography and used for measurement of radioactivity.

	Radioactivity cpm
Cytochrome b_5-antibody complex (CAC)	1410
CAC after hydrolysis	
supernatant	1302
supernatant, eluted from Dowex 50 W-x 8	1195

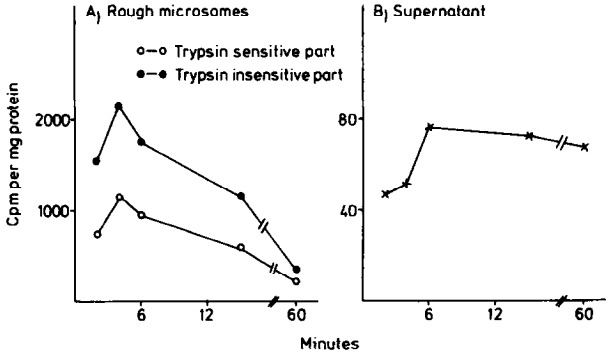

Fig. 4. Time course of [^{3}H]leucine incorporation into cytochrome b_5 in vivo. Rats were injected in the portal vein with 250 µCi [^{3}H]leucine/100 g body weight and were decapitated after various time intervals and rough microsomes and supernatant were prepared. A) Rough microsomes were treated with 50 µg trypsin (Boehringer)/mg protein at 30°C for 10 min. The reaction was stopped with trypsin inhibitor (Boehringer), twice as much as the amount of trypsin. After ultracentrifugation both fractions (2 mg protein/ml) were supplemented with 0.55 % deoxycholate, ultracentrifuged and the supernatants were treated with anti-b_5. B) The particle-free supernatant was used directly for precipitation with anti-b_5.

radioactivity in the holoenzyme is apparent and two-thirds of the counts are recovered in the trypsin insensitive compartment. The ratio between trypsin insensitive and sensitive portions decreases with time and at 60 minutes, when no more apoenzyme is labeled, the distribution in the two compartments is about equal. Interestingly, the amount of labeled b_5 found in the supernatant increases parallel to the decrease in rough microsomes (Fig. 4B).

The experiments described in this paper demonstrate that anti-b_5 antibodies against microsomal cytochrome b_5 also precipitate the cytochrome from Golgi and outer mitochondrial membranes. The enzyme appears to be synthesized exclusively on bound ribosomes, in agreement with previous experiments (21). Isolation and purification of enzyme labeled *in vivo* with glucosamine show the glycoprotein nature of this cytochrome from rat liver. The enzyme isolated from human liver has also been suggested to be a glycoprotein (22). Microsomal membranes contain four protein-bound monosaccharides: mannose, galactose, glucosamine and sialic acid (23). Two of these, glucosamine and galactose, appear to be attached to the hydrophilic part of cytochrome b_5. At least one moiety of each sugar is added to this protein at the ribosomal level, but it is possible that the oligosaccharide chain is completed in the rough microsomal membrane. Attachment of various sugar moieties to nascent polypeptide chains still associated with ribosomes has also been found in other systems (12,24,25). After detachment of cytochrome b_5 from ribosomes the newly synthesized enzyme seems to be distributed on the outer surface of the membrane. To understand the exact topology of this enzyme it will be necessary to perform differential labeling of the different parts of the polypeptide chain and to study its lateral movement and transport to other intracellular organelles.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Medical Research Council and the Magnus Bergwall Foundation.

REFERENCES

1. Ito, A. and Sato, R. (1968) J. Biol. Chem. 243, 4922-4923.
2. Spatz, L. and Strittmatter, P. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1042-1046.
3. Omura, T. and Takesue, S. (1970) J. Biochem. 67, 249-257.
4. Ozols, J. (1974) Biochemistry 13, 426-434.
5. Dallner, G. (1974) Methods Enzymol. 31, 191-201.
6. Ehrenreich, J.H., Bergeron, J.J.M., Siekevitz, P. and Palade, G.E. (1973) J. Cell Biol. 59, 45-72.
7. Sottocasa, G.L., Kuylenstierna, B., Bergstrand, A. and Ernster, L. (1967) J. Cell Biol. 32, 415-438.
8. DePierre, J. and Dallner, G. (1976) in Biochemical Analysis of Membranes (Maddy, A.H., ed.), pp. 79-131, Chapman and Hall, London.
9. Eriksson, L.C., DePierre, J.W. and Dallner, G. (1978) Pharmac. Ther. A., Vol. 2, pp. 281-317.
10. Autuori, F., Svensson, H. and Dallner, G. (1975) J. Cell Biol. 67, 687-699.
11. Blobel, G. and Potter, V.R. (1967) J. Mol. Biol. 26, 279-292.
12. Molnar, J. (1975) Mol. Cellular Biochem. 6, 3-14.
13. Margoliash, E., Nisoneff, A. and Reichlin, M. (1969) J. Biol. Chem. 245, 931-939.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
15. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
16. Hirokata, Y., Shigematsu, A. and Omura, T. (1978) J. Biochem. 83, 431-440.
17. Harms, E. and Reutter, W. (1974) Cancer Res. 34, 3165-3172.
18. Miyajima, N., Tomikawa, M., Kawasaki, T. and Yamashina, I. (1969) J. Biochem. 66, 711-732.
19. Kim, J.H., Shome, B., Liao, T. and Pierce, J.G. (1967) Anal. Biochem. 20, 258-274.
20. Negishi, M. and Omura, T. (1970) J. Biochem. 67, 745-747.
21. Harano, T. and Omura, T. (1977) Biochem. J. 82, 1541-1549.
22. Ozols, J. (1972) J. Biol. Chem. 247, 2242-2245.
23. Bergman, A. and Dallner, G. (1976) Biochim. Biophys. Acta 433, 496-508.
24. Kiely, M.L., McKnight, S. and Schimke, R.T. (1976) J. Biol. Chem. 251, 5490-5495.
25. Rothman, I.E. and Lodish, H.F. (1977) Nature 269, 775-780.