ULTRASTRUCTURAL LOCALIZATION OF CYTOCHROME b_5 ON RAT LIVER MICROSOMES BY MEANS OF HYBRID ANTIBODIES LABELED WITH FERRITIN

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INTRODUCTION

Subfractionation by density equilibration and by differential sedimentation in density gradients has resolved rat liver microsomes into several populations of subcellular components characterized by specific marker enzymes (1, 7). Vesicles derived from endoplasmic reticulum (ER)[1] were found to be heterogeneous, both in their physical characteristics and in their enzyme content. On the basis of these results, ER enzymes have been classified into two groups. Group b includes cytochromes b_5 and P 450, and other oxidoreductases related to these hemoproteins; group c includes glucose 6-phosphatase, several other microsomal hydrolases, and glucuronyltransferase. With respect to enzymes of group b, those of group c sediment faster and equilibrate at higher densities in various gradients (1, 7). Furthermore, their equilibrium density is reduced more markedly by treatments which detach ribosomes from microsomal vesicles (3). Since these differences never allowed a true separation, two alternative explanations have been envisaged. Either the two groups are associated together in the same membranes, but in such a manner that the ratio of group c to group b enzymes increases with increasing ribosome load; or each group is associated with a different part of the ER, that containing group c being on an average richer in ribosomes than that containing group b. We report here the results of a cytoimmunological study showing that cytochrome b_5 (Group b) is present in essentially all microsomal vesicles derived from ER. Together with the cytoenzymological data showing the widespread distribution of glucose 6-phosphatase (group c) throughout the ER (8), these results support the existence of a single ER system in hepatocytes.

[1] Abbreviations used in this paper: ER, endoplasmic reticulum; ab_5/aF-ferritin, antigen-antibody complex between ferritin and the anticytochrome b_5/antiferritin hybrid antibody.

MATERIALS AND METHODS

Highly purified cytochrome b_5 was prepared from rat liver as described by Omura et al. (10). Analysis

by immuno-diffusion showed the preparations to be immunologically pure. Ferritin (purchased from Fluka AG, Basel, Switzerland), was recrystallized six times in the presence of $CdSO_4$ (4). Rabbit antibodies were purified by affinity chromatography on cytochrome b_5 or ferritin, coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden) by means of cyanogen bromide (5), and were eluted by 2% formic acid. Hybrid molecules anticytochrome b_5/antiferritin were made from purified antibodies by the method of Nisonoff and Palmer (9), with minor modifications (11). The hybrid antibodies were isolated by successive immunoadsorbent chromatography on Sepharose-cytochrome b_5 and Sepharose-ferritin. An antigen-antibody complex between ferritin and ab_5/aF hybrid was prepared by slowly adding a dilute solution of ab_5/aF hybrid to a rapidly mixing solution of ferritin at neutral pH. The ferritin was in a fivefold molar excess over the amount of hybrid antibody added. Excess ferritin (about 60 mg) was separated from the ab_5/aF-ferritin complex (containing 10 mg of antibody protein) by adsorption of the complex onto a 2.5 cm × 8.5 cm column of SP-Sephadex equilibrated with 0.05 M acetate, pH 4.75. After a careful washing out of unreacted ferritin, the ab_5/aF-ferritin complex was eluted from the column with 0.1 M Tris-HCl, pH 7.4. Aggregates were eliminated by centrifugation (15 min at 40,000 rpm).

Microsomes (fraction P) were prepared from rat liver (2) and subfractionated by density equilibration in a sucrose gradient (7). Unfractionated microsomes (P), a light subfraction (P_1: density 1.123–1.140), and a heavy subfraction (P_2: density 1.245–1.267) were first treated with 25 mM Na-pyrophosphate, pH 7.4, in 0.25 M sucrose for detachment of the ribosomes (12), separated by chromatography on Biogel A-150m (Bio-Rad Laboratories, Richmond, Calif.), and then incubated with ab_5/aF-ferritin for 12 h at 2°C. The hybrid reagent was adjusted to provide a sixfold excess of cytochrome b_5 binding activity over the amount of membrane-bound cytochrome b_5 present in the fraction. After incubation the excess hybrid antibodies were removed by chromatography on Biogel A-150m. Controls were handled identically, but with an ab_5/aF-ferritin complex preincubated with a 12-fold excess of purified cytochrome b_5. Electron microscopy was performed as described by Wibo et al. (13). Enzyme assays were done according to published methods, with minor modifications (6).

RESULTS

Some biochemical properties of the subcellular preparations submitted to morphological examination after reaction with the ab_5/aF-ferritin are given in Table I. The enzymatic heterogeneity of microsomes is evidenced by the 4.7-fold higher ratio of glucose 6-phosphatase to NADH cytochrome *c* reductase in P_2 with respect to P_1 fraction. The activity of alkaline phosphodiesterase I, galactosyltransferase, and monoamine oxidase indicates that P_2 fraction consists essentially of elements derived from ER, whereas ER elements are markedly contaminated by other structures in P and P_1 fractions.

TABLE I

Biochemical Composition of the Microsomes and Microsomal Subfractions

Constituent	Fraction P	Fraction P_1	Fraction P_2
Protein	18.4	11.2	4.5
Cytochrome b_5	—	13.7	2.5
Glucose 6-phosphatase	72.5	8.5	5.2
NADH cytochrome *c* reductase	62.4	16.8	2.2
Alkaline phosphodiesterase I	44.8	12.7	0.1
Galactosyltransferase	71.2	38.1	0.2
Monoamine oxidase	21.5	37.4	0.3

Values are expressed in percent; they refer to the liver content for P and to the amount relative to the parent microsome fraction for P_1 and P_2. Recoveries for the enzymes and proteins ranged from 85 to 110%.

The morphological aspect of these preparations after incubation with the ab_5/aF-ferritin is shown in Fig. 1 *a–c*, that of one control (P_2 fraction) in Fig. 1 *d*. The latter is also representative of the controls made on P and P_1 fractions. (In similar experiments not presented here, labeling in the controls was the same after incubation with aF/aF-ferritin complexes as after incubation with ab_5/aF-ferritin complexes pretreated with cytochrome b_5.) The outer surface of many profiles is dotted with ferritin grains in all tests. On a quantitative basis, 96, 81, and 73% of the profiles were found specifically labeled in P_2, P, and P_1 fractions, respectively. Unlabeled profiles were usually of smaller size than the labeled ones in P_2 fraction; the reverse relationship between size and labeling occurred in P and P_1 fractions. Fractions P and P_1 contained some open membranes and these did not react with the ab_5/aF-ferritin. Some of the negative structures were identified as deriving from the Golgi or from the plasma membrane.

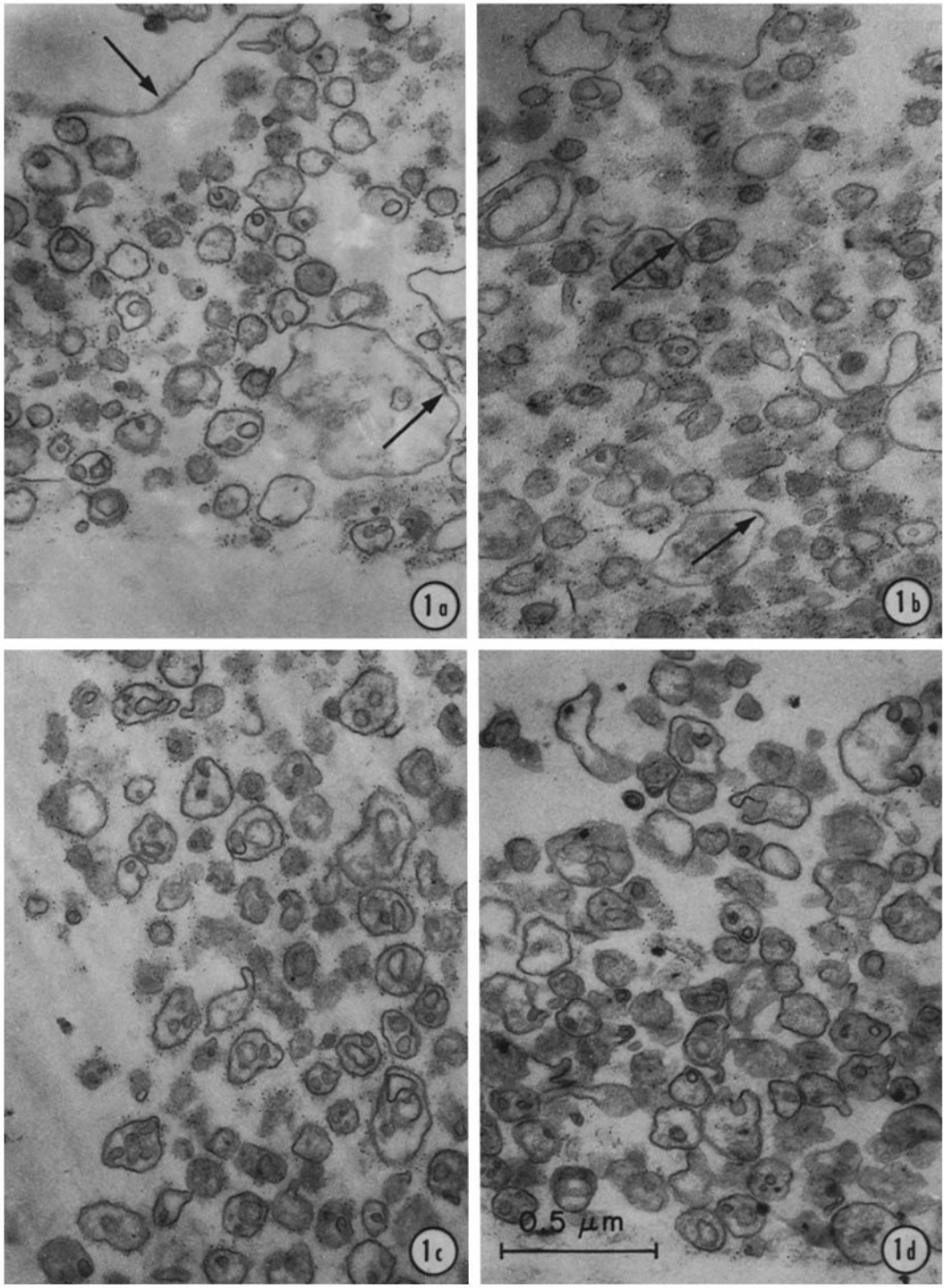

FIGURE 1. Electron micrographs of microsomes (*a*) and microsomal subfractions P_1 (*b*) and P_2 (*c*) incubated with the ab_5/aF-ferritin complex after detachment of ribosomes by Na-pyrophosphate, and (*d*) control of the P_2 subfraction. Many profiles are ferritin-free in the control. In contrast, most profiles are labeled with ferritin grains in the three test preparations, except for some large profiles and one open membrane fragment (arrows). × 47,000.

DISCUSSION

The occurrence of ferritin grains on almost all profiles in the P_2 microsomal subfraction shows that cytochrome b_5 is present in all the ER elements equilibrating between 1.245 and 1.267 in sucrose gradients. It is likely indeed that the few unlabeled profiles (4% of the total) derive from vesicles labeled in the neighboring sections. The smaller size of the unlabeled profiles is consistent with this interpretation. In view of the observations made on the P and P_1 fractions, we may extend this conclusion to the whole ER, since the proportion of unlabeled vesicles in these fractions is readily accounted for by the presence of non-ER contaminants. Detailed quantitative calculations supporting this conclusion will be presented in a subsequent publication.

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