PRESENCE OF NADPH-CYTOCHROME P-450 REDUCTASE IN RAT LIVER GOLGI MEMBRANES

Evidence Obtained by Immunoadsorption Method

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ABSTRACT

Light Golgi fractions (GF1+2) prepared from rat liver homogenates by a modification of the Ehrenreich et al. procedure (J. Cell Biol. 59:45) had significant NADPH-cytochrome P450 reductase (NADPH-cyt c reductase) activity if assayed immediately after their isolation. An antibody raised in rabbits against purified microsomal NADPH-cyt c reductase inhibited to the same extent the reductase activity of microsomal and Golgi fractions. To find out whether this activity is located in bona fide Golgi elements or in contaminating microsomal vesicles, we used the following 3-step immunoadsorption procedure: (a) antirabbit IgG (raised in goats) was conjugated to small (2–5 μm) polyacrylamide (PA) beads; (b) rabbit anti NADPH-cyt c reductase was immunoadsorbed to the antibody-coated beads; and (c) GF1+2 was reacted with the beads carrying the two successive layers of antibodies. The beads were then recovered by centrifugation, and were washed, fixed, embedded in agarose, and processed for transmission electron microscopy. Antireductase-coated beads absorbed 60% of the NADPH-cyt c reductase (and comparable fractions of NADH-cyt c reductase and glucose-6-phosphatase) but only 20% of the galactosyltransferase activity of the input GF1+2. Differential vesicle counts showed that ~72% of the immunoadsorbed vesicles were morphologically recognizable Golgi elements (vesicles with very low density lipoprotein [VLDL] clusters or Golgi cisternae); vesicles with single VLDL and smooth surfaced microsome-like vesicles were too few (~25%) to account for the activity. It is concluded that NADPH-cytochrome P450 reductase is a Golgi membrane enzyme of probably uneven distribution among the elements of the Golgi complex.

KEY WORDS anti NADPH cytochrome P450 reductase antibody - immunoadsorption - Golgi fraction - microsomal fraction - antibody-coated polyacrylamide beads

Reliable data on the biochemical composition of endoplasmic reticulum (ER) and Golgi membranes are required for any investigation of possible biogenetic interactions between these two types of intracellular membranes. The data should include a precise assessment of the degree of compositional overlap between the membranes in question. The existence of a number of enzymic activities (e.g., NADH-cytochrome b5 reductase, cytochrome b5 and 5'-nucleotidase) common to the ER and Golgi membranes is already recorded in the literature (1, 2, 9, 11, 13, 19, 20, 25), but recently obtained evidence suggests that the compositional overlap is greater than previously assumed. As reported in the preceding paper (12),
Golgi fractions exhibit relatively high glucose-6-
phosphatase (G-6-Pase) and NADPH-cyt P450 redu-
cetase activities, if assayed immediately upon
their isolation from rat liver homogenates. These
activities are rapidly lost on storage at 0°C, a
finding that explains the discrepancy between the
new results and previously published data (1).

These findings deserve to be carefully assessed,
since the enzymes mentioned are extensively used
as markers for microsomal (i.e., ER) membranes
(4, 5). With the data so far obtained (12), it
cannot be determined if the activities mentioned
are a result of bona fide enzymic components of
the Golgi membranes or to contaminating vesicles
of ER derivation, since our Golgi fractions, although
reasonably homogeneous, do contain a
small population of empty vesicles of unknown
(possibly ER) origin (6).

To resolve this alternative, we have used a
sensitive and specific immunoadsorption proce-
dure in which Golgi fractions were reacted with
polyacrylamide (PA) beads coated with two suc-
cessive layers of antibodies. The first layer con-
sisted of antirabbit immunoglobulin G (IgG)
(raised in goats), covalently linked to the surfaces
of the beads; the second layer, immunoadsorbed
to the first, consisted of antibody against rat
NADPH-cyt c reductase (raised in rabbits). For
convenience, the last antibody will be referred to
hereafter as "rabbit antireductase" or "antireduc-
tase." Morphological markers, especially the pres-
cence of clustered intravesicular very low density
lipoprotein particles (VLDL) (6), were used to
identify the intracellular origin of vesicles immu-
noadsorbed to the antireductase-coated beads.

This paper describes the preparation of antibody-
coated PA beads, and presents results which es-
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Preparation of Golgi Fractions

Golgi fractions were prepared from the livers of
ethanol-treated rats by the procedure of Ehrenreich et
al. (6), with the modifications already described in the
companion paper (12). In addition to a combined light
and intermediate Golgi fraction (GF1-2) and a residual
microsomal fraction (RdMF), a heavy Golgi fraction
(GF3) was obtained. It banded at the 0.86/1.15 M
sucrose interface in the discontinuous density gradient
used to isolate Golgi fractions from total microsomes.

Preparation of Anti-NADPH-Cyt C
Reductase IgG

NADPH-cyt c reductase was purified from trypic
digests of rat liver microsomes by the procedure of
Omura and Takesue (23). An antibody to the reductase
was raised in rabbits using 2 mg of purified enzyme as
antigen; the corresponding IgG fraction was prepared as
previously described (14). The specific antibody was
further purified by immunoabsorption on NADPH-cyt c
reductase insolubilized to Sepharose 4B (3), and was
eclut from the column with 0.2 M glycine-HCl buffer,
PH 2.8. By use of Ouchterlony's double diffusion pro-
cedure, the purified antibody was shown to be monospe-
cific, since it formed single precipitation lines with both
pure NADPH-cyt c reductase and microsomes solubi-
lized in 1% Triton X-100. Control IgGs were purified
by ammonium sulfate precipitation and O-(diethyl-
aminoethyl)cellulose chromatography (7) from sera obtained from nonimmunized rabbits.

Preparation of Antibody Conjugated Beads

PA beads of 2-5 μm diameter were used as solid
substrates for insulobilizing antibodies. Aminoethyl de-
vatives of such beads, assumed to have 30 μmol of
available terminal groups per gram, were reacted with
100-fold excess of succinic anhydride in water adjusted
to pH 6.0. After washing twice with water (by resuspen-
sion-centrifugation) to remove excess succinic anhydride,
the succinylaminoethyl PA beads were activated by the
addition of 25 μmol of the water soluble carbodimide,
1-ethyl-3(3-dimethylamino propyl) carbodiimide, per
gram beads at a pH adjusted to 4.7–5.0 with HCl. After
3-4 min, 7.5 mg (0.05 μmol) of goat antirabbit IgG
were added to the activated beads, the pH was immedi-
ately adjusted to 6.0-6.3 with NaOH, and the suspen-
sion was incubated at 4°C for 16 h with continuous
stirring (3). The antibody-conjugated beads were re-
covered by centrifugation (1,000 g × 5 min), washed
twice by suspension in 0.15 M NaCl-buffered to pH 7.5
with 10 mM Tris-HCl followed by sedimentation, and
finally resuspended in the same buffered saline. The
washed preparation contained 3 μg of goat-antirabbit
IgG/mg PA beads. Just before each immunoadsorption
experiment, PA beads conjugated with goat antirabbit
IgG were reacted with excess amounts (~20 μg protein/
mg beads) of rabbit antireductase and then washed
extensively with the same Tris-buffered saline.
Immunoadsorption of Golgi Fractions on Antibody-Coated Beads

PA beads (10-30 mg) conjugated with goat antirabbit IgG, and already reacted with either rabbit control IgG or rabbit antireductase, were incubated for 2 h at 0°C with 0.25 mg (protein equivalents) of GF1+2 or RdMF in 0.25 M sucrose-10 mM Tris-HCl buffer (pH 7.5)-0.15 M NaCl. At the end of the incubation the beads were pelleted by centrifugation (1,000 g x 7 min). The supernate was removed and used to determine nonsorbed protein and enzymic activities, and the pellet was washed twice by resuspension-centrifugation (above) and finally processed for electron microscopy as indicated below.

Specimen Preparation for Electron Microscopy

Antibody-coated PA beads, reacted with GF1+2 or RdMF and pelleted as above, were resuspended in 2 ml of incubation buffer and fixed in suspension with an equal volume of 2% glutaraldehyde-2% formaldehyde in 0.1 M cacodylate buffer, pH 7.2. After fixation for 30 min at 0°C, the beads were recovered by centrifugation at 1,000 g for 5 min, and resuspended in 3% OsO4 in the same buffer for 1 h postfixation at 0°C. At the end of this step, they were sedimented as above, washed twice with cold saline, and resuspended for embedding in 20% albumin or in 1% agarose at 60°C. The suspension was centrifuged at 8,000 g for 1 min in a microfuge (Beckman Instruments, Inc., Palo Alto, Calif.) before the agarose gelled, and the gelled pellets were cut into small blocks (~1 mm³) which were stained for 1 h at room temperature in 0.5% uranyl acetate in saline. After staining, the blocks were dehydrated in graded ethanols and embedded in Epon.

Aliquots of the original cell fractions and of the supernates obtained in the immunoadsorption experiments were processed for transmission electron microscopy by standard procedures (26).

The embedded blocks were sectioned with a diamond knife on a MT2 ultratome (Ivan Sorvall, Inc., Newtown, Conn.); the sections were stained with uranyl acetate and examined and micrographed in a Siemens 102 or a JEOL 100CX electron microscope.

Analytical Procedures

The assays used for protein and for NADPH-cyt c reductase, NADH-cyt c reductase, NADH-ferricyanide reductase, G-6-Pase and galactosyltransferase activities are given in the companion paper (12).

RESULTS

GF1+2 and GF3 and the corresponding RdMF were assayed for the enzymic activities listed in Table 1. G-6-Pase and NADPH-cyt c reductase activities were determined soon upon the isolation of the fractions, since they are rapidly inactivated by storage at 0°C. In agreement with the results given in the companion paper (12), we found that the specific activities of these two enzymes in GF1+2 were considerably higher than reported in the literature (1, 19); they amounted to ~15% (for G-6-Pase) and ~20% (for NADPH-cyt c reductase) of the corresponding figures for the residual microsomal fraction when assayed 3 h after the isolation of the fractions. The specific activities of the same enzymes were twice as high in GF3. For comparison, Table I includes data on NADH-cyt c reductase and NADH-ferricyanide reductase; the corresponding activities are generally assumed to be present in Golgi fractions (1, 2, 11, 13) and are known to be less susceptible to inactivation on storage (12). In GF1+2, their specific activities amounted to ~48% and ~53%, respectively, of the corresponding values in RdMF.

An antibody raised in rabbits against rat microsomal NADPH-cyt c reductase (purified as in Materials and Methods) was found to inhibit the corresponding reductase activity to the same extent in GF1+2 and GF3 as well as in RdMF (Fig. 1), indicating that the enzyme is immunologically identical in the three fractions, and that its active site is accessible on the cytoplasmic side of the vesicles that constitute all these fractions. An asymmetric positioning of the enzyme has already been established for microsomal membranes (13, 14, 15, 21). Our findings establish that a similar asymmetry applies for Golgi membranes.

The level of G-6-Pase and NADPH-cyt c reductase found in GF1+2 appeared too high to be ascribed to the questionable and, in any case, limited contamination of the fraction by ER-derived vesicles. Instead, the findings suggest that the two enzymes are present in Golgi elements as indigenous components of Golgi membranes, but direct evidence was obviously needed to test the suggestion. This kind of evidence could be obtained in the case of NADPH-cyt c reductase by the immunoadsorption procedure described in Materials and Methods.

The experiments were carried out according to the following protocol. PA beads conjugated in a first step to goat antirabbit IgG were reacted in a

3 NaCl was found to minimize nonspecific adsorption of subcellular components to beads.
TABLE I

Specific Enzyme Activities in Golgi and Residual Microsomal Fractions Isolated from Rat Livers

| Enzyme activities          | GF_{1+2} | GF_{2} | RdMF  |
|----------------------------|----------|--------|-------|
| Glucose-6-phosphatase$ (5) | 70 ± 23  | 153 ± 29| 458 ± 39|
| NADPH-cytochrome c reductase$ (7) | 37 ± 10 | 83 ± 8 | 170 ± 21|
| NADH-cytochrome c reductase$ (6) | 302 ± 45| 436 ± 37| 627 ± 52|
| NADH-ferricyanide reductase$ (5) | 2.18 ± 0.20| 2.70 ± 0.38| 4.04 ± 0.52|

* Number of experiments in parentheses.
$ nmol/min/mg protein
$ µmol/min/mg protein

When residual microsomes were reacted with antireductase-coated beads, only ≤10% of the NADPH-cyt c reductase was found in the supernate after sedimenting the beads. In control experiments (PA beads covered with nonspecific rabbit IgG), practically all NADPH-cyt c reductase activity remained in the supernate; loss by nonspecific adsorption to the PA beads was negligible (≤5%). When GF_{1+2} was reacted to antireductase-coated PA beads (Table II), 35–40% of the NADPH-cyt c reductase remained in the supernate and, by difference, 60–65% was immunoadsorbed to the beads. Nonspecific adsorption increased slightly (to ≤10%) for reasons that remain to be investigated. Morphological evidence (see below) suggested that the stickiness of VLDL particles liberated from broken Golgi vesicles might explain this increased nonspecific adsorption. Additional control experiments indicated that the specific adsorption of RdMF and GF_{1+2} elements to coated beads involved the interaction of membrane-bound NADPH-cyt c reductase with bead-bound antireductase.

The data in Table II shows that comparable fractions (35–40%) of the NADH-cyt c reductase and G-6-Pase activities of the input GF_{1+2} remained in the supernate in the experiments outlined, together with 75–80% of the protein. In contrast, ~80% of the galactosyltransferase activity of the input GF_{1+2} was not sedimentable at the end of the incubations. In most cases, nonspecific adsorption was <15% of the input activities. Taken together, the data suggest that the enzymes tested are differentially distributed among or within Golgi elements.

The samples processed for electron microscopy...
showed that the majority of the GFI+2 elements attached to antireductase-coated beads were identifiable as bona fide Golgi vesicles or vacuoles (Fig. 2), based on their size and shape and especially on the clusters of VLDLs they contained (Fig. 3). Differential counts (Table III) indicated that ~58% of the immunoadsorbed elements were profiles of large or medium sized Golgi vesicles loaded with VLDLs; ~16% appeared to be profiles of small vesicles or tubules marked by a single VLDL; ~11% were profiles of vesicles of varied size (mostly small) which did not contain VLDLs, and ~7% could not be ascribed to any one of these categories, because of extensive damage or unfavorable geometry (grazing sections of vesicles). The number of Golgi cisternae adsorbed to the antireductase-coated PA beads was small (14%), although the density of such cisternae (no./U vol) was high in both GFI+2 input and supernate in these immunoadsorption experiments. Free VLDLs were encountered in small numbers either attached to the outside (cytoplasmic) surface or adsorbed vesicles or bound directly to the surface of antibody-coated beads. PA beads coated with nonspecific rabbit IgG adsorbed very few vesicles (Fig. 2b), with a slight preference (over experimental) for vesicles containing no VLDLs.

| Antibody          | NADPH-cyt. c red. | NADH-cyt. c red. | G-6-Pase | Gal-transferase | Protein |
|-------------------|-------------------|------------------|----------|----------------|---------|
| Exp 1 Control     | 93.4              | 82.0             | -        | 85.4           | 93.8    |
| Antireductase     | 35.8              | 41.6             | -        | 81.5           | 77.5    |
| Exp 2 Control     | 90.7              | 86.2             | 78.1     | 91.6           | 88.0    |
| Antireductase     | 39.3              | 35.5             | 33.5     | 75.5           | 68.3    |

These values are taken from nine separate experiments. All figures represent % activities recovered in the supernate relative to those present in the original samples.

**Table II**

*Immunoadsorption of GFI+2 Elements on Antireductase Coated Beads; Distribution of Nonsedimentable Enzyme Activity*

![Figure 2a and b](image-url)

**Figure 2 a and b** Electron micrographs of light Golgi fraction incubated with antibody-conjugated beads. (a) GFI+2 vesicles immunoadsorbed to the surface of a PA bead coated with NADPH-cyt c reductase. Specimens embedded in albumin. The adsorbed vesicles from a quasi-continuous monolayer at the bead’s surface with only a few vesicles (arrows) adsorbed, as a second layer to the first. × 14,000. (b) GFI+2 vesicles adsorbed to the surface of a PA bead coated with nonspecific rabbit IgG (control) specimen embedded in agarose. Only a few vesicles appear attached to the bead’s surface. × 14,000.
FIGURE 3  Gallery of GF1+2 vesicles immunoadsorbed to the surface of antireductase coated PA beads. It illustrates the categories of vesicles described in the text and listed in Table III. Golgi cisternae in transverse sections = gcx; same in perpendicular sections: gcz; same obliquely sectioned gcα. Golgi vesicles containing clusters of VLDLs: gv (some of them are partially emptied in b and c; vesicles marked by a single VLDL: sl; smooth vesicles or tubules without VLDL: sv; unidentified elements (grazing sections): nl; unidentified elements on account of extensive damage: n2. In b, gc, marks a section through two superimposed Golgi cisternae: a, × 25,000; b, × 29,000; c, × 21,000; d, × 24,000.

TABLE III

| Type of vesicle                  | Control % | Experimental % | Experimental corrected |
|---------------------------------|-----------|----------------|------------------------|
| Golgi/ER smooth vesicles (no VLDL) | 24.6      | 17.0           | 11.7                   |
| Golgi/ER vesicles (1 VLDL)    | 20.0      | 18.3           | 16.6                   |
| Golgi vesicles (>1 VLDL)       | 30.7      | 42.4           | 58.5                   |
| Golgi cisternae                | 9.1       | 11.3           | 14.0                   |

For experimental (antireductase coated beads), 774 vesicles (and 61 free VLDLs) were counted on 24 beads. For Controls, the corresponding figures were 65 and 10 on 10 beads. In each case, the balance (to 100%) was accounted for by nonidentifiable profiles, mostly grazing sections of vesicles of all sizes. Each experimental figure was corrected by multiplying it with the factor experimental/control for the corresponding type of vesicle.

DISCUSSION

Two aspects of the work reported in this paper will be discussed. The first concerns the methodology, the second relates to the findings themselves.

Method

We have adapted the general procedure of affinity separation (3) by batch chromatography to a specific problem: the identification of the intracellular origin of vesicles that have a certain enzymic activity, i.e., NADPH-cyt c reductase. We have used the purified enzyme as an antigen and its antibody as a specific ligand. Since the results were to be assessed by electron microscopy, we searched for a solid substrate of appropriate shape, size, and surface characteristics that could withstand, without breakage or extensive alterations, the many preparatory steps involved in immunoadsorption and electron microscopy. We tested agarose-, polystyrene-, and different types of polyacrylamide beads and consistently obtained the best results with succinylaminoethyl derivatized PA beads.

We used an indirect immunochemical procedure in which antirabbit (goat) IgG covalently bound to PA beads was reacted with purified (rabbit) antireductase before interacting the com-
plex with the subcellular components of interest. This protocol has a number of advantages: (a) There is no loss in antireductase potency, since this antibody is not exposed to the conditions and reactions involved in conjugation to the bead’s surface. (b) Unbound antireductase can be efficiently and rapidly removed before reacting the beads with cell fractions, thus minimizing or preventing the aggregation of subcellular components in the medium and on the beads (reacting the antireductase to the cell fractions before reacting the resulting complexes to the PA beads [coated with antirabbit IgG] leads to extensive aggregation of either microsomal or Golgi vesicles). (c) After the last step of the immunoadsorption procedure the beads can be rapidly sedimented and washed free of unbound subcellular particles. (d) The insolubilized antirabbit IgG and the antireductase immunoadsorbed to it act as a sufficiently long arm to facilitate the attachment of relatively large vesicles to the PA beads.

With this protocol, satisfactory results can be obtained in a reasonably short time. Under optimal conditions, the beads are covered with a monolayer of individually adsorbed subcellular components. Their identification and quantitation of the results are much easier than in a simple immunoprecipitate. Embedding the beads in a coherent matrix facilitates handling and prevents losses during the rest of the processing. Of the media tested for embedding (agarose and different concentrations of albumin), agarose gave the best background and contrast conditions for electron microscopy (Fig. 2a and b).

Affinity separation by immunoadsorption (or other means) is a promising approach to cell fractionation. It has already been used to isolate subcellular components that bear a common antigen (17). And, in addition to the application discussed in this paper, it can be applied to the purification of cell fractions, the isolation and identification of particulate contaminants, and the subfractionation of preparations which may appear homogeneous by less specific criteria.

**Findings**

According to our biochemical data (Table II), 60–65% of the NADPH-cyt c reductase of GF1+z could be adsorbed on the surface of antireductase-coated beads, together with 20–30% of the fraction’s protein. Since in the case of antibody-antigen complexes we are dealing with equilibrium reactions, the percentage of GF1+z vesicles carrying the NADPH-cyt c reductase antigen is expected to be considerably higher (see, for instance, the degree of reductase inhibition by its antibody in Fig. 1).

By electron microscopy, ~58% of the GF1+z vesicles adsorbed to the surface of the antireductase-coated beads were found to be large vesicles with a dense content that included clusters of VLDLs. On account of these features, these elements were reliably identified as Golgi vesicles derived from either the trans side of the Golgi complex or the dilated rims of Golgi cisternae. Since Golgi cisternae account for ~14% of the immunoadsorbed particles, the percentage of reliably identified Golgi elements amounts to ~72% of the total population of vesicles bound to the beads. Small vesicles (or tubules) marked by single VLDL particles accounted for ~16% of the total population of GF1+z elements immunoadsorbed to PA beads and the corresponding value for vesicles without VLDLs was ~11%. Such elements are either peripheral Golgi vesicles or vesicles derived from the adjacent smooth ER. Even if all of them were of ER origin, their aggregate mass would not be sufficient to account for the reductase activity of GF1+z. Reductase specific activity is normalized to protein, and in our immunoadsorption experiments the closest approximation to protein content is volume. The contribution of small vesicles (with or without VLDL) to the reductase activity of GF1+z must be considerably smaller than ~26% since their average diameter is ~2–3 times smaller than those of Golgi vesicles marked with VLDL clusters.

Taken together, the biochemical data and the morphologic evidence indicate that the NADPH-cyt c reductase activity of this fraction is a result of enzyme indigenous to Golgi membranes, not to ER contaminants. This conclusion is in agreement with the degree of morphological homogeneity of GF1+z and with the instability of its reductase activity (12). The latter finding implies that the conditions to which the enzyme is exposed in Golgi fractions are different from those prevailing in microsomes.

Within the vesicle population of GF1+z, our immunoadsorption procedure distinguishes two groups. The first (~20% adsorbed) carries galactosyltransferase activity, whereas the second (~65% adsorbed) has NADH-cyt c reductase—and G-6-Pase—in addition to NADPH-cyt c reductase activity (Table II). Preliminary data obtained on the same fraction immunoadsorbed to PA beads coated with anticytochrome b5 IgG indicate that cytochrome b5 should be added to
the second group. The data suggest that the Golgi elements are functionally heterogeneous, but at present it is not possible to establish a morphological correlate to the type of heterogeneity revealed by our findings. Tentatively it can be assumed that the first group is represented by Golgi cisternae, primarily because they are underrepresented among the vesicles adsorbed to antireductase-coated beads. Heterogeneity of Golgi elements on other grounds than those mentioned above is already a well established concept (cf. reference 8).

Our data extend the list of enzyme activities shared in common by ER and Golgi elements to include NADPH-cyt c reductase and probably G-6-Pase; in addition, they strengthen the case for the presence of NADH cyt c reductase, and cytochrome b₅ in Golgi elements. Normalized to total protein content of the fractions, these activities appear less concentrated in Golgi fractions than in microsomes, but normalization to membrane lipids may alter the difference. Choline phosphotransferase and acyl-CoA:1,2-diacyl-sn-glycerol acyltransferase were previously considered as microsomal markers (27), but recently it was claimed that they are also present in Golgi fractions (16). The list of Golgi markers appears to be restricted at present to the enzymes involved in the biosynthesis of sulfatides (10) and in the glycosylation of glycoproteins and glycolipids (e.g., galactosyl- (1, 9, 19, 25), fucosyl- (24), and sialyltransf erase(18, 29). The evidence we have obtained does not actually challenge the concept of marker enzymes, it contributes data towards a more general correlate to the type of heterogeneity revealed by our findings. Tentatively it can be assumed that Golgi apparatus-rich fraction of liver.

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