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DISTRIBUTION AND TRANSPORT OF APO- AND HOLOCYTOCHROME b_5 IN THE ENDOPLASMIC RETICULUM OF RAT LIVER

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The transport and distribution of apo- and holocytochrome b_5 was investigated with the aid of specific antibodies. The holoenzyme was found to be localized mainly in the rough and smooth endoplasmic reticulum and in the Golgi system but some precipitation could also be obtained in the outer mitochondrial membranes and in the peroxisomes. The apoenzyme, however, could only be detected in the endoplasmic reticulum-Golgi system, which also was shown to be the sole site for incorporation of the prosthetic heme moiety. Time-course studies revealed that the labeled enzyme appeared both as apoenzyme and as holoenzyme in the rough endoplasmic reticulum 10 min after in vivo injection of radioactive leucine and that further transport to the smooth endoplasmic reticulum occurred within 10 min. The subsequent transport to other organelles, however, required a somewhat longer time and peak radioactivity in outer mitochondrial membranes was not attained until after 40 min.

Introduction

One of the most investigated microsomal enzymes is cytochrome b_5. This enzyme can be isolated in a highly purified form and is well characterized structurally; and its properties as an electron carrier have been very thoroughly investigated [1]. A large part of this enzyme is situated in the water phase on the outer surface of the microsomal membranes, and polypeptide containing 88 amino acid residues can thus be removed by tryptic treatment [2]. This part contains a prosthetic heme group and houses the electron carrier function. The remaining polypeptide carries a hydrophobic segment between amino acid residues 109 and 125, which is the part necessary for the association of the enzyme to the membrane [3]. This enzyme is interesting for biosynthetic studies for two reasons: first, the enzyme is found in highest concentration in rough and smooth microsomes but it is well established that cytochrome b_5 is also present in outer mitochondrial membranes and in peroxisomes [4]; second, contrary to other microsomal enzymes, this cytochrome occurs also as apoenzyme in measurable amounts [5]. In spite of the fact that the enzyme is well characterized from liver and other organs from several species, its main function is not yet understood. It serves as electron acceptor for the NADH-cytochrome b_5 reductase but the main electron acceptor to which cytochrome b_5 transfers electrons is not known. A minor function of the enzyme is participation in the microsomal fatty acid desaturase system [6] and it also interacts with the NADPH-dependent hydroxylation system [7]. This, however, does not explain why this enzyme is present in microsomal membranes in such a high concentration. The enzyme cannot be induced to any sizeable extent with the known inducers of the endoplasmic membranes and consequently induc-

tion cannot be used as a tool for investigating functional activities.

In this study we used immunoprecipitation together with in vivo and in vitro labeling procedures to isolate the enzyme and to investigate the presence and distribution of apoprotein. The redistribution of the apo- and holoenzyme was also followed and was found to exhibit a characteristic pattern.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 180–200 g were used in all experiments. The rats were starved for 20 h before death. [^{3}H]Leucine (188 Ci/mmol), ^{3}H-labeled amino-acid mixture, [^{59}Fe] (3–20 mCi/mg Fe) and 5-amino[4-^{14}C]levulinic acid (40–60 mCi/mmol) were purchased from Amersham International. NaCl solution was added to a final concentration of 0.9% to all labels used for injection and injections were made in the portal vein under pentobarbital anesthesia.

Fractionation and isolation

Rough and smooth microsomes, Golgi membranes, outer mitochondrial membranes and peroxisomes were prepared as described previously [8–10]. The purity of the isolated fractions was determined by measuring various marker enzymes. As is apparent from Table I, the isolated rough microsomal fraction was practically free from Golgi membranes but contained minor amounts, roughly 4 and 3%, respectively, of plasma membranes and outer mitochondrial membranes. The smooth microsomal fraction contained somewhat more Golgi vesicles approx. 3% together with 5% plasma membrane and 6% outer mitochondrial membranes. The amount of plasma membrane contamination in this fraction, however, is probably an overestimate since the values are based on AMPase activity measurements and it has been reported [12] that some AMPase is present in the endoplasmic reticulum. The Golgi fraction used contained some AMPase activity indicating the presence of small amounts of plasma membranes, also minor amounts of microsomes and outer mitochondrial membranes could be detected. The peroxisomes prepared in this investigation demonstrated a very high purity when measuring marker enzymes, only slight amounts of plasma membranes, less than 1.5%, could be detected while microsomal, Golgi and outer mitochondrial membranes where virtually absent (less than 0.5%). Erythrocyte ghosts were prepared according to Dodge et al. [13]. All membrane fractions were washed by recentrifugation in 0.15 M Tris-HCl buffer, pH 8.0. A particle-free supernatant was obtained by prolonged centrifugation [14]. Membrane-free bound ribosomes were prepared according to Blobel and Potter [15]. Nascent proteins were released from ribosomes with puromycin [16].

TABLE I

DISTRIBUTION OF MARKER ENZYMES IN LIVER SUBFRACTIONS

For measurement of CMP-NANA transferase desialylated fetuin was used as acceptor [11]. The values are the means of four experiments. NANA, *N*-acetylneuraminic acid.

	Glucose-6-phosphatase [a]	CMP-NANA transferase [b]	AMPase [a]	Monoamine oxidase [c]
Rough microsomes	2.2	4.2	0.03	6.5
Smooth microsomes	2.4	20.3	0.04	10.8
Golgi fraction	0.1	862.0	0.05	5.8
Plasma membranes			0.73	
Outer mitochondrial membranes	0.1	15.2	0.04	198
Peroxisomes	0.06	3.5	0.61	0.5

[a] μmol P_i/min per mg protein.
[b] pmol NANA transferred/10 min per mg protein.
[c] nmol benzaldehyde produced/min per mg protein.

The hydrophilic part of cytochrome b_5 was released with trypsin following the procedure of Omura and Takesue [17]. The intact cytochrome was isolated with detergent according to the method of Ozols [18] with minor modifications. This preparation was subsequently purified to electrophoretic homogeneity by chromatography on 8-aminooctyl-Sepharose [19].

Antibody preparation

Antibodies against cytochrome b_5 were raised in rabbits. Rabbits were pretreated by injecting 0.3 ml Freund's complete adjuvant (Gibco, U.K.), into each foot pad. This treatment caused the lymp nodes in the knee-joints to become swollen. The electrophoretically homogeneous antigen (50–70 μg) was immobilized by binding to CNBr-activated Sepharose and together with approx. 50 μl Freund's complete adjuvant was injected into the preswollen lymphatic glands. This treatment was repeated every 4th week and blood was collected from the ear vein 1 week after each booster. Ouchterlony immunodiffusion and rocket immunoelectrophoresis was used to test the specificity and titer of the antisera as described previously [20,21].

Antibody precipitation

Water soluble samples were mixed with an excess of antibodies in the presence of 20 mM Tris-HCl buffer, pH 7.5, before incubation. Non-water soluble samples, i.e., membrane fractions, were solubilized in 0.55% sodium deoxycholate (DOC)/20 mM Tris-HCl, pH 7.5, or 1% Triton/20 mM Tris-HCl, pH 7.5. The protein concentration was 2.5 mg protein/ml [22]. Incubations were performed on ice for 10 min before the addition of antibodies in excess. The antibody-antigen mixture was incubated at room temperature for 1 h, then overnight at +4°C. In order to separate the antigen-antibody complex from the remaining reaction mixture, samples were adsorbed on a protein A-Sepharose column equilibrated with 100 mM Tris-HCl buffer (pH 7.5)/200 mM NaCl. After adsorption the column was washed extensively with 100 mM Tris-HCl buffer (pH 7.5)/200 mM NaCl/1% Triton X-100 until the effluent contained no radioactivity and no measurable amount of protein (monitored by measuring the absorption at 280 nm). The column was subsequently eluted with 100 mM glycine-HCl buffer, pH 2.8, 200 mM NaCl and the eluate was neutralized with solid Tris base, dialyzed extensively against distitilled water and lyophilized. This material was then used for various analyses, for example, scintillation counting and SDS-polyacrylamide gel electrophoresis.

Heme incorporation

(1) Preparation of labeled heme. Purified porphyrin purchased from Sigma was converted to hemin in the presence of ^{59}Fe-labeled iron sulphate according to Morell et al. [23] with minor modifications. The labeled heme was stored in a diethyl ether solution and the specific radioactivity of the solution was determined by scintillation counting together with spectrophotometric analysis (the α-band) in 0.75 M NaOH/2 M pyridine.

(2) Incubation with labeled heme. Usually 50 μl to 4 mg membrane protein of the labeled stock solution was evaporated with nitrogen and subsequently resolubilized in 0.1 M NaOH containing enough unlabeled hemin to provide an excess during incubation. The resulting solution was diluted ten times with distilled water and the pH carefully adjusted to 9 with 0.5 M HCl. Membranes suspended in 0.25 M sucrose/50 mM Tris-HCl, pH 7.5 or solubilized in 0.25 sucrose/50 mM Tris-HCl, pH 7.5 and 0.55% deoxycholate were supplemented under continuous stirring with small portions of the labeled heme solution prepared as described above. Incubation was performed at 37°C for 30 min. Following incorporation of labeled heme, the membranes were subjected either to a direct precipitation with anti-cytochrome b_5-antibodies (solubilized microsomes) or to solubilization or trypsin treatment followed by antibody precipitation (see individual experiments).

Isolation of apoenzyme

When the apoenzyme was analyzed, microsomes were pretreated with heme followed by trypsin treatment and precipitation with antibody. A schematic representation of the procedure is given in Fig. 1.

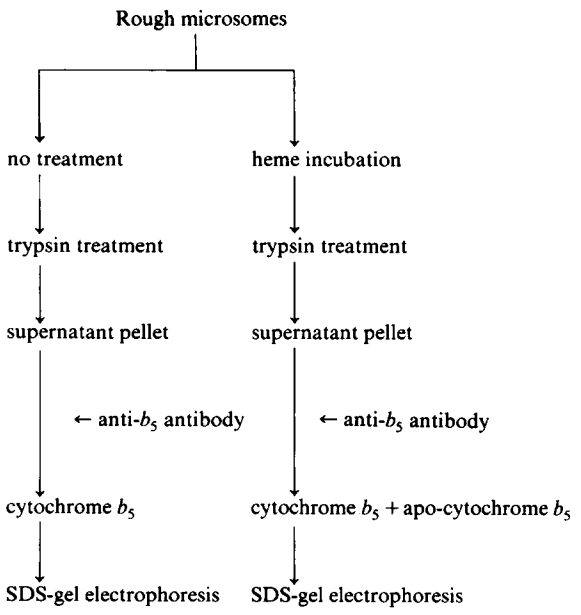

Fig. 1. Schematic representation of the procedure used to determine the amount of cytochrome b_5.

Trypsin treatment

In a typical case 5 mg of microsomal protein suspended in 0.25 M sucrose/50 mM Tris-HCl, pH 7.0 (2–2.5 mg/ml) was supplemented with 0.25 mg (50 μg/mg protein) trypsin (Boehringer) and incubation was carried out at 30°C for 10 min. The reaction was terminated by addition of 0.5 mg (100 μg/mg protein) trypsin inhibitor (Boehringer) to the reaction mixture followed by incubation at 30°C for an additional 15 min. The trypsin-sensitive part of the membrane was subsequently separated from the trypsin-insensitive part by centrifugation at 105 000 × g for 60 min.

Electrophoresis

Sodium dodecyl sulphate (SDS) gel electrophoresis was performed according to previously described methods [24,25]. The gels were stained with Coomassie brilliant blue and quantitative radioactivity measurement was carried out by slicing the gels in 1 or 2 mm slices followed by combustion in a Packard Oxidizer. Fluorography was carried out according to Bonner and Laskey [26] or by soaking the gels in ENHANCE (purchased from New England Nuclear) followed by water prior to exposure to the X-ray film (Kodak XR-2) at −70°C for 20 days.

Chemical and enzymic determinations

Protein was determined according to Lowry et al. [27] with bovine serum albumin as a standard.

Results

Enzyme purity

Isolation of cytochrome b_5 after detergent solubilization gives the complete polypeptide together with the hydrophobic segment. However, in comparison with the trypsin solubilized enzyme the purification procedure in this case was more difficult. By using the procedure described in Materials and Methods the final enzyme obtained gave only one single band on slab gel electrophoresis without apparent contaminants (Fig. 2). This enzyme preparation, therefore, was suitable for injection to obtain anti-cytochrome b_5 antibodies.

Antibody specificity

The antigen-antibody complex was isolated routinely by adsorption on Protein A-Sepharose. It appears on Fig. 3 that the antisera obtained was monospecific, since only one band corresponding to the enzyme band was visible on the gel beside the two immunoglobulin peptides. This gel at the same time demonstrated the effectiveness of the adsorption on protein A-Sepharose, since under our conditions no contaminating protein remained on the column after adsorption and elution. The fluorography of the enzyme in vivo labeled with protein precursors again demonstrated the purity of the precipitated enzyme which gave a single band on the fluorogram (Fig. 4). As in previous experiments, analysis on Ouchterlony plates was also performed and, again, one could observe only a single precipitin line (figure not shown).

Enzyme distribution

Isolation of cytochrome b_5 by immunoprecipitation from in vivo labeled rat liver demonstrated a distribution pattern in agreement with previous investigations (Table II). The enzyme was present in both rough and smooth microsomes and also in Golgi membranes. Labeled immunoprecipitate was

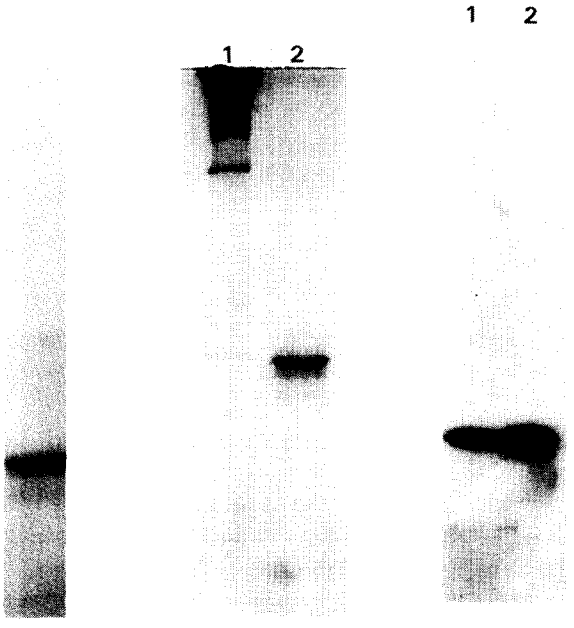

Fig. 2. (Left). SDS-slab gel electrophoresis of the purified antigen (cytochrome (b_5). Staining was performed with Coomassie brilliant blue.

Fig. 3. (Middle). SDS-slab gel electrophoresis of the cytochrome b_5 antibody-antigen complex. Lane 1: cytochrome b_5 antigen-antibody complex isolated on protein A-Sepharose chromatography from rough microsomes as described in Materials and Methods. Lane 2: 8 μg purified antigen (cytochrome b_5). Staining was performed with Coomassie brilliant blue.

Fig. 4. (Right). Fluorography of cytochrome b_5 antigen-antibody complex. Lane 1: Antigen-antibody complex isolated from rats labeled with 1 mCi ^{3}H-labeled amino acid mixture by injection into the portal vein 15 min before decapitation. Lane 2: Purified ^{125}I-labeled cytochrome b_5. Fluorography was performed as described in Materials and Methods.

also found in outer mitochondrial membranes and peroxisomes. Some precipitation could be obtained in the soluble supernatant, the significance of which, however, is not clear.

Apoenzyme

Contrary to several microsomal enzymes, cytochrome b_5 appears to be present in the membrane not only as holoenzyme but also in apoenzyme form. Sizeable amounts of this enzyme form are present in rough and smooth microsomes and also in Golgi membranes (Table III). The apoenzyme probably does not occur in the outer mitochondrial membrane and is also absent from the peroxisomes. The lack of interaction with erythrocyte membranes and also other control experiments demonstrated that the ^{59}Fe-labeled heme prepared in this investigation in combination with specific

TABLE II

INTRACELLULAR DISTRIBUTION OF ANTIBODY PRECIPITABLE CYTOCHROME b_5

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. Isolation of various membranes was carried out as described in Materials and Methods. The values are the mean values of five individual experiments ± S.E.

Fraction	Antibody precipitable cytochrome b_5 (cpm/mg protein)
Rough microsomes	920 ± 85
Smooth microsomes	1075 ± 60
Golgi membranes	610 ± 45
Outer mitochondrial membranes	361 ± 25
Peroxisomes	340 ± 30

TABLE III

PRESENCE OF APO-CYTOCHROME b_5 IN SUBCELLULAR MEMBRANES

Isolated, Tris-washed, membranes were incubated with heme containing ^{59}Fe, solubilized and treated with anti b_5-antibodies as described in Materials and Methods. After separation on SDS-gel electrophoresis the radioactivity in the antigen band was determined and the amount of antigen calculated. The values are given as mean values ± S.E. (n = 5).

Fraction	Heme incorporated (nmol/mg protein)
Rough microsomes	0.025 ± 0.002
Smooth microsomes	0.020 ± 0.003
Golgi fraction	0.015 ± 0.001
Outer mitochondrial membranes	0.006 ± 0.001
Peroxisomes	0.002 ± 0.001
Erythrocyte ghosts	0.001 ± 0.001

TABLE IV

EFFECT OF TRYPSIN TREATMENT ON HEME INCORPORATION INTO ROUGH MICROSOMAL 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. The total amount of cytochrome b_5 in the fractions was determined spectrophotometrically. The values are given as mean values ± S.E. ($n = 6$).

Rough microsomes	nmol/mg protein	
	Heme incorporated	Cytochrome b_5
Intact	0.027 ± 0.003	0.472
Solubilized	0.024 ± 0.002	0.467
Trypsin-treated	0.009 ± 0.001	0.152
Solubilized + trypsin-treated	0.005 ± 0.001	0.450

antibodies is most useful in studies which aim at the quantitative estimation of the apoenzyme.

In rough microsomes, the amount of apoenzyme accepting heme upon incubation makes up about 5% of the total cytochrome b_5 amount (Table IV). There is no membrane barrier in the interaction between the heme and the enzyme, since solubilization of the membrane prior to treatment did not cause any further association of heme to the specific protein. Trypsin treatment destroyed the majority of the apoenzyme and also released about 70% of the holoenzyme from the membrane. Solubilization of the microsomal membrane followed by trypsin treatment of the complete membrane mixture also destroyed the apoenzyme, but as is well established, the hydrolyzed holoenzyme was present in the supernatant with an intact spectrum.

Incubation with labeled heme demonstrated that both rough and smooth microsomes contained apoenzyme and the question arose as to whether the transformation to holoenzyme might occur at both locations. To investigate this question, rats were injected with radioactive α-aminolevulinic acid a precursor in the mitochondrial heme biosynthesis (Fig. 5). Incorporation occurred into cytochrome b_5 of both microsomal fractions in a parallel fashion, suggesting that the transfer of

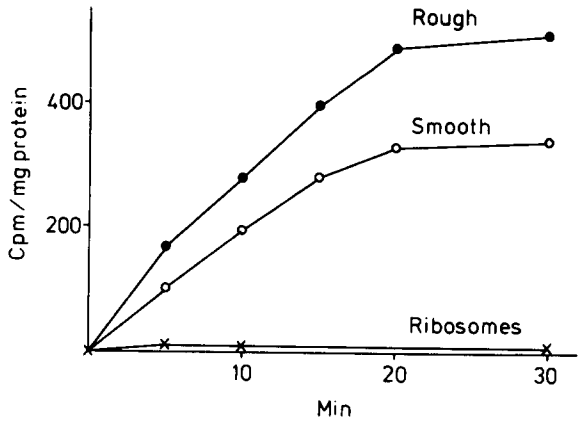

Fig. 5. Incorporation of α-amino[^{14}C]levulinic acid into cytochrome b_5. Rats were labeled in vivo by injecting 50 μCi α-amino[^{14}C]levulinic acid into the portal vein. Rough and smooth microsomes and bound ribosomes were prepared at different time points. Solubilized microsomes and puromycin released nascent proteins were precipitated with specific anti-cytochrome b_5 antibodies as described in Materials and Methods followed by radioactivity measurement in the antigen band after separation on SDS-gel electrophoresis.

heme to the apoenzyme takes place in both membranes. The total lack of incorporation into the apoenzyme at the ribosomal level indicates that the activation of the enzyme is a relatively late event in the biosynthetic process.

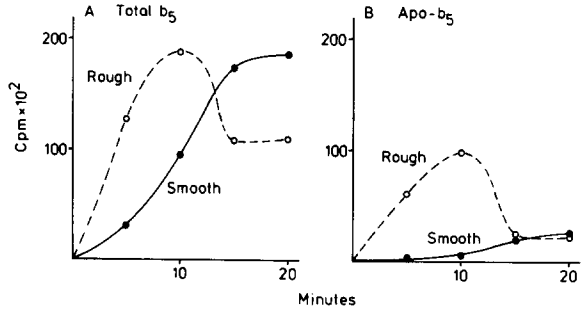

Fig. 6. Transport of newly synthesized cytochrome b_5 in the endoplasmic reticulum. 1 mCi tritium-labeled amino acid mixture was injected intraportally to each rat. The rats were decapitated after various time points and rough and smooth microsomes were prepared. Samples for apocytochrome b_5 measurements were incubated with heme. All samples were subsequently treated with trypsin before precipitation with anti-cytochrome b_5 antibodies, separation on SDS-gel electrophoresis and measurement of the radioactivity in the antigen band. (A) Total cytochrome b_5; (B) apocytochrome b_5.

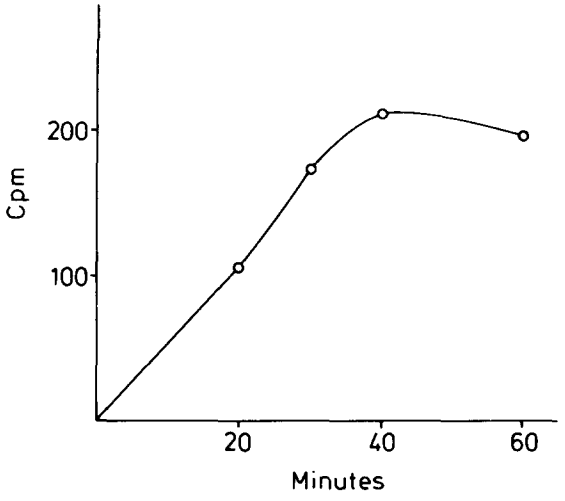

Fig. 7. Appearance of newly synthesized cytochrome b_5 in the outer mitochondrial membranes. Rats were labelled in vivo by injection of 1 mCi ^{3}H-labeled amino acid mixture into the portal vein. Outer mitochondrial membranes were prepared according to Sottocasa et al. [9]. Membrane samples from rats labeled for different time periods were solubilized in 1% Triton X-100 and cytochrome b_5 was precipitated with antibodies. The antigen-antibody complex was separated on SDS-gel electrophoresis and the antigen band was cut out for radioactivity measurement.

Transport in the endoplasmic reticulum

Transport of the apo- and holoenzyme was studied after pulse labelling through direct injection of radioactivity into the portal vein. During the first 5-min period, a high labelling in both the apo- and holoenzymes of the rough microsomes was observed and peak incorporation was reached after 10 min (Fig. 6 A and B). During the following 10 min the specific radioactivity in the apoenzyme of this fraction decreased rapidly. The picture was quite different in smooth microsomes. Here, the radioactivity in the holoenzyme increased after a delay, in comparison with the rough counterpart, and within the first 10 min almost no labelling appeared in the apoenzyme. A considerably longer time lag was encountered when measuring the incorporation of cytochrome b_5-bound radioactivity in outer mitochondrial membranes (Fig. 7), suggesting that the transport from the endoplasmic reticulum to other intracellular organelles is carried out in a more time-consuming manner. This pattern is characteristic and indicative for a transport of both types of cytochrome b_5 from rough to smooth microsomes and further to other organelles. The pattern described in Figs. 5 and 6 was obtained when radioactivity was determined in gel slices after separation of the enzyme from the antibodies with slab gel electrophoresis.

Discussion

The biosynthesis and transport of cytochrome b_5 in the rat liver were analyzed in this paper by using in vivo labelling and immunoprecipitation. The enzyme was present in microsomes also as an apoprotein.

Cytochrome b_5 is one of the few intrinsic membrane proteins of liver microsomes which can be purified to a high degree. This is valid not only for that portion which is released by trypsin together with a relatively few number of other proteins, but also for the detergent-solubilized form, which represents the complete molecule. On the other hand, because of its relatively small size and the similarity in amino acid composition of the enzyme in various species, the antigenicity of this protein is poor and it is difficult to obtain an antiserum with high titre.

The antigen-antibody complex in this study was adsorbed on protein A-Sepharose in order to isolate it from various contaminants. This procedure is effective for the isolation of cytochrome b_5, since on the following slab gel electrophoresis the only microsomal band visible was that of cytochrome b_5.

The intracellular distribution of cytochrome b_5 found with this labeling procedure has been previously well established by other studies. Our investigations showed that, beside rough and smooth microsomes, Golgi- and outer mitochondrial membranes and also peroxisomes house this hemoprotein. It has been reported [28,29] that cytochrome b_5 is present also in the plasma membrane and in the nuclear envelope. These organelles, however, were not investigated in the present work. Interestingly, the apoenzyme has a more restricted localization and is present only in the endoplasmic reticulum-Golgi membrane system. It therefore appears likely that the completion of the apoenzyme with heme may occur only in the endoplasmic membrame system and that only the holoenzyme is transported to other organelles. It appears less

probable that a part of the apoenzyme represents aged molecules in the breakdown process, but this possibility has not yet been excluded experimentally. One may speculate about the reason for the only enzyme in the microsomal membrane shown to be present in apoform being cytochrome b_5. One explanation may be that a signal built into the protein part or into the oligosaccharide chain of the molecule serves as a regulating factor in this matter.

The preferential appearance of the newly synthesized enzyme in the rough microsomal membranes is indicative of a proposed site of synthesis on bound ribosomes. The appearance of the apoprotein in this position is even more suggestive of the synthesis on bound ribosomes. The delay in its appearance in smooth microsomes and subsequently in outer mitochondrial membranes is in both cases consirable. Such a time course is characteristic for proteins moving from one compartment to another either by diffusion in the soluble phase, as secretory proteins, or by lateral displacement as membrane proteins. Furthermore, if cytochrome b_5 were synthesized on free ribosomes in the cytoplasma, one would expect results similar to those obtained by Borgese et al. [30]. These authors found a parallel incorporation of newly synthesized NADH-cytochrome b_5 reductase into several intracellular membranes. It is supposed that this enzyme is synthesized on free ribosomes.

Theoretically, the type of movement described above can occur also in the case of a protein originating from the soluble cytoplasm provided that receptors for the protein are present only in one type of membrane, here in rough microsomes. Also, if there is a great difference in the affinity of the receptor for the protein in different membranes, one could obtain this type of pattern. However, further and more elaborate experimental data are requried in order to make any final conclusions in this matter,

In conclusion, cytochrome b_5 is an intrinsic membrane protein with a comparatively broad distribution in the cell. The synthesis of the protein appears to take place on the bound ribosomes of the endoplasmic reticulum (although this circumstance is not yet conclusively established) from which the enzyme is incorporated into the rough membrane. Here conversion of the apoenzyme to holoenzyme takes place and the enzyme is transported, presumably in the membrane, to the smooth membrane and subsequently to other intracellular organelles. All enzyme molecules leaving the smooth microsomes for susbequent appearance in other membranes, however, appear to be in the holoenzyme form.

Acknowledgement

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