Journal of Bioenergetics and Biomembranes, Vol. 30, No. 5, 1998

The Sulfonylurea-Inhibited NADH Oxidase Activity of HeLa Cell Plasma Membranes has Properties of a Protein Disulfide–Thiol Oxidoreductase with Protein Disulfide–Thiol Interchange Activity

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Received February 9, 1998

Plasma membrane vesicles of HeLa cells are characterized by a drug-responsive oxidation of NADH. The NADH oxidation takes place in an argon or nitrogen atmosphere and in samples purged of oxygen. Direct assay of protein thiols by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent), suggests that protein disulfides may be the natural electron acceptors for NADH oxidation by the plasma membrane vesicles. In the presence of NADH, protein disulfides of the membranes were reduced with a concomitant stoichiometric increase in protein thiols. The increase in protein thiols was inhibited in parallel to the inhibition of NADH oxidation by the antitumor sulfonylurea LY181984 with an EC_{50} of ca. 30 nM. LY181984, with an EC_{50} of 30 nM, also inhibited a protein disulfide–thiol interchange activity based on the restoration of activity to inactive (scrambled) RNase and thiol oxidation. The findings suggest that thiol oxidation, NADH-dependent disulfide reduction (NADH oxidation), and protein disulfide–thiol interchange in the absence of NADH all may be manifestations of the same sulfonylurea binding protein of the HeLa plasma membrane. A surface location of the thiols involved was demonstrated using detergents and the impermeant thiol reagent *p*-chloromercuriphenylsulfonic acid (PCMPS). The surface location precludes a physiological role of the protein in NADH oxidation. Rather, it may carry out some other role more closely related to a function in growth, such as protein disulfide–thiol interchange coupled to cell enlargement.

KEY WORDS: NADH oxidase; NADH: protein disulfide reductase; protein disulfide–thiol interchange; plasma membrane; cancer; antitumor sulfonylureas; thiols; HeLa cells.

INTRODUCTION

Morré *et al.* (1995a) described a binding activity for the tritiated antitumor sulfonylurea [*N*-(4-methylphenylsulfonyl)-*N*′-(4-chlorophenyl)urea] ([^{3}H]LY181984) of isolated vesicles of plasma membranes from HeLa cells. The binding protein was subsequently shown to correlate with a 34-kD protein band associated with plasma membrane NADH oxidase activity (Morré *et al.*, 1995e, review by Lüthje *et al.*, 1997) and one or more reactive thiols protected against reaction with the thiol reagent *N*-ethylmaleimide (NEM) upon binding the antitumor active sulfonylurea LY181984 (Morré *et al.*, 1995d). The LY181984-binding protein with LY181984-inhibited NADH oxidase activity was demonstrated to be an ectoprotein of the plasma membrane based on analyses of plasma membrane vesicles of known orientation (Morré, 1995). Being an ectoprotein, the LY181984-inhibited NADH oxidase was shed into culture media, conditioned by growth of HeLa cells where

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0145-479X/98/1000-0477$15.00/0

the LY181984-inhibited NADH oxidase and the [^{3}H]LY181984 binding activity copurified with a 33.5-kD protein band (Morré *et al.*, 1996).

When inhibited by sulfonylurea, the cells failed to enlarge and, after approximately 72 h, underwent apoptosis as evidenced from morphological appearance and DNA fluorescence (Morré and Morré, 1995a). That the cell surface site involved inhibition of NADH oxidation and growth was confirmed through the use of an active antitumor analog to LY181984 linked to α-cyclodextrin to form an impermeant conjugate (Kim *et al.*, 1997). The impermeant sulfonylurea, on a molar sulfonylurea basis, was actually more efficacious in the inhibition of NADH oxidation by plasma membrane vesicles and of growth of HeLa cells than was the free sulfonylurea.

Since it was unlikely that an NADH site at the external surface of HeLa cells would ever come in contact with micromolar levels of reduced pyridine nucleotide, some function other than a role in NADH oxidation was sought.

One possibility for an alternative function was provided by protein disulfide–thiol interchange. An active site thiol of the NADH oxidase of the HeLa cell plasma membrane was first indicated by the sensitivity of the NADH oxidase activity of HeLa plasma cell membrane vesicles to reaction with the thiol reagent NEM (Morré *et al.*, 1995d) and other thiol reagents (Morré and Morré, 1995b). The protein disulfide–thiol interchange activity inhibited by the antitumor sulfonylurea LY181984 subsequently was demonstrated based on the restoration of activity to inactive (scrambled) pancreatic RNase (Morré *et al.*, 1997a). The changes in thiols and disulfides that occurred in stoichiometric proportions to the reducing equivalents coming from NADH suggested that protein disulfides serve as acceptors for growth factor-stimulated NADH oxidation by plasma membrane vesicles in plants, i.e., the protein functions as a NADH–protein disulfide reductase (Chueh *et al.*, 1997). The latter may be related to low levels of a protein disulfide isomerase-like activity reported at the cell surface (Mandel *et al.*, 1993) and potentially involved in lentivirus infection (Ryser *et al.*, 1994).

A function of the cell surface NADH oxidase–thiol interchange protein (NOX/TIP) in the cell enlargement phase of growth has been indicated. Normally, following division, cells must enlarge to reach some minimal size before they can divide again (Baserga, 1985). When the cell surface NOX/TIP is inhibited, cells are unable to enlarge normally and growth ceases (Morré and Morré, 1995a; Vaillant *et al.*, 1996; Wolvetang *et al.*, 1996; Dai *et al.*, 1997). When NOX activity is accelerated, cell enlargement also is accelerated and the cell cycle time is correspondingly shortened (Brightman *et al.*, 1988, 1992)

A role of the plasma membrane NADH oxidase in copper reduction at the mammalian cell surface also has been suggested (Van den Burg and McArdle, 1994). A role for cell surface copper reductases in neurodegenerative diseases is also an intriguing possibility (Multhaup *et al.*, 1996).

The "mitochondria theory of aging" (Miquel *et al.*, 1980) proposes that accumulation of somatic mutations of mitochondria DNA (mt DNA), induced by continuous exposure to free radical attack, leads to error in mtDNA-encoded polypeptide chains. The consequence of these alterations, which exclusively affect four mitochondrial enzyme complexes involved in energy conservation, would be defective electron transfer and oxidative phosphorylation (Lenaz *et al.*, 1997). One means to compensate for mitochondrial defects of aging and age-related diseases, would be an upregulation of plasma membrane electron transport from NADH to oxygen or to some natural membrane acceptor. It has recently been shown that the plasma membrane NADH oxidase by recycling NADH to generate ATP via glycolysis is responsible for the survival of cell lines lacking mitochondria (Vaillant *et al.*, 1996; Wolvetang *et al.*, 1996). The resultant electron transfer would provide a means to regenerate oxidized pyridine nucleotide to permit supplemental ATP regeneration by substrate-level phosphorylation.

In contrast to the NADH oxidase activities of HeLa (Morré *et al.*, 1995a) and hepatoma plasma membranes (Bruno *et al.*, 1992), which are in part constitutively activated, the NADH oxidase activity of nontransformed membranes is growth factor- and hormone-responsive (Brightman et al., 1988, 1992). An H_2O_2-generating NADH oxidase of human lung activated by transforming growth factor beta has been reported (Thannickal and Fanburg, 1995). Therefore, the more general catalysis of disulfide–thiol interchange among membrane proteins in the absence of NADH related to growth and growth control may be the more physiologically relevant function of the plasma membrane NADH oxidase (Morré, 1994).

MATERIALS AND METHODS

Growth of Cells

HeLa S cells with serum were grown in Joklik-modified minimal essential medium (S-MEM) supple-

mented with glutamine (244 mg/L), phosphate (1.3 g/L Na_2HPO_4), gentamicin sulfate (50 g/L), sodium bicarbonate (2 g/L), and 5% horse serum. Cells were collected by centrifugation for 15 min at 2500 rpm.

Purification of Plasma Membranes

The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 30 min to swell the cells. Homogenization was with a Polytron homogenizer for 40 s at 10,000 rpm using a ST-10 probe and 7-ml aliquots to achieve at least 90% cell breakage without breakage of nuclei. Cell breakage was monitored by light microscopy.

The homogenates were centrifuged for 10 min at 175 × *g* to remove unbroken cells and nuclei. The supernatant was centrifuged a second time at 1.4×10^6 g/min to prepare a plasma membrane-enriched fraction. Plasma membrane-enriched pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of 1 ml per pellet from approximately 5×10^8 cells. The resuspended membranes were then loaded onto the two-phase system constituted on a weight basis and consisting of 6.6% (w/w) dextran T-500 (Pharmacia) and 6.6% (w/w) polyethylene glycol 3350 (Fisher) in a 5 mM potassium phosphate buffer (pH 7.2) for the aqueous two-phase separation as described (Morré and Morré, 1989; Morré *et al.*, 1994). The purity of the plasma membrane was determined to be > 90% by electron microscope morphometry. The yield was about 2 mg plasma membrane protein from 10^9 cells.

Growth Measurements

Cells were treated in 35 × 10 mm plastic dishes in 2.5 ml culture medium. Sulfonylureas were dissolved in dimethyl sulfonide (DMSO) and added in 2.5 μl (of DMSO) to yield a final concentration of 0.1%. Epidermal growth factor (EGF) (10 nM) was added in 1000-fold concentrated aqueous solution 15 min later. Controls received 2.5 μl of DMSO. Growth was determined from cell numbers estimated after 24, 48, 72, and 96 h of treatment.

NADH Oxidase Activity

The assay for the plasma membrane NADH oxidase was in 50 mM Tris-Mes buffer (pH 7.0), 150 μM NADH, and 1 mM potassium cyanide, the latter to inhibit any mitochondrial NADH oxidases from contaminating the plasma membranes. The assay was started by the addition of 0.1 mg of plasma membrane protein (50 μl). The reaction was monitored by the decrease in the absorbance at 340 nm using a Hitachi Model U3210 or a SLM DW-2000 spectrophotometer, with stirring, at 37°C. The change of absorbance was recorded as a function of time by a chart recorder. The specific activity of the plasma membrane was calculated using a millimolar absorption coefficient of 6.21 cm^{-1}.

Assays were initiated by addition of NADH. Following the addition of NADH, and for each subsequent addition, the assays were continued for 10 min with steady state rates between 5 and 10 min being reported.

Incubation of Plasma Membrane Vesicles with LY181984

Plasma membranes (100–200 μg) resuspended in 200 μl of homogenization medium were incubated with or without varying concentrations of LY181984 for 0, 10, 20, or 30 min at 37°C. At the times indicated, aliquots of 50 μl were removed and centrifuged immediately for 3 min at 15,000 × *g* (Eppendorf Model 5414) for determination of NADH oxidase activity, thiols, or disulfides. The supernatants were removed and the tube completely drained of liquid. Thiols or disulfides were determined as follows.

Determination of Thiols Using 5,5′-Dithiobis-(2-nitrobenzoic Acid) (DTNB; Ellman's Reagent)

Plasma membranes (50 μg) resuspended in the incubation medium or in water following centrifugation as described above to recover the plasma membranes were combined with 50 μl of 10 mM DTNB and incubated 20 min at room temperature. The membranes were then diluted with 2.5 ml of 0.1 M sodium phosphate, pH 8.0. Absorbance was determined using a Shimadzu UV-160 (Columbia, MD) double-wavelength spectrophotometer at 412 nm with reference at 520 nm. Thiol content was estimated from a cysteine standard curve determined in parallel for each assay. Values for control samples without membranes were equivalent to the reagent blank. The absorbance of the reagent blank (= control samples) was subtracted for each set of determinations. Standards were unchanged over a 30-min incubation with or without LY181984.

Results were expressed as nmoles thiol/mg protein based on a cysteine standard. Reagent and plasma membrane blanks were subtracted to approximate absolute initial thiol levels exposed to DTNB at the cell surface on a protein basis. For the tables and figures, where time-dependent changes in thiols were measured, values at $t = 0$ were subtracted to facilitate comparisons among experiments on a protein basis.

Determination of Disulfides using NTSB

The procedure for disulfide determination was as outlined above with the exception that the plasma membranes (50 μg) were combined with 2.5 ml of 2-NTSB assay solution and incubated for 25 min at room temperature according to Thannhauser *et al.* (1984). The NTSB stock solution was prepared as follows: 0.1 g DTNB was dissolved in 10 ml of 1 M Na_2SO_3 at 38°C and the pH adjusted to 7.5. Oxygen was bubbled through the solution for 10 h. The assay solution was prepared by diluting the stock solution 1:100 with a solution containing 0.2 M Tris, 0.1 M Na_2CO_3, and 3 mM EDTA. The pH was adjusted to 9.5. Increase of absorbance upon liberation of NTSB was measured at 412 nm and expressed as nmoles disulfide/mg protein. Disulfide content was estimated from a cystine standard curve determined in parallel for each assay. Values at $t = 0$ were subtracted.

Protein

Proteins were determined by the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985). Standards were prepared with bovine serum albumin.

RESULTS

Activity Not Inhibited by Cyanide or an Argon or Nitrogen Atmosphere

Unlike those NADH oxidase activities where oxygen is the acceptor of electrons, the oxidation of NADH by isolated vesicles of plasma membranes from HeLa cells was largely unaffected by an argon or nitrogen atmosphere (Table I). A lack of inhibition by cyanide has served as an important criterion to distinguish the plasma membrane oxidase from that of mitochondria where NADH oxidation is cyanide sensitive (Moya-Camarena *et al.*, 1995). The activity also is insensitive to inhibition by rotenone or antimycin + myxothiazol, all tested at 1 μM final concentration. Purging the cuvette of oxygen or use of oxygen-purged solutions in combination with an argon or nitrogen atmosphere, as with an argon or nitrogen atmosphere alone, reduced but did not eliminate NADH oxidation.

Table I. Rate of NADH Oxidation by Isolated Vesicles of Plasma Membranes[a]

Addition	NADH oxidation (nmol/min/mg protein)
None	1.2 ± 0.06[b]
KCN	1.1 ± 0.03[b]
Nitrogen atmosphere	1.0 ± 0.05[b]
Argon atmosphere	1.1 ± 0.18[b]

[a] Plasma membranes prepared from HeLa cells. Note lack of response to cyanide and argon and nitrogen atmospheres.
[b] Values are averaged from at least three repetitions with different plasma membrane preparations ± S. D. Numbers followed by the same letter are not significantly different ($p < 0.001$).

Oxygen Consumption Measurements

Measurements of oxygen consumption using an oxygen electrode (Morré and Morré, 1989) also were indicative of some acceptor other than oxygen being involved (Table II). The activity was resistant, as well, to 150 μM salicylhydroxamic acid (SHAM) (Table II), an inhibitor of the alternate pathway of NADH oxidation exhibited by some mitochondria of most higher plants (Siedow and Moore, 1993) and many eukaryotic microorganisms (Sakajo *et al.*, 1991).

The HeLa plasma membrane vesicles did exhibit a slow rate of oxygen uptake. This oxygen uptake was inhibited by the sulfonylurea, LY181984 (Fig. 1), and may represent oxygen consumed in protein thiol oxidation (see below).

Inhibition of Oxidation of Thiols by LY181984 Determined by Reaction with DTNB

Direct determinations of thiols using DTNB showed a rapid and sustained decrease in the absence of NADH (Fig. 2). This presumably represented the autooxidation of protein thiols. The decrease was linear at about 2 nmol/min/mg protein and sustained for at

Table II. NADH Oxidation and Oxygen Uptake by Plasma Membrane Vesicles Prepared from HeLa Cells[a]

Addition	NADH oxidation (nmol/min/mg protein)	1/2 O_2 consumption (nmol/min/mg protein)
None	0	1.2 ± 0.2
+ NADH, 150 μM	3.0 ± 0.1	2.9 ± 0.1
+ NADH, 150 μM + SHAM, 100 μM	3.8 ± 0.4	4.4 ± 1.1

[a] Oxygen uptake was determined using an oxygen electrode (Siedow and Moore, 1993) in collaboration with the laboratory of Professor James Siedow, Duke University, Durham, North Carolina. Assays were in the presence of 100 μM GSH. Results are averages ± S.D. SHAM, salicylhydroxamic acid.

least 30 min of incubation. Upon addition of NADH, the decline in protein thiols was prevented and, in some instances, actually increased.

The stoichiometry of change in thiols comparing the absence and presence of NADH was found to be about 2 nmol/min/mg protein, which is approximately twice the rate of NADH oxidation of 1 nmol/min/mg protein. The decrease in disappearance of thiols resulting from NADH addition was inhibited by the active antitumor sulfonylurea LY181984 but not by the structurally related, but inactive, analog, LY181985. In the presence of the LY181984, thiol oxidation was inhibited (Fig. 2) as was the slowing by NADH of thiol disappearance (oxidation), again in stoichiometric proportions.

The dose response of the thiol oxidation showed a marked sensitivity of response to inhibition by LY181984 (Fig. 3). In the absence of NADH, the decrease in thiols over 15 min was 20 ± 4 nmol/mg protein (Fig. 3), which represented approximately 3% of the total membrane thiols determined after complete solubilization of the membrane with 1% Triton X-100

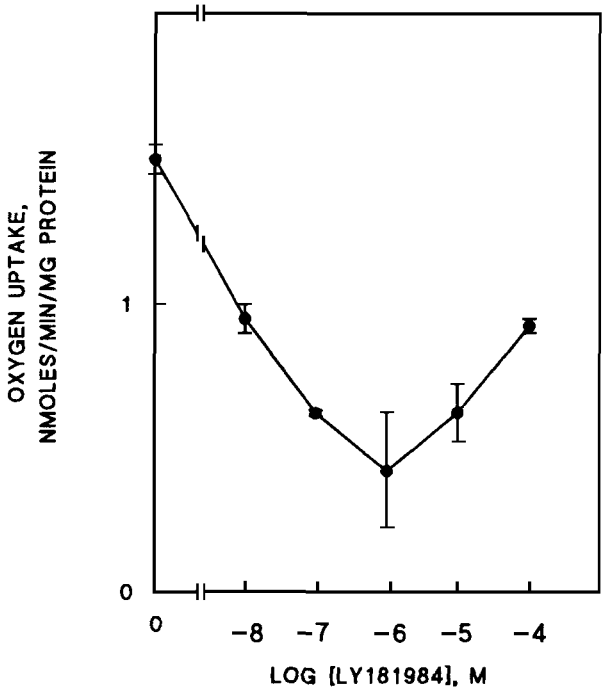

Fig. 1. Oxygen uptake by plasma membrane vesicles prepared from HeLa cells as a function of concentration of LY181984. Oxygen uptake was determined using an oxygen electrode as described in Table II. Results are averages of determinations from three different plasma membrane preparations ± S.D.

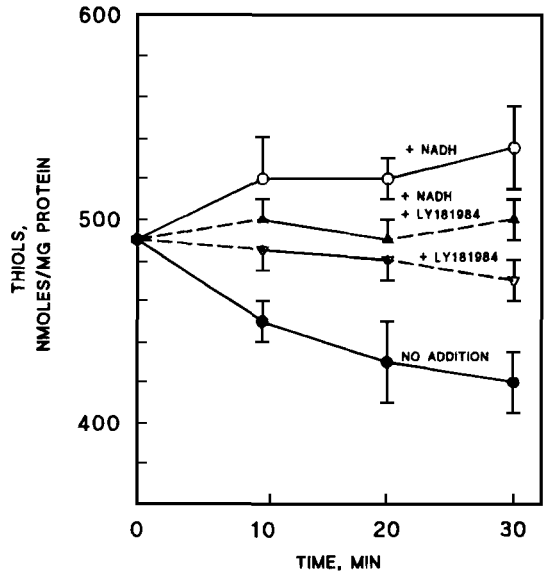

Fig. 2. Plasma membrane thiols accessible to reaction with DTNB (Ellman's reagent) of isolated plasma membrane vesicles as a function of time in response to 150 μM NADH and 10 μM LY181984 (active). Results with 10 μM LY181985 (inactive) were similar to NADH alone (not shown). The sulfonylureas were added in DMSO (0.1% final concentration) compared to 0.1% DMSO (no addition). Results are averages of determinations from five different plasma membrane preparations ± S.D.

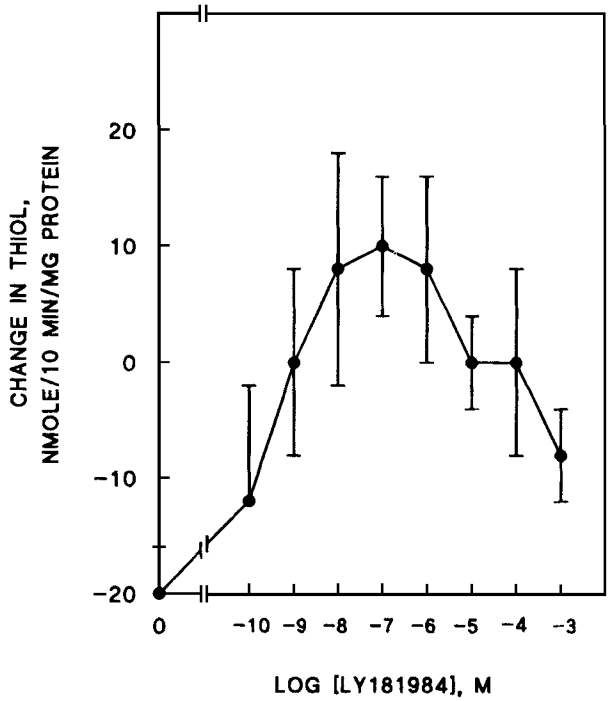

Fig. 3. Response of surface thiol content of HeLa plasma membrane vesicles treated in the absence of NADH as a function of LY181984 concentrations. Assays were performed with 0.15 mg of membrane protein. Membranes were prepared and stored at −70°C prior to assay. Results are averages from determinations from five different plasma membrane preparations ± S.D.

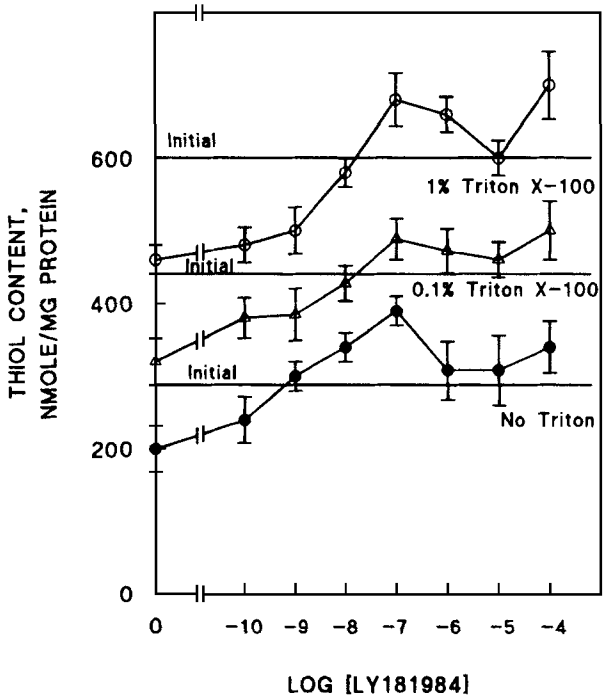

Fig. 4. LY181984-inhibited decrease in thiol content of plasma membrane vesicles isolated from HeLa cells as a function of LY181984 and Triton X-100 concentration. Intact membrane vesicles were incubated with and without LY181984 at the concentrations shown. The vesicles were then collected by centrifugation and the supernatants discarded. The vesicles were resuspended and different amounts of Triton X-100 (0, 0.1, and 1%) were added to progressively expose thiols both at the membrane surface (0% Triton X-100) and within the membrane (1% Triton X-100) prior to chemical determination of thiol content by reaction with DTNB. Triton X-100 at the concentrations used did not interface with the assay of thiols, although it was necessary to subtract a separate blank containing Triton X-100 and membranes only for each of the three Triton X-100 concentrations used. The values for no LY181984, which apply to all LY181984 concentrations, are indicated by the parallel lines. Values are averages from three experiments ± S.D.

(cf. Fig. 4). The decrease in membrane thiols was prevented by 1 nM LY181984 and, at higher concentrations, there was a small, but insignificant, net reduction in membrane thiols (Fig. 3).

Thiols Increased by Incubation with NADH Located at or Near the External Membrane Surface

The thiols increased by incubation with NADH appeared to be those accessible to DTNB at or near the membrane surface. The increase in DTNB-reactive thiols in response to LY181984 was essentially unchanged as the membranes were incrementally treated by concentrations of 0, 0.1, and 1% Triton X-100 (Fig. 4). In contrast, total thiols reactive with DTNB were incrementally increased by the treatment with detergent.

The membrane-impermeant thiol reagent PCMPS was employed as well. The NADH (Fig. 5) and thiol (not shown) oxidation with the isolated plasma membrane vesicles both were inhibited by reaction with this impermeant reagent to approximately the same extent (60%) as with LY181984. The plasma membrane vesicles used in these studies were determined previously to be > 80% right side-out (Morré, 1995).

Protein Disulfides Decreased by Treatment and NADH in Proportion to the Increase in Protein Thiols

When protein thiols plus disulfides were estimated by use of NTSB, according to the method of

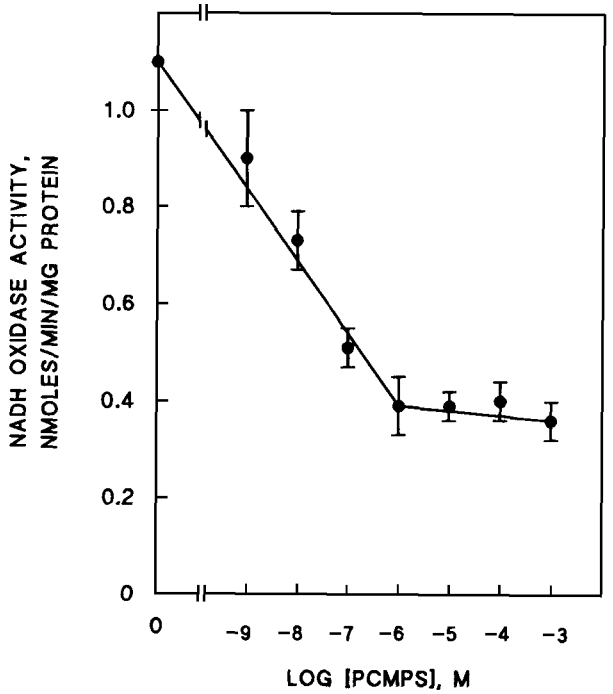

Fig. 5. The impermeant thiol reagent, PCMPS inhibited partially the NADH oxidase activity of isolated plasma membrane vesicles to nearly the same extent (60%) as did the LY181984. The EC_{50} for this inhibition was approximately 10 nM PCMPS. Results are averages of determinations from three different plasma membrane preparations ± S.D.

Thannhauser *et al.* (1984), the plasma membrane levels remained constant with NADH addition, indicating that protein disulfides changed in inverse proportion to changes in thiol.

Under steady-state conditions between 10 and 20 min, a net increase in thiols (and corresponding decrease in disulfides) of 2.8 ± 1.6 nmol/min/mg protein was measured when averaged from three experiments. This compared to a specific activity of NADH oxidation measured from the same membrane preparations of 1.6 ± 0.12 nmol/min/mg protein.

Inhibition by LY181984 of Growth of HeLa Cells and NADH Oxidation, Thiol Oxidation, and Protein Disulfide–Thiol Interchange with HeLa Plasma Membrane Vesicles Are Correlated

As summarized in Fig. 6, the inhibition of growth, NADH oxidation, thiol oxidation, and protein disulfide–thiol interchange by the active antitumor sulfonylurea LY181984 are correlated each with an EC_{50} of about 30 nM. This latter value correlates closely with the K_d of sulfonylurea binding to vesicles of HeLa cell plasma membranes of 30 nM, determined previously (Morré *et al.*, 1995c).

Reoxidation of Protein Thiols May Be Rate Limiting to NADH Oxidation

The oxidation of NADH by isolated vesicles of HeLa cell plasma membrane reached steady state after about 5 min of often very rapid NADH oxidation (Fig. 7). The steady-state rate of NADH oxidation then continued virtually indefinitely in a stirred cuvette. Thus it would appear that the steady-state rate of NADH oxidation is limited primarily by the availability of oxidized protein disulfide cosubstrate. In support of this contention are results of Table III showing that the rate of NADH oxidation is considerably stimulated in the presence of protein oxidants. Especially effective were dilute hydrogen peroxide or oxidized glutathione. These substances did not appear to be substrates as they did not show proportionality over a wide range of concentrations and responses that were given by chemically unrelated mild oxidizing agents such as *tert*-butyl hydroperoxide and *p*-chlorosuccinamide.

DISCUSSION

An NADH oxidase activity of unknown function has been observed for plasma membranes of HeLa cells as an activity inhibited by the antitumor sulfonylurea LY181984 (Morré *et al.*, 1995e). The activity was subsequently shown to be an ectoprotein (Morré, 1995) and a shed form was purified as ca. 34-kD [^{3}H]LY181984 binding peptide from culture media, conditioned by growth of HeLa cells (Morré *et al.*, 1996). The latter was suggested, based on antibody cross-reactivity, to correspond to a ca. 34-kD protein band of HeLa plasma membranes where reactivity of active thiol groups could be prevented by binding of LY181984 (Morré *et al.*, 1995e).

The oxidation of NADH by HeLa cell plasma membranes was not eliminated by an argon atmosphere depleted of oxygen. Thus some component other than oxygen present in the membrane might serve as the electron and proton acceptor for the sulfonylurea-inhibited activity. One possibility suggested by the present findings was protein disulfides. A similar conclusion was reached for the hormone-stimulated

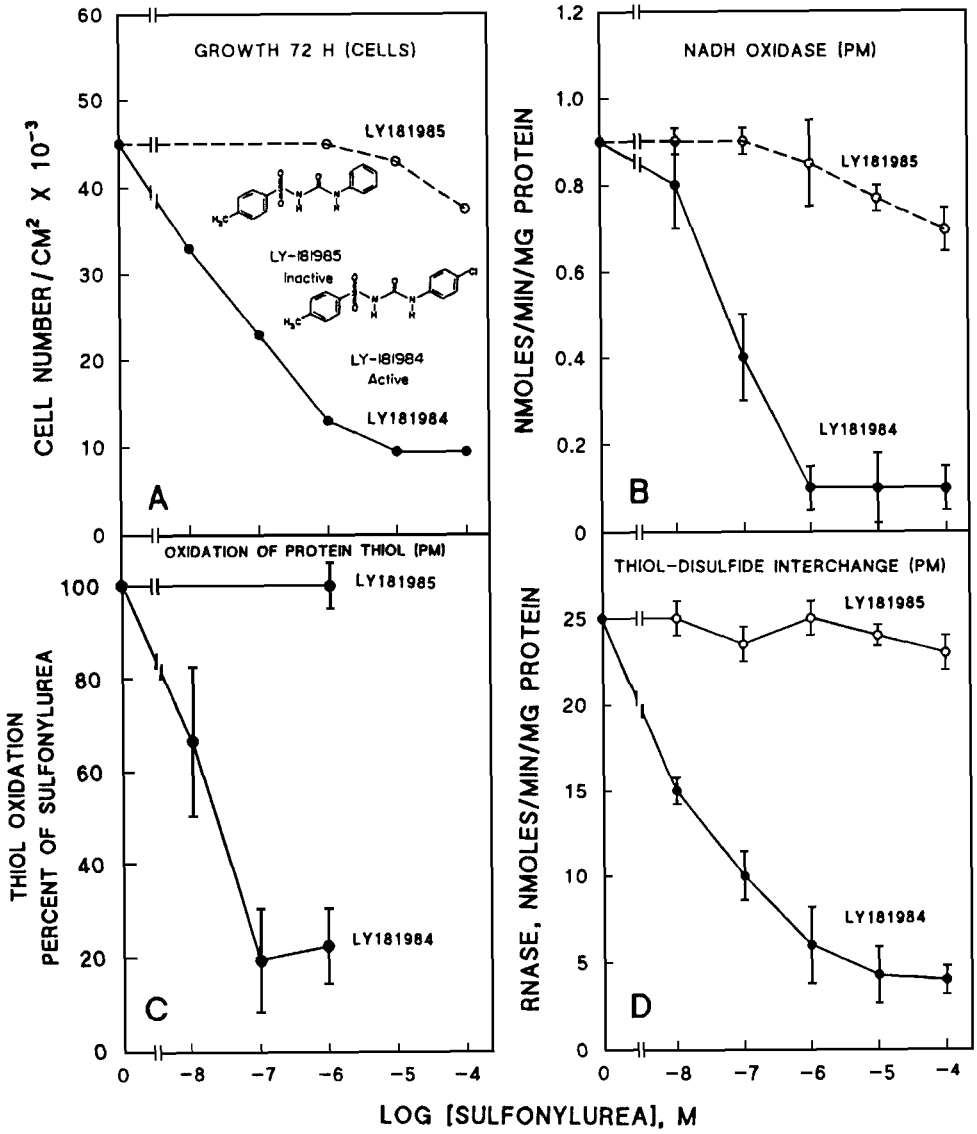

Fig. 6. The correlation between inhibition by the anticancer-active sulfonylurea (LY181984) growth of HeLa cells in culture (A), and oxidation of NADH (NADH oxidase) of isolated vesicles of HeLa plasma membrane (B), the oxidation of thiols measured in the presence of 0.1 mM NADH directly by reaction with DTNB (C), and a protein disulfide–thiol interchange activity estimated from the restoration of activity to scrambled and inactive bovine ribonuclease B (D). The antitumor-inactive LY181985 (one chlorine difference on the B ring) was largely without effect. Growth (A) was measured in the presence of 10 nM EGF as described (Morré *et al.*, 1997b). The inhibition of NADH oxidase (C) is from Morré *et al.* (1995e) and the inhibition of protein disulfide–thiol interchange activity (D) is from Morré *et al.* (1997a).

NADH oxidase of plant plasma membranes (Chueh *et al.*, 1997), which may represent the higher plant counterpart to the sulfonylurea-inhibited activity. In addition, HeLa plasma membranes were found to exhibit a sulfonylurea-inhibited restoration of activity to inactive scrambled RNase (Morré *et al.*, 1997b). Here, activity was restored as interchain disulfides were reformed under conditions of reduction followed by reoxidation under renaturing conditions. A role for the sulfonylurea-inhibited NADH oxidase in the restoration of activity to scrambled RNase would be consistent with experiments where the NADH oxidase activity was shown to be inhibited by thiol reagents (Morré and Morré 5b).

If the activity does, in fact, represent a protein disulfide–thiol interchange, then the external NADH oxidase activity is unlikely to represent a conventional

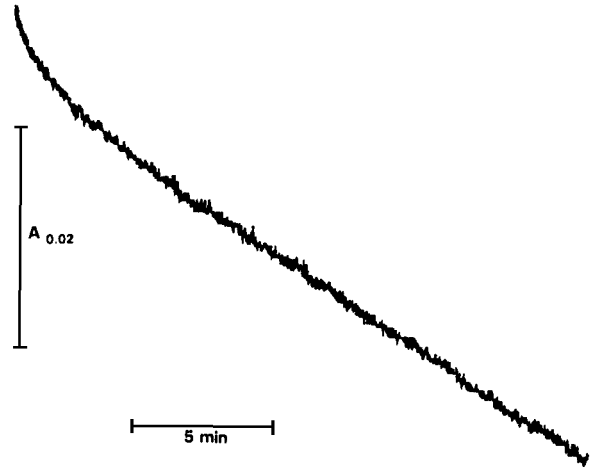

Fig. 7. Kinetics of NADH oxidation were determined as the disappearance of NADH measured at 340 nm with 430 nm as reference using an SLM Aminco DW-2000 spectrophotometer in the dual-wavelength mode of operation with continuous stirring and recording over 5- or 10-min intervals as shown. The kinetics exhibited an initial rapid rate of decline (equilibration phase) over the first 5 min followed by an apparent steady-state rate of NADH oxidation. Assays were with 0.25 mg protein.

NADH oxidase. The use of external NADH to measure the auxin-stimulated activity need not represent a normal flow of electrons from cytoplasm to cell surface but rather a convenient method of assay. This would be in keeping with the suggestion of Bienfait and Lüttge (1988) or Møller and Crane (1990) that one of the functions of plasma membrane redox components may be to reduce thiol groups of membrane proteins.

The binding of sulfonylurea as well as inhibition of HeLa cell growth and the inhibition of NADH oxidation by intact cells was enhanced both by sequential treatment with EGF (Morré *et al.*, 1997a) or by reducing conditions (Morré *et al.*, 1995d, 1997b). The growth data of Fig. 6A were obtained by sulfonylurea treatment in combination with EGF. However, similar results have subsequently been obtained by the use of reducing conditions afforded by 1 μM reduced glutathione (Morré *et al.*, 1997b). With oxidizing conditions, specific binding of [^{3}H]LY181984 (Morré *et al.*, 1995d) and inhibition of HeLa cell growth by LY181984 (Mooré *et al.*, 1997b) were greatly reduced or eliminated, compared to results in the presence of reduced glutathione.

A shed form of the sulfonylurea-responsive NADH oxidase, either inhibited or stimulated by the sulfonylurea, appears in the circulation with cancer patients (Morré and Reust, 1997). Under reducing conditions, the activity of patient sera was predominantly inhibited by the sulfonylurea (Morré *et al.*, 1997b). On the other hand, under oxidizing conditions, the activity of patient sera was predominantly stimulated by the sulfonylurea (Morré *et al.*, 1997b).

The reactions catalyzed by the NADH oxidase (NOX) protein (NADH: protein disulfide–thiol oxidoreductase activity or protein disulfide–thiol interchange activity) are summarized in Fig. 8. In the presence of NADH, the activity appears to be that of

Table III. Response of the NADH Oxidase Activity of Plasma Membranes Vesicles from HeLa cells to Oxidizing and Reducing Conditions[a]

Addition	NADH oxidation (nmol/min/mg protein)
None	0.9 ± 0.15 (6)
Dithiothreitol, 1 μM	0.75 ± 0.1 (4)
Hydrogen peroxide, 0.003%	1.2 ± 0.15 (4)
Hydrogen peroxide, 0.03%	1.2 ± 0.2 (4)
Reduced glutathione (GSH), 100 μM	0.7 ± 0.1 (6)
Oxidized glutathione (GSSG), 100 μM	1.0 ± 0.1 (6)

[a] Results are averages ± S. D. The number of determinations are given in parentheses. The ratio of activity of GSSG/GSH (n = 6) was 1.4 ± 0.1 and highly significant ($p < 0.001$). The ratio of 0.003% hydrogen peroxide to 1 μM DTT (n = 4) was 1.6 ± 0.3 and also highly significant ($p < 0.001$).

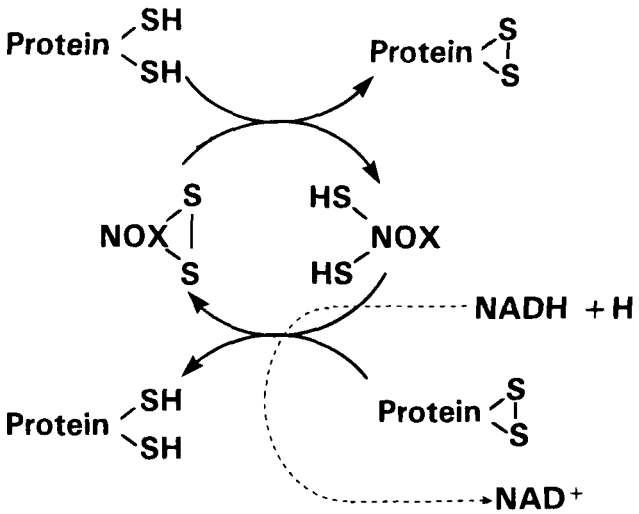

Fig. 8. Two reactions catalyzed by the plasma membrane-associated protein disulfide–thiol interchange and protein disulfide–thiol oxidoreductase activities (NOX protein). The interchange activity (solid arrows) which occurs in the absence of NADH is postulated to play a role in cell growth. The reductase activity (dotted arrow) is suggested to be the reaction measured by the rate of NADH oxidation in the presence of potassium cyanide and in the absence of oxygen and appears to be catalyzed by the same protein as the interchange activity.

a NADH–protein disulfide reductase (dotted arrow). This reaction is inhibited by LY181984. What appears to be rate limiting to this reaction is the reoxidation of protein thiols to form disulfides. For this reason, it may be that the reaction is promoted by the addition of dilute solutions of hydrogen peroxide, oxidized glutathione, or other mild oxidizing agents. With purified enzyme, the activity is stimulated as well by an external source of protein disulfide, e.g., oxidized serum albumin. Also inhibited by LY181984 under reducing conditions was the portion of the protein disulfide–thiol interchange involved in the reoxidation of protein thiols to form disulfide bonds. This is the same reaction that appears to be rate limiting to NADH oxidation. Under oxidizing conditions, NADH oxidation was unaffected or stimulated by the sulfonylurea, which is interpreted to indicate that the partial interchange reaction normally rate limiting to NADH oxidation and protein disulfide interchange is that which serves to reoxidize protein thiols. This reaction also appears to be rate limiting to the restoration of activity to scrambled RNase under reducing conditions where sulfonylurea inhibition is most evident (Morré *et al.*, 1997b).

The nature of the postulated native protein cosubstrate or cosubstrates of the NADH–protein disulfide–thiol oxidoreductase with NADH oxidase activity (NOX) or protein disulfide–thiol interchange activity of the HeLa plasma membrane is unknown. As part of an earlier theoretical consideration of the role of NOX activity in cell enlargement, a mechanism was proposed where the NOX protein catalyzed the formation and breakage of disulfide bonds between interacting proteins related to physical membrane displacements associated with vesicle budding and/or cell enlargement (Morré, 1994). According to this model, the reaction would be limited to some subset of plasma membrane proteins accessible to the NOX protein, perhaps at, or near, the plasma membrane surface. This supposition would be supported by the observations with the effect of Triton X-100 and using the impermeant thiol reagent.

The observation that the drug-responsive form of the NOX activity designated tNOX or tTIP (for tumor-specific thiol interchange protein) appears to be confined to plasma membranes of transformed cells adds yet a further dimension to the present discussion (Morré *et al.*, 1995b). While nontransformed cells and tissue contain a NOX activity designated CNOX or CTIP, this activity is normally not drug responsive (Morré *et al.*, 1995d) or is the CNOX activity inhibited by thiol reagents except at very high concentrations (Kim *et al.*, 1997) in contrast to tNOX, which is particularly sensitive to inhibition by thiol reagents (Morré and Morré, 1995b).

The resistance to thiol reagent inhibition was utilized by Vaillant *et al.* (1996) to distinguish the plasma membrane NADH oxidase from NADH: (acceptor) [ferric salt, 2,6-dichloroindophenol (DCIP), ferricyanide] oxidoreductases of the plasma membrane. The latter were inhibited 88 to 98% by 20 μM PCMPS whereas the NADH oxidase was unaffected at this PCMPS concentration. In contrast to results with rat liver plasma membranes where NADH oxidation was unaffected by PCMB at a concentration of 10 μM, the activity of rat hepatoma plasma membranes was ca. 50% inhibited by 10 μM PCMPS whereas with plasma membranes of HeLa cells, all three thiol reagents tested, DTNB, PCMPS, and NEM, resulted in 50% inhibition of NADH oxidase activity between 0.1 and 1 μM, again without inhibiting NADH oxidation by plasma membrane vesicles isolated from rat liver (Morré and Morré, 1995b).

The circulating form of tNOX/tTIP in serum exhibited a similar pattern of response (Morré and Reust, 1997). When samples of sera from 201 patients with active, late-stage leukemia, lymphoma, or solid tumors (breast, lung, prostate, ovarian, colon, etc.) were analyzed, nearly all exhibited a sulfonylurea-responsive NOX activity. In contrast with sera from healthy volunteers or patients with diseases other than cancer, none exhibited a sulfonylurea-responsive NOX activity. While the sign of the drug response (inhibition vs. stimulation) was dependent upon the redox environment, the absolute specificity was not. Sera from healthy volunteers did not respond to the sulfonylurea either when oxidized, when reduced, or in the absence of added reductant or oxidant (Morré *et al.*, 1997b). Thus, while both normal and cancer cells exhibited a NOX activity at their surface, the cancer form (tNOX) found at the HeLa cell surface was uniquely drug- and thiol reagent-sensitive. Only a portion of the activity, approximately 50%, was drug- and thiol reagent-sensitive and thus may occur at the HeLa cell surface together with CNOX. It was, however, the drug-responsive tNOX that was inhibited by low concentrations of thiol reagents and, as such, its substrate and kinetic properties may be expected to differ somewhat from those of the drug-unresponsive and thiol reagent-resistant CNOX form of the activity.

ACKNOWLEDGMENTS

We thank Lian-Ying Wu for preparing the plasma membranes, Julie Gibson, Kelly Loughlin, Sui Wang, and Lian-Ying Wu for conducting some of the NADH oxidase assays, and Rebecca Bosley, Dagmar Sedlak, Matthew Kelker, and Keri Safranski for thiol determinations. Financial support from Lilly Research Laboratories, Indianapolis, Indiana, Portola Sciences, Portola Valley, California, and Pi Beta Psi Sorority (DMM) is gratefully acknowledged.

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