Purification and Characterization of a Doxorubicin-inhibited NADH-quinone (NADH-ferricyanide) Reductase from Rat Liver Plasma Membranes*

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Plasma membrane-associated redox systems play important roles in regulation of cell growth, internal pH, signal transduction, apoptosis, and defense against pathogens. Stimulation of cell growth and stimulation of the redox system of plasma membranes are correlated. When cell growth is inhibited by antitumor agents such as doxorubicin, capsaicin, and antitumor sulfonulearase, redox activities of the plasma membrane also are inhibited. A doxorubicin-inhibited NADH-quinone reductase was characterized and purified from plasma membranes of rat liver. First, an NADH-cytochrome b5 reductase, which was doxorubicin-insensitive, was removed from the plasma membranes by the lysosomal protease, cathepsin D. After removal of the NADH-cytochrome b5 reductase, the plasma membranes retained a doxorubicin-inhibited NADH-quinone reductase activity. The enzyme, with an apparent molecular mass of 57 kDa, was purified 200-fold over the cathepsin D-treated plasma membranes. The purified enzyme had also an NADH-coenzyme Q0 reductase (NADH: external acceptor (quino) reductase; EC 1.6.5..) activity. Partial amino acid sequence of the enzyme showed that it was unique with no sequence homology to any known protein. Antibody against the enzyme (peptide sequence) was produced and affinity-purified. The purified antibody immunoprecipitated both the NADH-ferricyanide reductase activity and NADH-coenzyme Q0 reductase activity of plasma membranes and cross-reacted with human chronic myelogenous leukemia K562 cells and doxorubicin-resistant human chronic myelogenous leukemia K562R cells. Localization by fluorescence microscopy showed that the reaction was with the external surface of the plasma membranes. The doxorubicin-inhibited NADH-quinone reductase may provide a target for the anthracycline antitumor agents and a candidate ferricyanide reductase for plasma membrane electron transport.

Membrane-bound oxidoreductases and enzymatic electron transfer chains are common to all living organisms (1, 2). In eukaryotic cells, chemically and functionally different electron transport systems are localized in different subcellular membranes. Some of the electron carriers may be loosely bound to the membrane or even soluble in the cytoplasm; others are structured as integral proteins of the membrane (1). The mitochondrial inner membrane and outer membranes, endoplasmic reticulum, Golgi apparatus, plasma membrane, and membranes derived from these structures all contain redox systems. Evidence for a growth-related NADH-ferricyanide (FeCN\(^3\); Fe(CN)\(_6\)) reductase traces its origin to the work of Ellem and Kay (3). When melanoma cells were cultured at low concentrations of fetal calf serum, cell growth was <20% of the optimum growth. If low concentrations of impermeable ferricyanide were added (0.01–0.1 mM), the growth was increased to 80% of the maximum rate with optimum fetal calf serum (3). HeLa cells also were stimulated to proliferate by micromolar concentrations of ferricyanide in serum free-media (2). Indeed, there appears to be a direct connection between increases in transplasma membrane electron transport activity and cell growth (1). Stimulation of growth by external oxidants is not limited to iron compounds because HeLa cell growth also is stimulated by hexamine ruthenium III (4) and by indigotetrasulfonate (5).

Inhibitors of the NADH-ferricyanide reductase and NADH oxidase of plasma membranes have been reported. The most interesting inhibitors from the view of both selectivity for tumor cells and significance are certain antitumor drugs such as anthracyclines (6, 7), cis-platinum (8), bleomycin (9), capsaicin (9), and antitumor sulfonulearase (11).

The growth response of several cell types in culture to both external impermeable oxidants and antitumor drugs indicates that the electron transport system of the transplasma membrane transcends iron uptake for nutritive purposes. A broader role of plasma membrane electron transport related to mechanisms of cell growth and possibly cell differentiation is indicated. In this study, we report isolation and characterization of NADH-ferricyanide reductase from rat liver plasma membrane, which is inhibited by the antitumor agent doxorubicin.

EXPERIMENTAL PROCEDURES

Materials

NADH, Trizma base, Hepes, cathepsin D, protein A-Sepharose, doxorubicin HCl, cyanogen bromide, α-phenylmethylsulfonyl fluoride, EDTA disodium salt, and potassium cyanide were from Sigma Chemical Co. Triton X-100 and CHAPS were from Roche Molecular Biochemicals. Nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and polyvinylidene difluoride membrane (ProBlott) were from Promega (Madison, WI). Potassium ferricyanide (KF,Fe(CN)\(_6\)) was from Fisher Scientific Products. Fetal bovine serum was from Invitrogen. Penicillin G, streptomyein, and L-glutamine were from Whittaker Bioproducts.

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(Walkersville, MD). Fluorescein isothiocyanate-labeled goat F(ab')2, antibody to rabbit IgG and rhodamine-labeled goat F(ab')2 antibody to rabbit IgG were from Proto Immunoresearch (San Francisco, CA). Hydroxyapatite column (TSK Gel HA-1000; 75 × 7.5-mm inner diameter) was from Tosohaas (Montgomeryville, PA). Anion-exchange column (LiChromat 1000 DEAE, 50 × 10-mm inner diameter) was from EM Science (Gibbstown, NJ). Gel filtration column (TSK G3000SWXL; 300 × 7.8-mm inner diameter) was from Supelco (Bellefonte, PA). SulfoLink column resin was from Pierce. Male Wistar rats or male Holtzman rats were from Harlan (Indianapolis, IN) and were used for the preparation of plasma membranes.

Preparation of Rat Liver Plasma Membranes by Aqueous Two-phase Partition
Rat liver homogenates were prepared as described by Bruno et al. (12). Homogenates then were resuspended in 1 mM sodium bicarbonate solution and fractionated by using a 16-g aqueous two-phase system (13, 14). The contents of the two-phase system were mixed thoroughly and separated by centrifugation. Plasma membranes partitioned into the upper phase were collected and stored at −70°C until used.

Enzyme Assays
For assay of NADH-ferricyanide reductase activity, the assay medium contained 50 mM Tris-HCl, pH 7.2, 200 μM NADH, 200 μM K₃Fe(CN)₆, and 0.02 to 0.1 mg of protein and/or inhibitors as indicated in a final volume of 2.5 ml. Ferricyanide reduction was monitored at 420 nm with a reference beam at 600 nm using a SLM Aminco DW 2000 dual wavelength spectrophotometer or by the decrease in the absorbance at 420 nm using Hitachi U3210 spectrophotometers. The assay medium contained all the reagents except potassium ferricyanide which was preincubated for 5 min at 37°C, and the assay was started by the addition of potassium ferricyanide and continued for two consecutive 5-min periods. Decrease in absorbance during the second 5-min period was used to calculate the specific activity of ferricyanide reductase. A blank rate was determined in the absence of proteins and subtracted. The extinction coefficient for ferricyanide reduction was 1.00 mM⁻¹ cm⁻¹.

For assay of NADH-quinone reductase activity, enzyme activity was measured as described above, except that 200 μM coenzyme Q0 was added in place of potassium ferricyanide, and the decrease in absorbance at 410 nm was measured. The extinction coefficient for coenzyme Q0 was 0.80 mM⁻¹ cm⁻¹.

Treatment of Plasma Membranes with Cathepsin D
Rat liver plasma membranes were resuspended in 15 ml of 100 mM Tris-HCl, pH 5.9, containing 2.5 μM of cathepsin D unit and incubated at 37°C for 4 h with occasional agitation. After incubation, the pH was adjusted to 7.0 with 1 N HCl, and the preparations were centrifuged at 100,000 × g for 1 h to collect the membranes. The membrane pellet was resuspended in 20 ml of 100 mM Tris-HCl, pH 7.0.

Solubilization of Proteins from Plasma Membranes
Peripheral proteins and loosely bound cytosolic proteins were first removed by incubating plasma membranes (∼76 mg of protein) in a medium containing 50 mM Tris-HCl, pH 7.2, and 1 mM EDTA with gentle stirring at 4°C, followed by centrifugation for 60 min at 100,000 × g. After centrifugation, pellet was collected and treated with cathepsin D. The cathepsin D-treated plasma membranes were resuspended and solubilized in 20 ml of Tris-HCl, pH 7.0, containing 1 mM α-phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.5% Triton X-100, and 10% glycerol. The plasma membranes were incubated at 4°C for 4 h with stirring and then centrifuged at 100,000 × g for 1 h. The supernatant was collected.

Purification of NADH-quinone Reductase
NADH-quinone reductase was purified from rat liver plasma membranes by hydroxyapatite, anion-exchange, and gel filtration chromatographies, as follows.

Purification by Hydroxyapatite Chromatography—Triton X-100-solubilized proteins of cathepsin D-treated rat liver plasma membranes were fractionated by hydroxyapatite HPLC. Samples were loaded onto a hydroxyapatite column (TSK Gel HA-1000). All purifications including subsequent steps were performed at room temperature by using a Waters HPLC system. The column was prewashed with elution buffer (0.5 mM NaH₂PO₄, pH 6.8, 10% glycerol, and 0.3% CHAPS) and pre-equilibrated with elution buffer (10 mM NaH₂PO₄, pH 6.8, 10% glycerol, and 0.3% CHAPS). After loading of the sample, proteins were eluted with a linear gradient of equilibration buffer to elution buffer in 60 min at a flow rate of 0.7 ml/min with monitoring absorbance at 280 nm. NADH-ferricyanide reductase activity was assayed in each fraction, and fractions with enzymatic activity were pooled and concentrated for further purification.

Purification by Anion-exchange Chromatography—The pool from hydroxyapatite chromatography was further purified by anion-exchange HPLC. The pool was loaded onto an anion-exchange column (LiChro- spher 1000 DEAE). The column was prewashed with elution buffer (50 mM Tris-HCl, pH 7.5, 0.3% CHAPS, and 1 M NaCl) and pre-equilibrated with equilibration buffer (50 mM Tris-HCl, pH 7.5, and 0.3% CHAPS). With a linear gradient, rat liver plasma membranes were eluted with 100 mM Tris-HCl buffer to elution buffer in 100 min at a flow rate of 0.5 ml/min. Fractions with the highest NADH-ferricyanide reductase activity were concentrated and further purified.

Purification by Gel Filtration Chromatography—The pool from anion-exchange chromatography was purified by gel filtration HPLC by using TSK G3000SWXL column. Proteins were eluted with 20 mM Tris-HCl, pH 7.0, containing 100 mM NaCl and 0.3% CHAPS at a flow rate of 0.5 ml/min. Fractions were collected and assayed for NADH-ferricyanide reductase activity.

Determination and Analysis of Partial Amino Acid Sequence of NADH-quinone Reductase
Partial amino acid sequence of NADH-quinone reductase was determined from a peptide generated by cyanogen bromide cleavage. About 7 μl of purified NADH-quinone reductase were cleaved with cyanogen bromide, and peptides were separated by SDS-PAGE as described by Shagger and Jagov (15). After SDS-PAGE, peptides were transferred to polyvinylidene difluoride membrane. One of the cleaved peptides was subjected to N-terminal amino acid sequencing. The sequencing was carried out using an automated pulsed-liquid protein sequencer, courtesy of Dr. Gerald W. Becker (Eli Lilly Research Laboratories, Indianapolis, IN). The sequence obtained was compared with known protein sequences using the BLAST program of the NCBI/GenBank™ data base.

Preparation of Anti-NADH-quinone Reductase Antibody
A synthetic peptide (CMVADKANIDK) derived from the amino acid sequence of NADH-quinone reductase was used to generate rabbit anti-NADH-quinone reductase antibodies. Synthesis and analysis of the peptide and production of the peptide antibody were performed by Immunodynamics, Inc. (La Jolla, CA). Briefly, the synthesized peptide was conjugated to carrier proteins, keyhole limpet hemocyanin and bovine serum albumin. Antibodies were made by immunizing two rabbits and maintaining them for a period of 10 weeks. Rabbis were bled at t = 3 weeks and t = 6 weeks. Bleeds were taken at t = 5 weeks, t = 7 weeks, and t = 9 weeks and monitored for specificity and reactivity. The antibody titer by ear-bleed titrations was determined. After bleedings were pooled, and antibody was affinity-purified. For affinity purification, the antibody was precipitated with 33% ammonium sulfate, resuspended, dialyzed against 20 mM Tris-HCl, pH 8.0, containing 30 mM NaCl and 0.02% NaN₃, and passed through the DEAE-Affi-Gel blue column. The antibody, unbound to the column at 30 mM NaCl, was collected and further purified by the peptide-linked affinity resin, which was prepared by linking poly-L-lysine to the activated affinity resin (Subsolink) according to the procedure provided by the manufacturer. Antibody was applied to the affinity column, and anti-NADH-quinone reductase antibody was eluted with 100 mM glycine, pH 2.8, and neutralized by 1 mM Tris-HCl, pH 8.0.

Immunoprecipitation of NADH-quinone Reductase
Rat liver plasma membranes (2.5 mg of protein) were solubilized in 400 μl of lysis buffer (10 mM Tris, 10 mM NaCl, 1 mM dithiothreitol, 1 mM α-phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40, pH 8.0) at 4°C for 16 h with shaking and centrifuged at 100,000 × g for 30 min, and supernatant was collected. Supernatant (150 μl containing 550 μg of protein) was diluted to 1.2 ml with Tris-phosphate-buffered saline (10 mM Tris, 10 mM sodium phosphate, and 140 mM NaCl, pH 8.0) and precleared with 100 μl of protein A-Sepharose (70 μg resin/ml) by incubation for 4 h at 4°C. Three aliquots of precleared solution (300 μl; 120 μg of protein) were prepared. Two of these aliquots were incubated with the affinity-purified anti-NADH-quinone reductase antibody (20 μl) for 4 h at 4°C; the other aliquot was incubated with 20 μl of 10 mM Tris-Cl buffer, pH 8.0, or preimmune IgG as controls. Protein A-Sepharose (200 μl) was added and incubated for an additional 2 h at 4°C. The solution was centri-
The total NADH-ferricyanide reductase activity of rat liver plasma membranes prepared by aqueous two-phase partition was largely not inhibited by doxorubicin and was even stimulated at high concentrations (Fig. 1, ○). Because plasma membranes contain more than one redox enzyme, the doxorubicin-inhibited activity may have been masked by the NADH-ferricyanide reductase that is not inhibited by doxorubicin. To test this possibility, the NADH-cytochrome b₅ reductase was removed from the membranes without loss of enzymatic activity by cathepsin D (23, 24). When the plasma membranes were treated with cathepsin D, the enzyme activity was reduced to 7% of the initial rate. The activity that remained, however, was strongly inhibited by doxorubicin, with maximum inhibition at 1 µM doxorubicin (Fig. 1, ○). A 10-fold higher concentration was less inhibitory (Fig. 1, □). The cathepsin D-treated plasma membranes were used as the starting material for purification and characterization of the doxorubicin-inhibited NADH-ferricyanide reductase.

**Purification of NADH-quinone Reductase**—The major activity of NADH-ferricyanide reductase was eluted at concentrations between 0.14 and 0.18 M sodium phosphate upon hydroxyapatite chromatography (fractions 6–9 in Fig. 2A). Fractions 6–9 were pooled, concentrated, and purified by anion-exchange chromatography. NADH-ferricyanide reductase was eluted at concentrations between 0.22 and 0.28 M NaCl (Fig. 2B). Fractions 3–5 had enzyme activity, and fraction 4 had the highest enzyme activity. Fraction 4 was concentrated, applied to a gel
filtration column, and eluted. Enzymatic activity was concentrated in fractions 2–4 (Fig. 2C).

The protein composition and purity of fractions 2–4 of gel filtration chromatography were analyzed by SDS-PAGE. Equal amounts were subjected to SDS-PAGE, and band intensity was compared with enzyme activity. A 57-kDa protein band (arrows in Fig. 3A) correlated with NADH-ferricyanide reductase activity (Fig. 3B). Although minor protein bands were visible, no correlation between band intensity of the minor bands and enzyme activity was noted.

The purified enzyme also reduced CoQ0 (NADH-CoQ0 reductase) (NADH: external acceptor (quinone) reductase; EC 1.6.5...), indicating that the natural substrate for the enzyme may be coenzyme Q (ubiquinone). NADH-ferricyanide reductase was purified 198-fold with a yield of 5% over that of cathepsin D-treated plasma membranes (Table I). Purification of NADH-CoQ0 reductase was 186-fold, and the yield was 5% (Table II).

Fractions 3 and 4 from gel filtration chromatography were pooled, concentrated, cleaved by cyanogen bromide, and electrotransferred to polyvinylidene difluoride membrane. They yielded a peptide band with the N-terminal sequence: Val-X-Asp-Lys-Ala-Asn-Ile-Asp/Ser/Glu-Lys-Gln/Glu-Asp/Thr/Glu-X-Asp/Gln-X-Val. Peptide sequence homology search (December 2001) showed no sequence homology with known proteins, but a segment of the peptide sequence had homology with aldehyde dehydrogenase, pyruvate:ferredoxin oxidoreductase, and trans-2-enoyl-acyl carrier protein reductase II (Fig. 4A).

**Fig. 2.** Purification of NADH-FeCN/quinone reductase by hydroxyapatite chromatography (A), anion-exchange chromatography (B), and gel filtration chromatography (C). Protein elution was monitored at 280 nm. The unit of enzyme activity measured was a decrease in absorbance of potassium ferricyanide at 430 nm/min for 100 μl of each fraction assayed. Numbers inside the chromatogram indicate the fraction numbers collected, and dashed lines (—-) indicate the gradient of sodium phosphate (A) and sodium chloride (B) concentration.

**Fig. 3.** SDS-PAGE (8%) of fractions from gel filtration chromatography. A, 90 μl of each fraction with NADH-FeCN reductase activity was loaded per lane, and protein bands were visualized by silver staining. The lanes are (from left to right) molecular mass standards, sample fraction 2, fraction 3, and fraction 4. B, NADH-FeCN reductase activity of corresponding fractions. The band intensity of the 57-kDa protein (arrows in A) correlated with the enzyme activity. The unit of enzyme activity measured was a decrease in absorbance of potassium ferricyanide at 420 nm/min for 100 μl of each fraction assayed.

**Western Blot Analysis and Immunoprecipitation of NADH-quinone Reductase**—Antibody was generated by using peptide (CMVADKANIDK) deduced from the partial amino acid sequence, and the antibody was affinity-purified. Cysteine was added to the N terminus for the purpose of conjugating the peptide to activated affinity resin. Because cyanogen bromide cleaves after methionine at the N terminus, methionine was added to the deduced amino acid sequence. Alanine replaced the unknown amino acids because alanine has low antigenicity. For Western blot analysis, rat liver plasma membranes were electrophoresed, and proteins were transferred to a nitrocellulose membrane. Proteins were reacted with affinity-purified antibody and detected with alkaline phosphatase-conjugated anti-rabbit antibody using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. Affinity-purified antibody reacted with a 57-kDa protein (arrow in Fig. 4B).

After immunoprecipitation of NADH-quinone reductase from cathepsin D-treated and solubilized plasma membranes, sample was centrifuged, and the supernatant was collected for
Doxorubicin-inhibited NADH-quinone Reductase

Table I

| Fraction                          | Total protein (mg) | Specific activity (FeCN reduced) (nmol/mg protein/min) | Purification (fold) | Total activity (µmol/min) | Yield (%) |
|----------------------------------|--------------------|--------------------------------------------------------|---------------------|---------------------------|-----------|
| RLPM*                            | 76.3               | 910                                                    | 1                   | 69                        | 100       |
| Cathepsin D-treated RLPM         | 32.9               | 52                                                     | 1                   | 1.71                      | 32        |
| Triton X-100-solubilized solution | 9.04               | 61                                                     | 1.17                | 0.55                      | 32        |
| Gel filtration chromatography    | 0.009              | 10300                                                  | 198                 | 0.09                      | 5         |

* RLPM, rat liver plasma membrane.

Table II

| Fraction                          | Total protein (mg) | Specific activity (CoQ0 reduced) (nmol/mg protein/min) | Purification (fold) | Total activity (µmol/min) | Yield (%) |
|----------------------------------|--------------------|--------------------------------------------------------|---------------------|---------------------------|-----------|
| RLPM*                            | 76.3               | 76                                                     | 5.8                 | 100                       |
| Cathepsin-D-treated RLPM         | 32.9               | 71                                                     | 1                   | 2.34                      | 35        |
| Triton X-100-solubilized solution | 9.04               | 91                                                     | 1.28                | 0.82                      | 35        |
| Gel filtration chromatography    | 0.009              | 13200                                                  | 186                 | 1.12                      | 5         |

* RLPM, rat liver plasma membrane.

Table III

| Treatment                          | NADH-FeCN oxidase (nmol/mg protein/min) | NADH-CoQ0 oxidase (nmol/mg protein/min) |
|------------------------------------|----------------------------------------|----------------------------------------|
| None                               | 85                                     | 135                                    |
| Antibody                           | 30                                     | 75                                     |

Fig. 4. Comparison of the amino acid sequence (A) and Western blot analysis of NADH-quinone reductase (B). A, a segment of the peptide sequence had sequence homology with aldehyde dehydrogenase, pyruvate:ferredoxin oxidoreductase, and trans-2-enoyl-ACP reductase II. B, plasma membranes were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Proteins were reacted with the affinity-purified antibody and detected with the alkaline phosphatase-conjugated anti-rabbit IgG antibody in the presence of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. Lane std, protein molecular mass standards; lane 1, plasma membranes of rat liver. A 57-kDa protein band reacted with the affinity-purified antibody (arrow).

The measurement of reductase activity. Both the NADH-ferri cyanide reductase and NADH-CoQ0 reductase activities were depleted from the supernatant, although the depletion was incomplete (Table III). Both the lack of complete depletion by immunoprecipitation and the lack of complete inhibition by doxorubicin may be due to residual NADH-cytochrome b5 reductase remaining in the cathepsin D-treated membranes.

**Localization of NADH-quinone Reductase**—Localization of NADH-quinone reductase was studied by fluorescence microscopy. K562R cells were incubated with affinity-purified antibody, followed by incubation with rhodamine-labeled anti-rabbit IgG. Detection was by fluorescence microscopy. The antibody reacted with plasma membranes of doxorubicin-resistant human chronic myelogenous leukemia (K562R) cells, and the reaction was localized to the external surface of the plasma membrane as patches (Fig. 5A), which indicates that the purified NADH-quinone reductase was from plasma membranes.

**Quantitation of NADH-quinone Reductase by Flow Cytometry**—K562 and K562R cells were reacted with affinity-purified antibody, followed by reaction with fluorescein isothiocyanate-labeled anti-rabbit IgG. Quantification was measured by flow cytometry. No fluorescence channel shift occurred with cells incubated with preimmune IgG (3.44 ± 0.61 in K562 cells and 3.89 ± in K562R cells) (Fig. 5B, a and c), but fluorescence channel shift occurred with cells incubated with affinity-purified antibody (7.09 ± 1.36 in K562 cells and 24.24 ± 1.16 in K562R cells) (Fig. 5B, b and d). K562 cells expressed NADH-quinone reductase on the plasma membrane, and K562R cells expressed NADH-quinone reductase at a level 1 order of magnitude greater than that of K562 cells.

**Discussion**

Although physiological roles of the redox system are not yet completely understood, functions in which plasma membrane redox systems have been implicated are proton extrusion and control of internal pH (25), generation of superoxide (26), reduction of ferric iron and iron uptake (27, 28), control of cell growth and proliferation (5, 29, 30), and biological timekeeping (31, 32). Comprehensive reviews of the plasma membrane redox system are found elsewhere (33–35).

In this study, we have described purification and characterization of doxorubicin-inhibited NADH-quinone (CoQ0) reductase from rat liver plasma membranes. Although redox enzymes have been purified previously from plasma membranes of rat liver, pig liver, Ehrlich tumor, HeLa cells, or human erythrocytes (22, 36–39), this is the first report of purification and characterization of a doxorubicin-inhibited NADH-quinone (CoQ0) reductase from rat liver plasma membranes. The findings demonstrate it to be a unique reductase that is distinct from NADH-cytochrome b5 reductase. Preparation of plasma membranes by two-phase partition yielded highly pure plasma membranes, which is essential to prevent contamination of endogenous membranes for characterization and purification of plasma proteins (40).

NADH-cytochrome b5 reductase (34 kDa) has been known to be present on the cytoplasmic surface of the plasma membrane (23), and it has an NADH-ferri cyanide reductase activity, but it was not inhibited doxorubicin (22). Cathepsin D has been used to remove NADH-cytochrome b5 reductase from plasma mem-
branes of erythrocytes as a soluble form without losing its activity (24). Selective digestion of NADH-cytochrome b₅ reductase by cathepsin D and resistance of doxorubicin-inhibited NADH-quinone reductase to cathepsin D digestion were critical to unmask and identify the doxorubicin-inhibited NADH-quinone reductase of rat liver plasma membranes. After cathepsin D treatment, the remaining NADH-ferricyanide reductase activity was about 7% of the initial activity of the untreated plasma membranes, and the activity was inhibited by doxorubicin.

Anthracycline antitumor drugs inhibit cell growth by interaction with DNA or RNA. However, there are analogs of doxorubicin that do not react well with DNA, yet they inhibit cell growth. Israel et al. (41) have shown that N-trifluoroacetyl-14-valerate doxorubicin (AD32) does not intercalate with DNA but does inhibit cell growth (41), NADH-ferricyanide reductase (42) and of plasma membranes. It has been shown that doxorubicin is cytotoxic even without entering cells and targets the plasma membrane (43). Transferrin conjugates of doxorubicin are cytotoxic without intercalating nuclear DNA (19), target primarily plasma membranes (44), and inhibit plasma membrane oxidoreductase of K562 cells (45). Sun et al. (6, 7, 42) reported that ferricyanide stimulated HeLa cell growth and that doxorubicin and its anthracycline derivatives inhibited cell growth as well as NADH-ferricyanide reductase activity.

Three chromatographic steps enabled us to purify cathepsin D-resistant and doxorubicin-inhibited NADH-quinone reductase from rat liver plasma membranes with an apparent molecular mass of 57 kDa, which differs from the 32-kDa NADH dehydrogenase purified from Ehrlich tumor cells (36), the 34-kDa coen-

FIG. 5. Localization (A) and quantification of NADH-quinone reductase (B). A, K562R cells were incubated with affinity-purified antibody, followed by incubation with rhodamine-labeled goat anti-rabbit antibody. Localization was analyzed by fluorescence microscopy. The antibody reacted with the external surface of the plasma membrane as patches. B, K562 and K562R cells were incubated with affinity-purified anti-NADH-FeCN/quinone reductase antibody, followed by incubation with fluorescein isothiocyanate-labeled goat anti-rabbit antibody. Quantitation was by flow cytometry. Pre-immune IgG did not react with NADH-FeCN/quinone reductase (a and c), but affinity-purified anti-NADH-FeCN/quinone reductase antibody reacted with NADH-FeCN/quinone reductase (b and d). Reaction with K562R cells was even greater.
zyme Q reductase from pig liver plasma membranes (39), the 40-kDa oxido- 
reductase purified from human erythrocyte plasma membrane (37), the 33.5-kDa protein with capsaicin-inhibited 
NADH-quinone reductase activity from total defined culture media conditioned 
by growth of HeLa cells (38), the 45-kDa protein from plasma membranes of spinach leaves (46), and the auxin-stimu-
lated NADH oxidase purified from soybean plasma membranes (36, 52, and 72-kDa complex) (47). The partial amino 
acid sequence of the NADH-quinone reductase revealed no se-
quence homology to any known proteins and appears to be 
unique.

The purified NADH-ferricyanide reductase also had an 
NADH-quinone reductase activity, which suggests that the 
natural substrate of the enzyme is coenzyme Q. Coenzyme Q 
has been found in the plasma membranes of HeLa cells (48– 
50), and the redox system utilizing coenzyme Q as a substrate 
has been reported to be present in the plasma membrane (29, 
39, 51, 52). NADH-diferri transferrin reductase is also present in 
the plasma membranes (28, 53), and its activity is inhibited 
by doxorubicin (6). Affinity-purified antibody showed that the 
NADH-quinone reductase was localized to the plasma mem-
brane of both K562 and K562R cells.

When these studies are combined with the fact that four of 
the most commonly used anticancer drugs (doxorubicin, bleo-
mycin, actinomycin D, and cis-platinum) can inhibit the 
NADH oxidase purified from soybean plasma mem-
brane (37), the 33.5-kDa protein with capsaicin-inhibited 
zyme Q reductase from pig liver plasma membranes (39), the 
Peroxidase oxidase of the plasma membrane electron transport 
systems of the plasma membrane are not simply 
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