

LOCALIZATION AND BIOSYNTHESIS OF NADH-CYTOCHROME b_5 REDUCTASE, AN INTEGRAL MEMBRANE PROTEIN, IN RAT LIVER CELLS

II. Evidence That a Single Enzyme Accounts for the Activity in Its Various Subcellular Locations

J. MELDOLESI, G. CORTE, G. PIETRINI, and N. BORGESE

From the Department of Pharmacology and the CNR Center of Cytopharmacology, University of Milan, 20129 Milan, Italy, and the Department of Biochemistry, University of Genoa, Genoa, Italy

ABSTRACT

NADH-cytochrome b_5 reductases of rat liver microsomes, mitochondria, and heavy and light Golgi fractions (GF_3 and GF_{1+2}) were compared by antibody inhibition and competition experiments, by peptide mapping, and by CNBr fragment analysis. The water-soluble portion of the microsomal enzyme, released by lysosomal digestion and purified by a published procedure, was used to raise antibodies in rabbits. Contaminant antimicrosome antibodies were removed from immune sera by immunoabsorption onto the purified antigen, and the $F(ab')_2$ fragments of the pure antireductase antibody thus obtained were found to inhibit the NADH-cytochrome c reductase activity equally well in the four membrane fractions investigated, with similar dose-response relationships. Moreover, the purified water-soluble fragment of microsomal reductase, which by itself is very inefficient in reducing cytochrome c , competed for antibody binding with the membrane-bound enzymes, and therefore prevented the inhibition of their activity, not only in microsomes but also in the other fractions. The reductases isolated from detergent-solubilized microsomes, mitochondria, GF_3 , and GF_{1+2} by immunoabsorption had identical mobilities in SDS polyacrylamide gels. The corresponding bands were eluted from gels, fragmented with pepsin or CNBr treatment, and the two families of peptides thus obtained were analyzed by two-dimensional mapping and SDS polyacrylamide gel electrophoresis, respectively. Both analyses failed to reveal differences among reductases of the four fractions. These findings support the hypothesis that NADH-cytochrome b_5 reductase in its various subcellular locations is molecularly identical.

NADH-cytochrome b_5 reductase is an integral membrane protein composed of a hydrophilic portion, which contains the active site of the enzyme and is exposed at the cytoplasmic surface of the membrane, and a smaller hydrophobic sequence, which anchors the protein to the mem-

brane (6, 13, 18). This hydrophobic portion is located toward the C terminus (14) and can be cleaved from the hydrophilic moiety by treatment with lysosomal cathepsin D (13, 18, 21). The resulting water-soluble fragment has been termed lysosome-solubilized reductase (l-reductase), while the detergent-solubilized form (d-reductase) corresponds to the entire molecule (13).

In the first article of this series (4), we confirmed and extended previous reports that NADH-cytochrome b_5 reductase is distributed in several subcellular compartments: the endoplasmic reticulum, (ER),¹ the mitochondrial outer membrane, and the Golgi complex. Because of its multiple intracellular distribution, NADH-cytochrome b_5 reductase might be expected to be a useful tool for the study of biogenetic relationships among membranes. However, a necessary prerequisite of such studies is the demonstration that the enzyme activity in its various locations is due to a single enzyme protein. Data in the literature on activities with multicompartment distribution indicate that this is not necessarily the case. The microsomal and lysosomal forms of β -glucuronidase, although characterized by considerable kinetic and immunological similarities, have been reported to be different in both carbohydrate and amino acid composition (22). Even larger differences have been observed in the case of cytochrome b_5 because a form of this enzyme, localized in the mitochondrial outer membrane, is distinguishable from the microsomal form in spectral and immunological properties as well as in primary structure (7). Spectral and acceptor specificity peculiarities have been reported for liver mitochondrial cytochrome P_{450} with respect to its microsomal counterparts (23), and the lysosomal, Golgi, and cytosolic forms of α -D-mannosidase have been found to differ greatly in several physicochemical, kinetic, and immunological features (16). In the case of rat liver NADH-cytochrome b_5 reductase, kinetic and immunological studies have revealed a considerable similarity between the microsomal

¹ Abbreviations used in this paper: BAWP, butanol:acetic acid:water 1:10:89; DOC, sodium deoxycholate; ER, endoplasmic reticulum; F(ab')₂, divalent antibody fragments prepared by pepsin digestion; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; l-reductase, water-soluble fragment of NADH-cytochrome b_5 reductase cleaved by lysosomal enzymes; d-reductase, detergent-solubilized NADH-cytochrome b_5 reductase; SDS, sodium dodecyl sulfate.

and mitochondrial forms (10, 20). Moreover, our preliminary immunological findings suggest that the same might also be true of the enzyme localized in the Golgi complex (3). Thus, it seems possible that, in this case, the enzyme activity in its various subcellular locations is due to a single protein. In the present study, this hypothesis has been put to further test by carrying out an immunological study with a purified and well-characterized antibody and by a structural comparison of the variously located reductases by analysis of peptides obtained by pepsin or CNBr treatment.

MATERIALS AND METHODS

General

Sucrose solutions and apparatus were the same as described in the first article of this series (4). Other solutions indicated in abbreviated forms are: BAWP, *n*-butanol:acetic acid:water:pyridine, 7.5:6:6:5; PBS, phosphate-buffered saline; Pyridine-acetic acid buffer, pyridine:acetic acid:water, 1:10:89; Tris-barbital-glycine buffer, 31.5 mM sodium barbital, 5.6 mM barbital, 370 mM glycine, 186 mM Tris.

Cell Fractionation

Preparation of microsomes, mitochondria, and Golgi fractions were carried out as described in the first article of this series (4), but the bands collected from the discontinuous sucrose gradient were diluted with 2 vol of 3 mM imidazole-HCl, pH 7.4, instead of water. Cell fractions were resuspended in small volumes of buffered 0.25 M sucrose.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE of TCA precipitates or of nonprecipitated material was carried out on 1-mm-thick slabs or in 5 × 120-mm tubes essentially as described by Maizel (12). Samples were solubilized by boiling for 2 min with 2 vol of a mixture containing 4% SDS, 0.8 M sucrose, 8% mercaptoethanol, 20 mM Tris-phosphate buffer, pH 6.7, and 0.0005% bromophenol blue. Before solubilization, TCA precipitates were washed with 1% TCA. If the samples turned yellow after addition of the solubilization mixture, 3 M Tris was added in microliter portions until they turned blue. After electrophoresis, gels were stained with Coomassie Brilliant Blue or dried and autoradiographed.

Purification of NADH-Cytochrome b_5 Reductase

Rat liver microsomal NADH-cytochrome b_5 reductase, solubilized by lysosomal digestion, was purified starting with 500–1,000 g of liver by the method of Takesue and Omura (19), but Sephadex A-50 was used instead of diethylaminoethyl cellulose. After dialyzing away salts, the enzyme was lyophilized and stored at -20°C . A 550-fold purification of NADH-FeCN reductase activity as compared with the starting MR was obtained, with 15–20% recovery of the activity.

To further purify the reductase, in some preparations the material enriched with enzyme collected from the Sephadex A-

50 column was taken with an Amicon ultrafiltration apparatus (with a PM-10 filter) (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) to a protein concentration of 5 mg/ml, and 1 ml was loaded onto a Sephadex G-100 column (1 × 50 cm) equilibrated with 20 mM Tris-HCl, pH 8.5. The enzyme prepared with this additional gel filtration step will be referred to as "purified l-reductase" (Fig. 1), and the more contaminated enzyme carried only through the Sephadex A-50 step will simply be called "l-reductase."

Immunization of Rabbits

Rabbits were immunized with l-reductase by four successive injections at weekly intervals. The first two (1 mg) were emulsified in complete Freund's adjuvant and administered subcutaneously in the back by multiple injections. The third and fourth injections (0.2 mg) were intravenous.

At intervals of 15 d, boosters consisting of 50 µg of l-reductase were administered intravenously, and blood was collected 5 d later.

Purification of Antireductase IgG or F(ab')₂ by Affinity Chromatography

l-reductase or purified l-reductase was immobilized to CNBr activated Sepharose 4B (0.6 g per mg of enzyme). Under these

conditions, essentially all the enzyme was coupled, as shown by the lack of NADH-FeCN reductase activity in the supernate after sedimentation of the reacted Sepharose beads. The reductase-Sepharose conjugate was packed in columns and stored at 4°C in PBS that contained 0.02% NaN₃.

To purify antireductase antibodies, an amount of rabbit antiserum approaching the total capacity of the reductase-Sepharose conjugate was loaded on the columns, which were then incubated in an end-over-end mixer at 4°C for 2 h. The columns were then placed upright and washed with PBS until the absorbance at 280 nm of the eluate fell to <0.05. Immunoabsorbed antibodies were detached with 0.2 M glycine-HCl buffer, pH 2.2. The antibody-containing fractions were immediately neutralized with 1 M K₂HPO₄, concentrated to 2–3 mg/ml, and equilibrated in PBS that contained 0.02% NaN₃ by means of an Amicon ultrafiltration apparatus (PM-10 filter). After use, columns were neutralized with PBS and reused indefinitely.

For purification of bivalent F(ab')₂ antireductase (9), the antiserum was brought to pH 4 by the addition of 50% acetic acid. Pepsin (7.5 mg/10 ml of antiserum) was added, and the antiserum was incubated for 12 h at 37°C, neutralized by the addition of 1 N NaOH, cleared by centrifugation (10,000 rpm, 10 min, 40 rotor), and subjected to affinity chromatography as described above, with the purified l-reductase-Sepharose 4B conjugate as immunoabsorbent.

Isolation of d-Reductase from Membrane Fractions by Affinity Chromatography

Antireductase antibodies, partially purified by affinity chromatography with the l-reductase immunoabsorbent, were immobilized to CNBr-activated Sepharose 4B (8 mg of antibody/g of dry Sepharose). Measurement of the protein content of the supernates after incubation indicated that essentially all the antibody was bound. The antibody-Sepharose conjugate was packed in columns and stored as described above.

To extract reductase from cell fractions (MR, mitochondria, GF₃, and GF₁₊₂), membranes were solubilized by the addition of sodium deoxycholate (DOC), Triton X-100, and NaCl to final concentrations of 0.5, 2, and 0.9%, respectively. After the samples had been cleared by ultracentrifugation (30,000 rpm, 40 min, 40 rotor), NADH-FeCN reductase assays demonstrated that >90% of the activity was solubilized. The samples were loaded onto immunoabsorbent columns preequilibrated with PBS that contained DOC-Triton X-100 at the concentrations described above. After washing, the DOC and Triton were replaced with 0.2% Lubrol PX (which is soluble at low pH). The columns were then eluted with glycine-HCl buffer (0.2 M, pH 2.2) that contained 0.2% Lubrol. The protein-containing fractions were neutralized with 1 M K₂HPO₄ and pooled, and the Lubrol was partially eliminated by dialysis for 48 h against 60% ethanol using dialysis tubing with a 12,000 mol wt cutoff. After the ethanol was removed by dialysis against water, the protein was precipitated by the addition of TCA (final concentration 10%).

In the experiments reported in this paper and in the third paper in this series,² the same batch of immunoabsorbent, characterized by a reductase binding capacity of 25 µg of reductase/



FIGURE 1 SDS-PAGE of purified l-NADH-cytochrome b₅ reductase isolated from a lysosomal digest of rat liver microsomes. 40 µg of the purified enzyme preparation were electrophoresed onto 10% polyacrylamide cylindrical gels. Gels were stained with Coomassie Brilliant Blue.

² Borgese, N., G. Pietrini, and J. Meldolesi. 1980. Localization and biosynthesis of NADH-cytochrome b₅ reductase, an integral membrane protein, in rat liver cells. III. Evidence for the independent insertion and turnover of the enzyme in various subcellular compartments. *J. Cell Biol.* In press.

mg of conjugated antibody, was used. The capacity of the columns was determined by measuring the NADH-FeCN reductase activity of the material not bound by the immunoabsorbents. The enzyme eluted from the column by the pH 2.2 buffer could not be assayed because it was irreversibly inactivated by the low pH.

Peptide Mapping of Iodinated Reductase

TCA precipitates prepared as described above were resuspended in PBS that contained 2% SDS and labeled with ^{125}I by the chloramine-T procedure (8). The iodinated proteins were separated by SDS-PAGE. The position of d-reductase was determined by autoradiography, and the corresponding band was eluted by incubation of gel fragments in small volumes of PBS-0.1% SDS for 2 d (two buffer changes) and precipitated with 12% TCA in the presence of 50 μg of cold bovine serum albumin. The precipitate was washed twice with acidified acetone, dried under vacuum, suspended in 100 μl of formic acid-acetic acid buffer, pH 2.1, and digested with pepsin (2%, wt/wt) for 18 h at 37°C. The digested material was dried under vacuum, resuspended in a small volume of pyridine-acetic acid buffer, pH 3.5, and cleared by centrifugation in a Beckman Microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Fingerprints were obtained by the method of Mole (15) as modified by Corte et al. (5). 50,000–100,000 cpm of each experimental digest were spotted on silica gel plates side by side with a similar amount of purified l-reductase standard, iodinated, and processed in parallel. After electrophoresis in pyridine acetate buffer, pH 3.5, (300 V for 6 h), the plate was cut in half and chromatography was carried out at right angles in BAWP. The plates were then dried and autoradiographed on Kodak RPX Omat films.

Analysis of CNBr Fragments of Iodinated Reductase

Samples of d-reductase, prepared, iodinated, purified, and TCA-precipitated as described above, were dissolved in 60 μl of 70% formic acid and treated with CNBr as reported by Lai (11). After reaction at room temperature for 22 h the samples were diluted to 1 ml with distilled water, and CNBr was removed by repeated evaporation. The samples were then resuspended in 5% SDS and divided into two aliquots, to one of which was added the complete solubilization mixture for SDS-PAGE (described above). To the other, the same mixture without β -mercaptoethanol was added. Reduced and nonreduced samples were analyzed on 15% SDS polyacrylamide slab gels. A sample of microsomal l-reductase was processed and analyzed in parallel to d-reductases.

Crossed Immunoelectrophoresis

Antibody preparations were analyzed by crossed immunoelectrophoresis in the presence of 1% Lubrol (1, 2). MR (~1 mg of protein), solubilized with 2% Lubrol and cleared by ultracentrifugation, were supplemented with 5–10 μg of soluble l-reductase and loaded onto 10 \times 10-cm, 1% agarose gels made up in Tris-barbital-glycine buffer containing 1% Lubrol. Electrophoresis in the first dimension was carried out for 1.5 h at 250 V in a horizontal electrophoresis apparatus with water cooling. Strips (5 \times 2 cm) containing the electrophoresed samples were cut and transferred to 5 \times 7-cm glass plates. The remaining 5 \times 5-cm area was covered with gel that contained antibody and 1% Lubrol. After electrophoresis in the 2nd dimension (100 V, overnight), the gels were thoroughly washed with PBS, and

reductase-containing immunoprecipitate arcs were revealed by the NADH-dependent reduction of tetrazolium blue according to the procedure of Raftell and Perlmann (17). Finally, the gels were washed in water, dried, and stained with Coomassie Brilliant Blue.

Enzyme Assays

Assays of NADH-FeCN reductase and rotenone-insensitive NADH-cytochrome *c* reductase activities and protein determination were carried out as described in the first article in this series (4).

For antibody inhibition experiments, the amount of cell fraction necessary for the enzyme assay was incubated in a small volume of PBS (20–30 μl) with antireductase F(ab')₂ and, in some experiments, with purified l-reductase. After incubation for 10 min at room temperature, the enzyme assay mixture was added, and rotenone-insensitive NADH-cytochrome *c* activity was immediately assayed. Further details are given below and in the figure legends.

Materials

In addition to the materials listed in the first article, the following reagents were purchased from the indicated sources: agarose for gel electrophoresis, Marine Colloids, Inc., Rockland, Maine; pepsin, Worthington Biochemical Corp., Freehold, N. J.; tetranitrotetrazolium blue chloride: Fluka AG, Buchs, Switzerland; SDS, Sigma Chemical Co., St. Louis, Mo.; acrylamide, bisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine, Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.

RESULTS

Production and Purification of Antireductase Antibodies

Immunization of rabbits with preparations of l-reductase purified by the original method of Takesue and Omura (19) yielded antisera directed not only against the enzyme but also against various other microsomal constituents. These contaminating antibodies were not always adequately revealed by the Ouchterlony double diffusion test (3). However, when crossed immunoelectrophoresis against a total MR Lubrol extract enriched in l-reductase was used, several precipitation arcs were consistently revealed in addition to the one stained specifically for the enzyme by reduced tetrazolium blue (Fig. 2A). Although the immune response of the various rabbits tested varied considerably (with many animals producing only low-titer antisera and a few giving better or even high responses [Fig. 2]), contaminant antibodies were always present. At least one contaminant antibody was also found when the purified l-reductase, which appears to be nearly 100% pure (Fig. 1), was injected into rabbits in very low amounts (a total of 150 μg distributed over four injections) to reduce to a minimum the dose of contaminants.

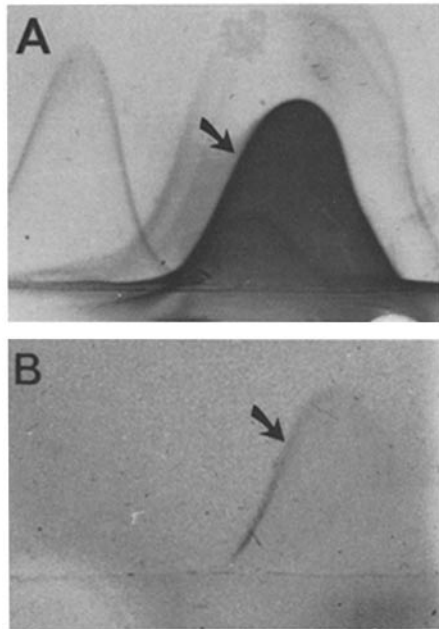


FIGURE 2 Crossed immunoelectrophoresis of antireductase antibodies against Lubrol-solubilized MR with added l-reductase. A Lubrol extract of MR (A, 0.67 mg of protein; B, 0.41 mg of protein) was mixed with l-reductase (A, $\sim 12 \mu\text{g}$; B, $\sim 4 \mu\text{g}$) and electrophoresed in the first dimension. The separated antigens were then electrophoresed into the antibody-containing gel (A, 3.5 ml of 1.1% agarose-1% Lubrol with 250 μl of whole antireductase antiserum; B, 3.5 ml of 1.1% agarose-1% Lubrol with 150 μl of antireductase antibodies [0.3 mg/ml] purified by immunoabsorption against purified l-reductase). Material was electrophoresed toward the anode because preliminary experiments showed that no precipitation arcs were formed when electrophoresis was performed in the opposite direction. The arrow indicates the tetrazolium blue-positive arc. The other arcs were revealed by Coomassie Brilliant Blue staining. The difference in the position of the reductase arc in A and B is only apparent and is the result of slightly different conditions during electrophoresis in the first dimension.

One must therefore conclude that the contaminants of the l-reductase preparation are highly immunogenic when compared with the enzyme itself.

To obtain a pure antibody, we attempted to use as antigen the material eluted from the l-reductase band of SDS-PAGE slabs. However, this approach failed because no antireductase antibody was produced by any of the five rabbits injected with the SDS-treated material.

Because these attempts to obtain pure antibodies

directly from immunized rabbits were unsuccessful, heterogeneous antibody preparations were purified by immunoabsorption. When the antiserum whose immunoelectrophoretogram is illustrated in Fig. 2A was passed through an affinity column bearing immobilized purified l-reductase, the contaminating arcs disappeared from the crossed immunoelectrophoretogram, which revealed only a reductase positive arc. Preparations such as the one illustrated in Fig. 2B, pure within the sensitivity of our analyses, were used for the inhibition and competition experiments. However, to prevent the possibility of unspecific binding of antibodies by their Fc fragments, antisera were subjected to pepsin proteolysis and the resulting antireductase $\text{F}(\text{ab}')_2$ fragments were purified by immunoabsorption.

Antibody Inhibition of NADH-Cytochrome *c* Reductase Activity

The $\text{F}(\text{ab}')_2$ antibody fragments, purified as described in the preceding section, were used to inhibit NADH-cytochrome *c* reductase activity in the various subcellular fractions. Fig. 3 shows an experiment in which aliquots of fresh MR, mitochondria, GF_3 , and GF_{1+2} fractions exhibiting the same total rotenone-insensitive NADH-cytochrome *c* reductase activity were preincubated with increasing amounts of the $\text{F}(\text{ab}')_2$ preparation before the enzyme assay was carried out. At equal antibody:enzyme activity ratios the same degree of inhibition was induced in the four fractions, suggesting that the activity might be due to one single enzyme. However, the possibility existed that the results were due not to the equal susceptibility to the same antibody of the enzyme in the various membranes but to the contamination of the $\text{F}(\text{ab}')_2$ preparation with trace amounts of antibodies against the mitochondrial and Golgi enzymes. These antibodies, possibly because of a higher affinity, could effect a specific inhibition of their antigens with dose-response curves apparently identical to that of the microsomal enzyme.

To test this possibility, we carried out the competition experiments summarized in Fig. 4 using the purified microsomal l-reductase to compete for antibodies with the membrane-bound reductase. In these experiments, we took advantage of the fact that l-reductase, because it is not membrane-bound, is so inefficient in reducing cytochrome *c* (13) that, at the concentration used, its NADH-cytochrome *c* reductase activity was undetectable

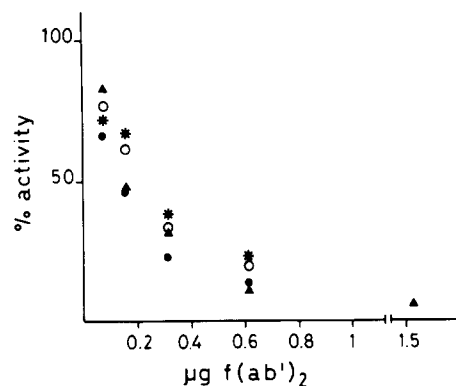


FIGURE 3 Effect of increasing amounts of antimicrosomal reductase antibodies on the NADH-cytochrome *c* reductase activity of fixed amounts of microsomes, mitochondria, and Golgi fractions. The indicated amounts of F(ab')₂ fragments of antimicrosomal reductase antibodies, purified by immunoadsorption, were incubated in PBS with fixed amounts of fresh cell fractions exhibiting an NADH-cytochrome *c* reductase activity of $0.11 \pm 0.01 \Delta OD$ at $550 \text{ nm ml}^{-1} \text{ min}^{-1}$ in a final volume of $20 \mu\text{l}$. After 10 min at room temperature, the incubated mixtures were brought to 0.8 ml with 60 mM phosphate buffer, pH 7.5, and the NADH-cytochrome *c* reductase activity was assayed. Values shown are expressed as percent of activity in controls incubated without antibodies. F(ab')₂ fragments prepared from nonimmune sera were without effect. (●) MR. (*) Mitochondria. (○) GF₃. (▲) GF₁₊₂.

(not shown). Fixed amounts of fresh MR, mitochondria, GF₃, and GF₁₊₂ exhibiting the same total NADH-cytochrome *c* reductase activity were mixed with increasing amounts of purified microsomal l-reductase and then with a fixed amount of F(ab')₂ antireductase in a concentration that yielded an ~80% inhibition in the absence of added l-reductase. We found that the purified microsomal l-reductase prevented the antibody inhibition not only on its homologous fraction but also on mitochondria and Golgi fractions, with the same dose-response relationships. This result indicates that it is highly unlikely that the parallel inhibition of different enzymes is due to different antibodies, and corroborates the conclusion that the antibody raised against microsomal reductase has the same affinity for the MR, mitochondria, and Golgi enzyme.

Peptide Mapping

The other approach we used to comparatively characterize the microsomal, mitochondrial, GF₃,

and GF₁₊₂ NADH-cytochrome *b*₅ reductase was the analysis of the purified enzyme molecules by peptide mapping. Detergent extracts of the four fractions were loaded onto affinity columns bearing antireductase antibodies. For convenience, in these experiments we did not use the pure antibody described in the preceding section but a preparation obtained by immunoadsorption of immune sera onto immobilized l-reductase (i.e., a preparation carried only to the Sephadex A-50 step). This preparation contains a number of contaminants as revealed by crossed immunoelectrophoresis (not shown). It was, therefore, not a surprise that, when aliquots of the immunoeleuates obtained from the detergent-solubilized fractions were resolved by SDS slab PAGE, they gave rise to several bands (Fig. 5). In all the four fractions, a band appeared at the position expected for uncleaved NADH-cytochrome *b*₅ reductase (d-reductase). From the work of Spatz and Strittmatter (18), it is known that d-reductase migrates in SDS-PAGE just behind l-reductase and more rapidly than predicted from its molecular weight. The d-

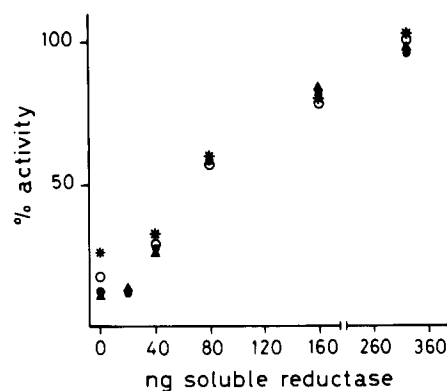


FIGURE 4 Competition for antimicrosomal reductase antibodies between added purified l-NADH-cytochrome *b*₅ reductase isolated from microsomes after lysosomal digestion and membrane-bound reductase endogenous to microsomes, mitochondria, and Golgi fractions. Aliquots of fresh cell fractions, exhibiting an NADH-cytochrome *c* reductase activity of $0.11 \Delta OD$ at $550 \text{ nm ml}^{-1} \text{ min}^{-1}$, were mixed with purified l-reductase in the amounts indicated on the abscissa and then with $0.622 \mu\text{g}$ of F(ab')₂ fragments of purified antireductase antibodies in a final volume of $30 \mu\text{l}$. Values are expressed as percent activity of samples incubated without antibody and without l-reductase. Addition of l-reductase to membrane samples in the absence of antibody was without effect. Incubation conditions, assay, and symbols as in Fig. 3.

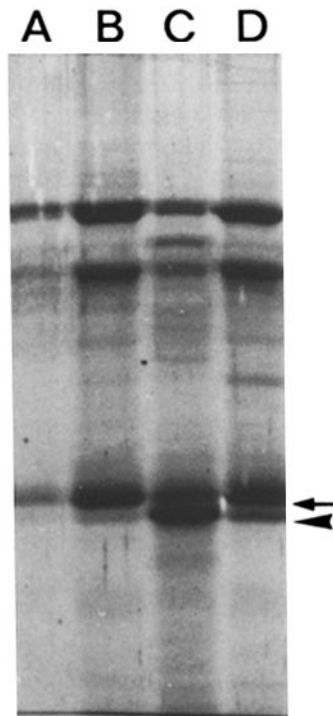


FIGURE 5 SDS slab PAGE of immunoeleuates obtained from detergent-solubilized microsomes, mitochondria, and Golgi fractions. Freshly isolated fractions were solubilized with DOC-Triton X-100, and the detergent extracts were applied to affinity columns bearing a preparation of nonpurified antimicrosomal reductase antibodies. After elution of the columns with Lubrol PX-containing buffer, the samples were dialyzed against 60% ethanol and divided into two unequal portions, both of which were precipitated with TCA. The smaller of these portions was analyzed by SDS slab PAGE (acrylamide concentration, 10%); the other was iodinated and processed for peptide mapping as described in the legend to Fig. 6. (A) GF₁₊₂. (B) GF₃. (C) Mitochondria. (D) MR. The expected position of d-reductase is indicated by an arrow; that of l-reductase by an arrowhead.

reductase band was accompanied by another band with the same mobility as the l-reductase standard, which was particularly prominent in the eluate of mitochondria.

Aliquots of the immunoeleuates were iodinated with ¹²⁵I before the SDS-PAGE step. After their gel pattern was revealed by autoradiography, the bands migrating as d-reductase were eluted and analyzed. The large band, corresponding to l-reductase in the mitochondrial pattern, was also analyzed. In each mapping, one experimental sample was processed in parallel with a sample of ¹²⁵I-

labeled, purified l-reductase standard. The distribution of labeled peptides was revealed by autoradiography.

The results are illustrated in Fig. 6. It is clear that all maps, whether of the l-reductase standard (Fig. 6*a-d*) or of the experimental samples (Fig. 6*A-D*), are very similar to each other. At least eight major peptides and a similar number of fainter spots were revealed in all cases. The minor differences that appeared were more quantitative than qualitative and were also seen in maps obtained in different runs of the same sample (compare the intensity of the spot indicated by the arrowhead in *c* and *d* of Fig. 6, both of which refer to the purified l-reductase standard). Thus, they cannot be attributed to molecular heterogeneity of the analyzed proteins but might depend on minor differences in the experimental conditions and in the pepsin cleavage pattern. The map of the mitochondrial band migrating ahead of d-reductase in SDS-PAGE (Fig. 6*B*) was also not appreciably different from that of the standard. The peptide separated in this band can therefore be identified as l-reductase. Its appearance is not surprising because of the well-known sensitivity of the peptide bond between the soluble and hydrophobic portions of the reductase molecule to cathepsin D (13, 21, 19), an enzyme that is expected to be released from contaminating lysosomes (present especially in the mitochondrial fraction) by detergent treatment.

Analysis of CNBr Fragments

Because no difference between the peptide maps of d- and l-reductase were revealed, it must be concluded that this analysis was limited to the water-soluble fragment of NADH-cytochrome *b*₅ reductase, and that the hydrophobic peptide(s) obtained from d-reductase by pepsin digestion were not detected most probably because of their insolubility in the solvents used for the analysis. Therefore, we attempted to gain information also on the hydrophobic moiety of NADH-cytochrome *b*₅ reductase by analyzing the SDS-PAGE mobilities of the CNBr fragments obtained from the [¹²⁵I]d-reductase of the various cell fractions and from microsomal [¹²⁵I]l-reductase standard. The results can be seen in Fig. 7. Under the conditions employed, a large number of bands in the molecular weight region from 34,000–10,000 could be observed. The prominent band indicated by the arrow had the same mobility as uncleaved d-reductase, and the faster running bands presumably

correspond to fragments resulting from different degrees of CNBr cleavage. Samples were analyzed both after treatment with β -mercaptoethanol (Fig. 7B) and without reduction (Fig. 7A). Among the changes induced by β -mercaptoethanol was the disappearance of a band migrating slower than d-reductase, which, because of its slow migration, can probably be accounted for by disulfide bridging. Neither in reduced nor in nonreduced samples were appreciable differences between fragments of the d-reductases isolated from MR, mitochondria, GF₃, and GF₁₊₂ revealed, indicating a correspondence in the location of methionine as well as a similar distribution of cysteine residues among the fragments. When compared with the fragments obtained from d-reductases, the pattern of the microsomal l-reductase standard (lane 1 of Fig. 7A and B) was distinctly different, with peptides showing on the average a lower molecular weight, as expected.

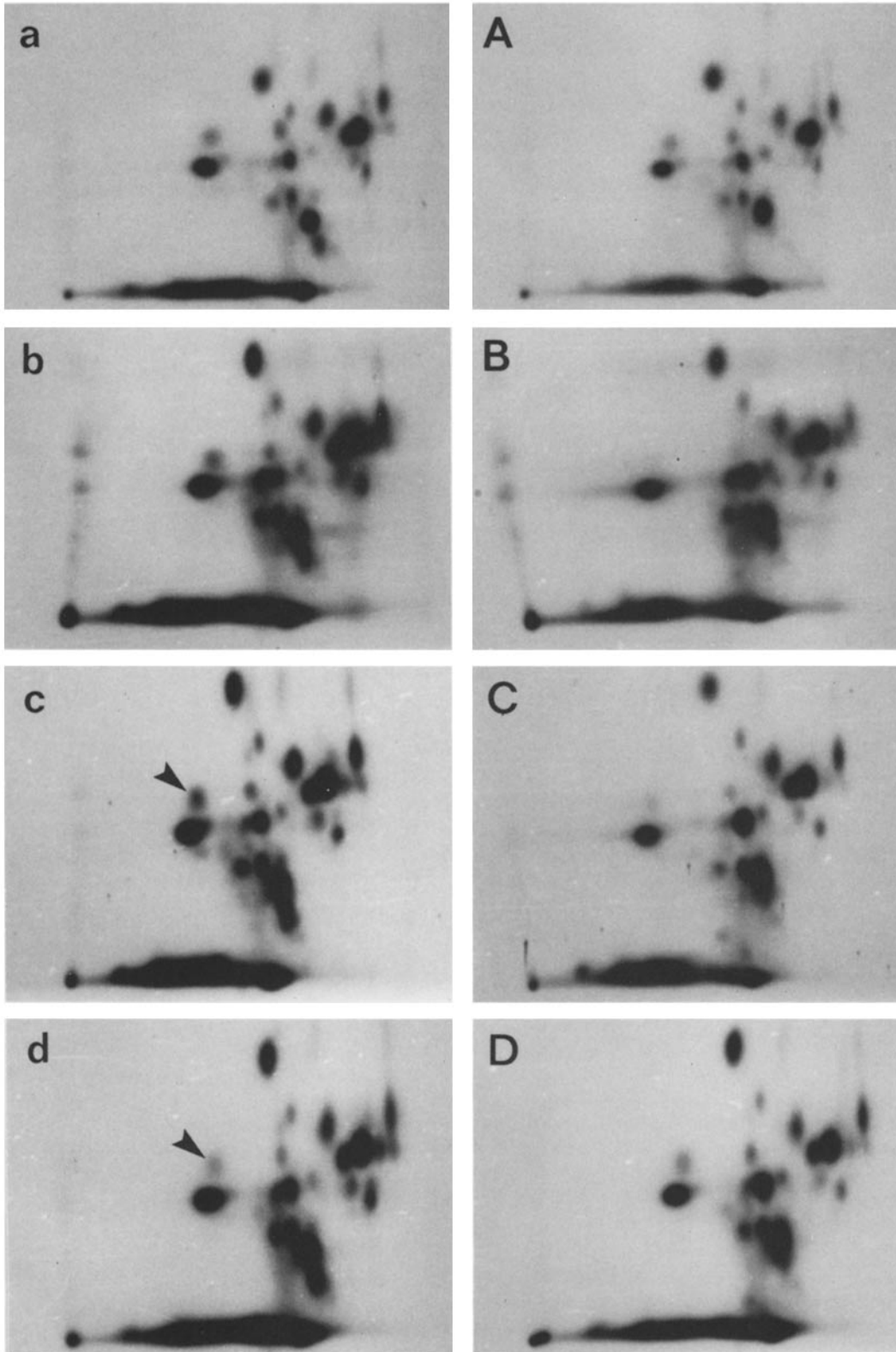
DISCUSSION

The question as to whether rat liver NADH-cytochrome *b*₅ reductase, an enzyme activity with a multicompartiment distribution, is due to a single enzyme in its various subcellular locations has already been considered in the past. In 1970, Takasue and Omura reported that an immune serum raised against the water-soluble fragment of the microsomal enzyme was equally effective in inhibiting the NADH-cytochrome *c* reductase activity of MR and mitochondria (20). Recent work from the same laboratory (10) showed that the reductases of the two subcellular fractions have a number of common features: apparent molecular weight, isoelectric point, pH optimum, and susceptibility to solubilization from membranes by cathepsin D. In preliminary experiments (3), we found that antisera raised against the microsomal enzyme also inhibit the NADH-cytochrome *c* reductase of the two Golgi fractions, GF₃ and GF₁₊₂. Although these results suggested that NADH-cytochrome *b*₅ reductase is accounted for by the same enzyme in its three subcellular locations, further experimental evidence was needed to prove this point. In fact, it is not clear that the antibodies used in these previous immunological studies were pure because they were characterized only by the Ouchterlony double diffusion test. In our experience, antisera that showed multiple arcs when analyzed by crossed immunoelectrophoresis gave in some cases a single precipitation line in the double diffusion test. Therefore, the possibility

cannot be excluded that the sera used in previous studies contained not one antireductase antibody directed against identical enzyme molecules located in the three types of membranes but three different antireductases, each directed against its specific antigen. To explore this possibility, we purified our immunoglobulins to the level that only one NADH-tetrazolium reductase-positive arc was revealed by crossed immunoelectrophoresis against solubilized microsomes and found that the F(ab')₂ fragments of these immunoglobulins, exactly as the contaminated starting immune serum, equally inhibited the NADH-cytochrome *c* reductase activity of the four fractions investigated. We also carried out the competition experiments with the purified microsomal l-reductase, which again failed to reveal any difference in the antibody inhibition of NADH-cytochrome *c* reductase in its various locations.

The immunological data discussed clearly indicate that the NADH-cytochrome *b*₅ reductase molecules of ER, mitochondria, and Golgi complex are indistinguishable in their binding to antireductase leading to the inhibition of the enzyme activity. This, however, does not necessarily mean that in its various locations the enzyme is molecularly the same. In fact, cases of enzymes that exist in multiple forms, identical in some portions and different in the rest of the molecule have been reported. This is the case for rat liver microsomal and lysosomal β -glucuronidases as reported by Tulsiani et al. (22). Moreover, the brush-border aminopeptidases of pig intestine and kidney, studied by sophisticated immunological techniques by Vannier et al. (24), were found to share six antigenic determinants; however, other determinants (six in the intestinal and four in the renal enzyme) were heterologous.

To investigate the possibility that NADH-cytochrome *b*₅ reductase in its various locations is only partially homologous, we studied the peptide maps obtained after pepsin hydrolysis. From the results it can be concluded (a) that purification of d-reductase from cell fractions by immunoadsorption followed by SDS-PAGE is a valid procedure because the peptide maps obtained were indistinguishable from those of the microsomal l-reductase purified by conventional biochemical techniques, and (b) that d-reductase molecules purified from different cell fractions have the same mobility in SDS-PAGE and indistinguishable peptide maps. The peptide mapping technique, however, had a clear limitation. It gave information only on



the water-soluble fragment of d-reductase, presumably because peptides derived from the hydrophobic portion were insoluble in the aqueous solutions used for the analysis. To also obtain information concerning the hydrophobic portion, we compared the CNBr fragments of d-reductases by SDS-PAGE. Again, no differences between the enzyme purified from the different fractions were revealed, but in this case the pattern of l-reductase was distinctly different.

Taken together, therefore, our results strongly corroborate the hypothesis that a single enzyme accounts for the reductase activity in the various membranes investigated. This conclusion, however, appears to be more soundly based for the water-soluble fragment of reductase than for the hydrophobic portion. In fact, the immunological experiments and the peptide maps refer to the hydrophilic portion of the enzyme only. The information concerning entire molecules relies on a technique that has a lower resolution inasmuch as it depends only on the position of methionine residues. It should also be mentioned that, because of the limited amount of purified enzyme we had available, the structural analyses were carried out on ^{125}I -labeled protein, and thus only peptides containing tyrosines were visualized. If one considers the relatively low molecular weight of the reductase and the relatively large number of peptides that appeared in the maps and CNBr patterns, the possibility that many other peptides remained unlabeled seems unlikely. However, any heterogeneity of these unlabeled peptides would have gone undetected.

In conclusion, the hypothesis that rat liver NADH-cytochrome b_5 reductase in its various sub-cellular locations is due to the same protein molecule survives even after a comprehensive test including immunological and structural analyses.

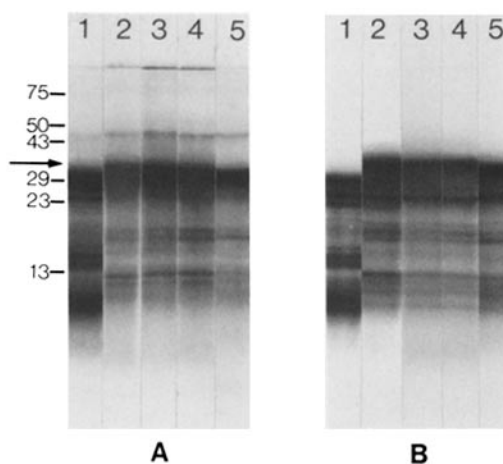


FIGURE 7 SDS-PAGE of CNBr fragments obtained from NADH-cytochrome b_5 reductases purified from MR, mitochondria, GF_3 , and GF_{1+2} . Iodinated d-reductase, obtained from detergent extracts of MR, mitochondria, GF_3 , and GF_{1+2} as in Figs. 5 and 6, were treated with CNBr and analyzed on 15% SDS polyacrylamide gels. An iodinated microsomal l-reductase standard was also analyzed. (A) Nonreduced samples. (B) Samples reduced with β -mercaptoethanol. Lanes contained: 1, microsomal l-reductase; 2-5, d-reductases obtained from MR (lane 2), GF_{1+2} (lane 3), GF_3 (lane 4), and mitochondria (lane 5). Arrow indicates the position of d-reductase. Molecular weight standards used were IgM μ chain, 75,000; IgG γ chain, 50,000; ovalbumin, 43,000; Ig λ chain, 29,000; IgK chain, 23,000; bovine pancreatic ribonuclease, 13,700.

In the final paper of this series, we report the results of studies on the biosynthesis and turnover of this enzyme.²

The photographic assistance of Mr. P. Tinelli and Mr. F. Crippa is gratefully acknowledged.

Received for publication 22 May 1979, and in revised form 8 January 1980.

FIGURE 6 Peptide mapping of NADH-cytochrome b_5 reductase purified from MR, mitochondria, GF_3 , and GF_{1+2} . Detergent extracts of cell fractions were immunoabsorbed onto a Sepharose-antireductase-IgG conjugate. The resulting immunoeuate was iodinated by the chloramine-T method and electrophoresed by SDS slab PAGE. The bands corresponding to d-reductase and the mitochondrial band running as l-reductase (arrow and arrowhead in Fig. 5, respectively) were cut out, eluted, digested with 2% pepsin, and analyzed. Each experimental sample was electrophoresed on the same plate with a sample of purified soluble microsomal reductase, iodinated in parallel. After electrophoresis, each plate was cut into halves and subjected to chromatography. Peptides were revealed by autoradiography. In each map pair, the purified microsomal l-reductase is shown on the left (a-d). (A) Mitochondria, d-reductase. (B) Mitochondria, l-reductase. (C and D) GF_{1+2} and GF_3 d-reductase, respectively. The pattern of the microsomal d-reductase was indistinguishable from that of the microsomal l-reductase standard. Arrowheads in c and d point to a spot whose intensity varies also among the maps of the purified standard. Electrophoresis toward the cathode, from bottom to top; chromatography from left to right.

REFERENCES

1. AXELSEN, N. H., J. KRØI, and B. WEEKE. 1973. Quantitative Immuno-electrophoresis. Methods and Applications. Universitetsforlaget, Oslo.
2. BJERRUM, O. J. 1977. Immunochemical investigation of membrane proteins. Methodological survey with emphasis placed on immunoprecipitation in gels. *Biochim. Biophys. Acta.* **472**:135-195.
3. BORGESSE, N., and J. MELDOLESI. 1976. Immunological similarity of the NADH-cytochrome *c* electron transport system in microsomes, Golgi complex and mitochondrial outer membrane of rat liver cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **63**:231-234.
4. BORGESSE, N., and J. MELDOLESI. 1980. Localization and biosynthesis of NADH-cytochrome *b₅* reductase, an integral membrane protein, in rat liver cells. I. Distribution of the enzyme activity in microsomes, mitochondria, and Golgi complex. *J. Cell Biol.* **85**:501-515.
5. CORTE, G., P. TONDA, E. COSULICH, C. P. MILSTEIN, A. BARGELLES, and M. FERRARINI. 1979. Characterization of IgD. I. Isolation of two molecular forms from human serum. *Scand. J. Immunol.* **9**:141-149.
6. DE PIERRE, J. W., and G. DALLNER. 1975. Structural aspects of the membrane of the endoplasmic reticulum. *Biochim. Biophys. Acta.* **415**:411-472.
7. FUKUSHIMA, K., and R. SATO. 1973. Purification and characterization of cytochrome *b₅*-like hemoprotein associated with outer mitochondrial membrane of rat liver. *J. Biochem. (Tokyo).* **74**:161-173.
8. GREENWOOD, F. C., W. M. HUNTER, and J. S. GLOVER. 1963. The preparation of ¹²⁵I-labelled human growth hormone at high specific activity. *Biochem. J.* **89**:114-123.
9. KRAEHNBUHL, J. P., L. RACINE, and R. E. GALLARDY. 1975. Localization of secretory IgA, secretory component, and α chain in the mammary gland of lactating rabbits by immunoelectron microscopy. *Ann. N. Y. Acad. Sci.* **254**:190-202.
10. KUWAHARA, S., Y. OKADA, and T. OMURA. 1978. Evidence for molecular identity of microsomal and mitochondrial NADH-cytochrome *b₅* reductases of rat liver. *J. Biochem. (Tokyo).* **83**:1049-1069.
11. LAI, C. Y. 1968. Studies on the structure of rabbit muscle aldolase. I. Cleavage with cyanogen bromide: an approach to the determination of total primary structure. *Arch. Biochem. Biophys.* **128**:202-211.
12. MAIZEL, J. V. 1971. Polyacrylamide gel electrophoresis of viral proteins. *In* Methods in Virology. K. Maromrosch, and H. Koprowsky, editors. Academic Press, Inc., New York. **5**:179-246.
13. MIHARA, S., and R. SATO. 1972. Partial purification of NADH-cytochrome *b₅* reductase from rabbit liver microsomes with detergents and its properties. *J. Biochem. (Tokyo).* **71**:725-735.
14. MIHARA, S., R. SATO, R. SAKAKIBARA, and H. WADA. 1978. Reduced nicotinamide dinucleotide-cytochrome *b₅* reductase: location of the hydrophobic membrane-binding region at the carboxyl terminal end and the masked amino terminus. *Biochemistry.* **17**:2829-2834.
15. MOLE, L. E. 1975. A genetic marker in the variable region of rabbit immunoglobulin heavy chain. *Biochem. J.* **151**:351.
16. OPHEIM, D. J., and O. TOUSTER. 1978. Lysosomal α -D-mannosidases of rat liver. Purification and comparison with the Golgi and cytosolic α -D-mannosidase. *J. Biol. Chem.* **253**:1017-1023.
17. RAFTELL, M., and P. PERLMANN. 1969. Antigen development in sub-microsomal fractions of rat liver. *Exp. Cell Res.* **57**:119-128.
18. SPATZ, L., and P. STRITTMATTER. 1973. A form of reduced nicotinamide adenine dinucleotide-cytochrome *b₅* reductase containing both the catalytic site and an additional hydrophobic membrane-binding segment. *J. Biol. Chem.* **248**:793-799.
19. TAKESUE, S., and T. OMURA. 1970. Purification and properties of NADH-cytochrome *b₅* reductase solubilized by lysosomes from rat liver microsomes. *J. Biochem. (Tokyo).* **67**:267-276.
20. TAKESUE, S., and T. OMURA. 1970. Immunological similarity between NADH-cytochrome *c* reductases of mitochondrial outer membrane and microsomes. *Biochim. Biophys. Res. Commun.* **40**:396-401.
21. TAKESUE, S., and T. OMURA. 1970. Solubilization of NADH-cytochrome *b₅* reductase from liver microsomes by lysosomal digestion. *J. Biochem. (Tokyo).* **67**:259-266.
22. TULSIANI, D. R. P., H. SIX, and O. TOUSTER. 1978. Rat liver microsomal and lysosomal β -glucuronidases differ in both carbohydrate and amino acid composition. *Proc. Natl. Acad. Sci. U. S. A.* **75**:3080-3084.
23. UEMURA, T., and E. CHIESARA. 1976. NADH-dependent aryl hydrocarbon hydroxylase in rat liver mitochondrial outer membrane. *Eur. J. Biochem.* **66**:293-307.
24. VANNIER, C., D. LOUVARD, S. MAROUX, and P. DESNUELLE. 1976. Structural and topological homology between porcine intestinal and brush border aminopeptidases. *Biochim. Biophys. Acta.* **455**:185-199.