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A growth factor- and hormone-stimulated NADH oxidase from rat liver plasma membrane

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NADH oxidase activity (electron transfer from NADH to molecular oxygen) of plasma membranes purified from rat liver was characterized by a cyanide-insensitive rate of 1 to 5 nmol/min per mg protein. The activity was stimulated by growth factors (diferric transferrin and epidermal growth factor) and hormones (insulin and pituitary extract) 2- to 3-fold. In contrast, NADH oxidase was inhibited up to 80% by several agents known to inhibit growth or induce differentiation (retinoic acid, calcitriol, and the monosialoganglioside, G_{M3}). The growth factor-responsive NADH oxidase of isolated plasma membranes was not inhibited by common inhibitors of oxidoreductases of endoplasmic reticulum or mitochondria. As well, NADH oxidase of the plasma membrane was stimulated by concentrations of detergents which strongly inhibited mitochondrial NADH oxidases and by lysolipids or fatty acids. Growth factor-responsive NADH oxidase, however, was inhibited >90% by chloroquine and quinone analogues. Addition of coenzyme Q_{10} stimulated the activity and partially reversed the analogue inhibition. The pH optimum for NADH oxidase was 7.0 both in the absence and presence of growth factors. The K_m for NADH was 5 μM and was increased in the presence of growth factors. The stoichiometry of the electron transfer reaction from NADH to oxygen was 2 to 1, indicating a 2 electron transfer. NADH oxidase was separated from NADH–ferricyanide reductase, also present at the plasma membrane, by ion exchange chromatography. Taken together, the evidence suggests that NADH oxidase of the plasma membrane is a unique oxidoreductase and may be important to the regulation of cell growth.

Introduction

The presence of a plasma membrane NAD(P)H oxidoreductase was first hypothesized according to the membrane flow theory [1], which suggested that the components of the plasma membrane were derived from the endoplasmic reticulum (ER) and transported through the Golgi apparatus to the plasma membrane. A transplasma membrane NADH–receptor oxidoreductase activity was subsequently found in many cell types from both plants and animals [2]. The most extensively studied was a NAD(P)H–acceptor oxidoreductase demonstrated with cells [3], or with fractions of plasma membrane purified from liver [4] using impermeant iron compounds such as ferricyanide, as acceptors. The plasma membrane NADPH oxidase of leukocytes is an example of a plasma membrane enzyme for which oxygen is the natural acceptor [5]. This oxidase in leukocytes generates superoxide as a defense against bacteria.

A NADH–acceptor oxidoreductase activity using oxygen as electron acceptor has been demonstrated with isolated plasma membranes as well [6,7]. An increase in this activity by transferrin was demonstrated for plasma membranes isolated from liver but not from hyperplastic liver nodules [8]. At least a portion of the transferrin-stimulated oxidation of NADH previously

Abbreviations: BCA, bicinchoninic acid; CHAPS, 3([3-cholamidopropyl]dimethylamonio)-1-propanesulfonate; DMNMQ, 6-naphthylmercapto-2,3-dimethoxy-1,4-benzoquinone; HOQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; MDMEQ, 6-hexadecyl-2-methoxy-3-ethoxy-5-methyl-1,4-benzoquinone; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; Fe_2TF, diferric transferrin; G_{M3}, sialylgalactosylglucosylceramide; G_{D1a}, sialylgalactosyl-*N*-acetylgalactosaminyl(sialyl)galactosylglucosylceramide; G_{T1a}, sialylsialylgalactosyl-*N*-acetylgalactosaminyl(sialyl)galactosylglucosylceramide; lyso PC, lysophosphatidylcholine; PMSF, α-phenylmethylsulfonyl fluoride.

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ascribed to NADH–diferric transferrin reductase [9] may now be correctly ascribed to this plasma membrane NADH oxidase.

In this report we characterize the NADH oxidase of rat liver plasma membrane as an enzyme with many properties that differ from those of NADH oxidoreductases or the NADPH oxidase also associated with the plasma membrane or NADH oxidases that may be present in mitochondria, for example. Among these unique properties is the activation of the NADH oxidase by growth factors and hormones.

Materials and Methods

Purification of rat liver plasma membrane. The 5000 $\times g$ pellet from the preparation of the Golgi apparatus [10] was the starting material. The fluffy layer which contains the Golgi apparatus fraction was mixed and withdrawn with a 1 mm diameter pipette, and was excluded from the plasma membrane preparations. Cold 1 mM $NaHCO_3$ (5 ml) was added to each tube and the friable yellow-brown upper part of the pellet was resuspended with a pen-brush, leaving the reddish tightly packed bottom part of the pellet undisturbed. The resuspended material was transferred to a centrifuge tube, and a second 5 ml of cold $NaHCO_3$ was added to collect the remaining friable material. The combined resuspended material was homogenized in aliquots of 5 ml each in a 30 ml stainless steel (Duragrind) homogenizer 20 times by hand. The homogenates were combined, diluted with cold 1 mM $NaHCO_3$ (1 : 1 dilution), and centrifuged at 6000 $\times g$ in a HB 4 rotor for 15 min. The supernatant was discarded and the pellet was used for the two-phase separation.

The two-phase system contained 6.4% (w/w) Dextran T-500 (Pharmacia), 6.4% (w/w) poly(ethylene glycol) 3350 (Fisher), and 5 mM potassium phosphate buffer (pH 7.2) [11]. The homogenate (1 g) was added to the two-phase system and the weight of the system was brought to 8 g with distilled water. The tubes were inverted vigorously for 40 times in the cold (4°C). The phases were separated by centrifugation at 750 rpm (150 $\times g$) in a Sorvall HB 4 rotor for 5 min. The upper phases were carefully withdrawn with a pasteur pipette, divided in half and transferred into 40 ml plastic centrifuge tubes and diluted with cold 1 mM $NaHCO_3$ by filling the tubes. The plasma membrane was collected by centrifugation at 10000 $\times g$ in a HB 4 rotor for 30 min. Proteins were determined using bicinchoninic acid (BCA) as an assay reagent [12] with bovine serum albumin as standard.

Electron microscopy. The plasma membrane pellets were fixed in 2% glutaraldehyde in phosphate buffer (pH 7.2). The glutaraldehyde was removed by rinsing three times with 0.1 M phosphate buffer (pH 7.2), and the pellet was dehydrated in an acetone series. Samples were embedded in Epon [13], sectioned, stained with lead, and examined and photographed with a Philips EM/200. For morphometry, a transparent overlay containing lines one cm apart was placed over a micrograph of 35000-times enlargement [14]. Intercepts of membranes with lines on the overlay were counted both for total membrane and for each cell component to be analyzed. Results are reported as intercepts with the plasma membrane per 100 total intercepts with all membranes present. The upper phase from the aqueous two-phase partition system contained 90% plasma membrane as determined from morphometric analyses of micrographs of membrane preparations. The lower phase contained mostly rough endoplasmic reticulum and the inter-phase contained mitochondria (Table I). The yield of the plasma membrane was 2–5 mg per 10 g rat liver as measured by the BCA protein assay.

TABLE I

Composition by electron microscope morphometry of the plasma membrane-rich upper phase and the plasma membrane depleted lower phase after aqueous two-phase partition

Values are means from three different plasma membrane preparations representative of those used in the study ± standard deviations. n.d. = None detected. Trace = < 0.5%.

Cell component	Profiles per 100 membrane profiles	
	Upper phase	Lower phase
Plasma membrane	90 ± 4	1 ± 1
Mitochondria	4 ± 2	3 ± 2
Endoplasmic reticulum	3 ± 1	32 ± 4
Nuclear envelope	trace	2 ± 2
Lysosomes	n.d.	trace
Peroxisomes	trace	trace
Other	3 ± 2	1 ± 0

NADH oxidase activity. The assay for the plasma membrane NADH oxidase was in 40 mM Tris-Mes buffer (pH 7.0), 150 μM NADH in the presence of 1 mM potassium cyanide (KCN), the latter to inhibit any mitochondrial NADH oxidases contaminating the plasma membranes. The assay was started by the addition of 0.1 mg of plasma membrane protein. The reaction was monitored by the decrease in the absorbance at 340 nm with 430 nm as reference, using an SLM DW-2000 spectrophotometer in the dual wavelength mode of operation or a Hitachi Model U3210. 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 an absorption coefficient of 6.21 mM^{-1} cm^{-1} and expressed as nmol/min per mg protein.

O_2 consumption by isolated plasma membrane fractions was measured spectrophotometrically as follows. Hemoglobin from horse blood (0.2 mg, Sigma) was

added to plasma membranes (about 25 μg protein) in solution buffered with 25 mM Tris-Mes (pH 7). The reaction was started by the addition of 150 μM NADH. The simultaneous oxidation of NADH and loss of the oxygenated form of hemoglobin were monitored as the difference between 436 and 344 nm. Reaction rates were recorded with a linear recorder. An absorption coefficient of 65 mM^{-1} cm^{-1} was used for calculation.

Solubilization and purification. Rat liver plasma membranes were prepared by aqueous two-phase partition [11] and stored at −70°C. The protein concentration was adjusted to 2 mg/ml with buffer A (25 mM Tris-HCl (pH 8), 10% glycerin, 1 mM EDTA, and 1 mM PMSF). After 30 min incubation at 4°C, the solution was centrifuged at 95000 × *g* for 30 min to recover the plasma membrane from extracted peripheral proteins in the supernatant. A total of approximately 50 mg protein resuspended in 25 ml of buffer A was mixed with equal volume of CHAPS (4 mg/ml) in buffer A, and the mixture was incubated at 4°C for at least 2 h with stirring. The solubilized plasma membrane was then centrifuged (1 h at 95000 × *g*) and the supernatant used for further purification.

For initial purification by ion exchange chromatography, about 10 g of pre-swollen DEAE-cellulose resin (DE-52 Whatman) was equilibrated with buffer A containing 0.5 mM CHAPS, and packed into a 3 × 15 cm column. Solubilized plasma membrane proteins were loaded and the column was washed with more than one column volume of buffer A with 0.5 mM CHAPS. The column was eluted with 50 ml steps of sodium chloride in buffer A plus 0.5 mM CHAPS (25 mM, 50 mM, 75 mM, 125 mM, 150 mM) and 2 ml fractions were collected.

Results

The plasma membrane preparations utilized in this study have been characterized extensively based on both morphological and enzymatic criteria [11,15]. From morphometric analysis using electron microscopy, the preparations contain 90 ± 4 percent plasma membrane. Contaminants include mitochondria (4%), endoplasmic reticulum (3%) and trace amounts of nuclear envelope, Golgi apparatus, lysosomes, peroxisomes and unidentified membranes (combined total of 3%). Based on analyses of marker enzymes, the contamination by endoplasmic reticulum was estimated to be 3%, that of mitochondria 15% and that of Golgi apparatus 1%. The yield of plasma membranes was estimated to average 18% based on recovery of enzyme markers.

The orientation of the plasma membranes was determined from ATP latency and binding of concanavalin A linked to peroxidase (Con A). Based on morphological analyses [11], the majority of the preparations consisted of plasma membrane sheets stabilized by junctional complexes rather than sealed closed vesicles. Therefore, both membrane surfaces were exposed with nearly equal probability in the preparations and the majority of the plasma membranes present were represented by sheets with both membrane surfaces accessible to impermeant reagents such as ATP, Con A or NADH [11].

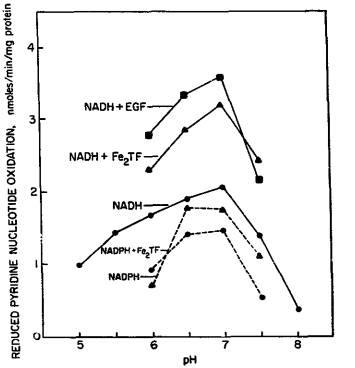

Fig. 1. pH dependence of reduced pyridine nucelotide oxidation by rat liver plasma membranes. Shown are basal rates of oxidation of NADH and the diferric transferrin (Fe_2TF)- and EGF-stimulated rate of NADH oxidation together with the rate of NADPH oxidation and the lack of Fe_2TF stimulation of NADPH oxidation.

Basal activity (in the absence of growth factor) of the NADH oxidase of purified plasma membrane ranged from 0.8 to 2.0 nmol/min per mg protein when measured in 50 mM Tris-Mes buffer (pH 7.0), at 37°C, with 150 μM NADH and in the presence of 1 mM potassium cyanide. The enzyme functions maximally around pH 7.0 in the absence of growth factor (Fig. 1). At pH 7.0 the activity with NAD(P)H as electron donor was approx. 80% that with NADH. The enzyme has no obvious ion requirements. EDTA or EGTA (1 mM) were without effect. Calcium was slightly stimulatory at low concentrations but strongly inhibited at higher, non-physiological concentrations (Fig. 2). The K_m for NADH was 5.2 μM and the V_{max} was 4.4 nmol/min per mg protein (Table II). The activity had an absolute requirement for oxygen. No activity was observed in a system purged of oxygen under argon. The stoichiometry of oxygen reduced to NADH oxidized yielded a $e^-/\frac{1}{2}O_2$ ratio of 2.

Plasma membrane NADH oxidase activity was stimulated over 2-fold by the growth factor diferric trans-

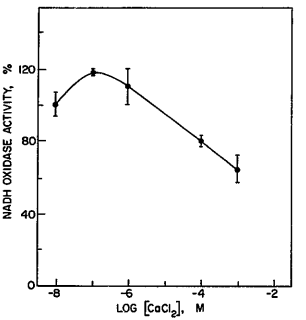

Fig. 2. Response of NADH oxidation by rat liver plasma membranes to added Ca^{2+} added as $CaCl_2$. All assays contained 50 mM Tris-Mes buffer (pH 6.5), 1 mM KCN and 150 μM NADH and 0.1 mg membrane protein. The reactions were initiated by the addition of membrane and calcium was added to the established rate of NADH oxidation.

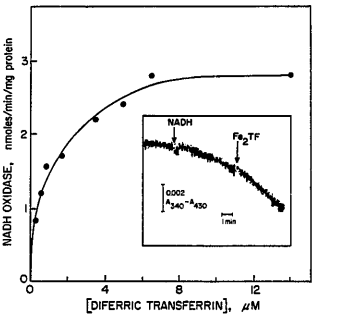

Fig. 3. Oxidation of NADH by rat liver plasma membrane in the presence of diferric transferrin. The inset shows a spectrophotometric trace where reaction was initiated by addition of NADH. After about 5 min 3.4 μM diferric transferrin was added and the accelerated rate was monitored. In the values presented in the figure, the NADH oxidase activity prior to transferrin addition was subtracted from the rate of NADH oxidation after transferrin addition. Absorbance was measured at 340 nm with reference at 430 nm.

ferrin (Fig. 3). The stimulation increased with increasing concentration of transferrin to a maximum at 10 μM. Reaction rates were linear and the response to growth factor occurred without perceptible lag (Fig. 3 inset). Plasma membrane NADH oxidase activity also was responsive to epidermal growth factor, insulin and pituitary extract (Table III). The pH optimum remained at 7.0 when the enzyme was stimulated by diferric transferrin or EGF (Fig. 2). EGF and diferric transferrin increased the K_m from 5 μM to 12 μM and 19 μM, respectively. Retinoic acid and calcitriol, two compounds, which induce differentiation of cells in culture, inhibited NADH oxidase activity at low doses (10^{-7} M and 10^{-10} M, respectively, Table III).

TABLE II

Kinetic parameters of the NADH oxidase of rat liver plasma membranes

NADH oxidase activity was determined at pH 6.5 (37°C) over the range of NADH concentrations 6 to 120 μM. Growth factors, EGF (33 nM) and transferrin (10 μM) were added to establish rates of NADH oxidation and the stimulated rates determined after 10 min. Values for the unstimulated activity are from different membrane preparations ± standard deviations.

Growth factor	K_m (μM)	V_{max} (nmol/min per mg protein)
None	5.2 ± 2.2	4.3 ± 0.9
Epidermal growth factor	11.5	5.8
Diferric transferrin	19.3	8.6

The hormone and growth factor-responsiveness of the plasma membranes declined with increasing animal age. Values presented in Table III are for near optimum concentrations of growth factor or hormone determined from complete dose response curves for plasma membranes prepared from male rats of the Holtzman strain, 100 to 150 g in weight.

Because of literature reports of growth inhibitions by simple sialic acid-containing glycosphingolipids

TABLE III

Effects of growth factors and differentiation agents on NADH oxidase activity of plasma membrane

NADH oxidase activity of plasma membranes isolated from rat liver was assayed directly or after storage at − 70°C. Assays contained 50 mM Tris-Mes (pH 7), 1 mM KCN, 100 μg membrane at 37°C. Effectors were added at the beginning of the reaction or after a basal rate had been established and changes in NADH oxidase activity determined. Values are averages of duplicate or triplicate determinations with different plasma membrane preparations from 100 to 150 g rats.

Addition	Concn.	NADH oxidase (nmol/min per mg protein)
None		0.72 ± 0.06
Epidermal growth factor	27 nM	1.08 ± 0.03
Insulin	0.5 ng/ml	1.17 ± 0.04
Pituitary extract	2.5 μl/ml	1.48 ± 0.15
Retinoic acid	0.1 μM	0.54 ± 0.08
Calcitriol	0.1 nM	0.16 ± 0.04

TABLE IV

Response of redox activities of plasma membranes purified from rat liver to gangliosides and long chain bases

Note the preferential inhibition of the NADH oxidase by the monosialoganglioside G_{M3}. Gangliosides dissolved in ethanol were added to the membranes in buffer mixed virorously, and the ethanol was removed by evaporation under nitrogen. Long chain bases were added from a stock solution in bovine serum albumin (10 mg/ml BSA) and control activities were in the presence of equivalent concentrations of BSA.

Additive	Concn.	Percent of control activity	
		NADH oxidase	NADH–ferricyanide oxidoreductase
Monosialoganglioside, G_{M3}	10 μM	50	92
Disialoganglioside, G_{D1a}	10 μM	85	82
Trisialoganglioside, G_{T1b}	10 μM	105	92
Mixed brain gangliosides	10 μM	70	88
Sphingosine	120 μM	51	76
N-Stearylamine	150 μM	21	86

(gangliosides), several gangliosides were tested for effects on NADH oxidase (Table IV). The activity of the NADH oxidase was inhibited about 50% by the monosialoganglioside G_{M3} but not by the disialoganglioside G_{D1a} or the trisialoganglioside G_{T1b} at 10 μM. Mixed brain gangliosides from the bovine which contain G_{M3}, G_{D1a} and G_{T1b} in approximate molar ratios of 1:125:85 inhibited the NADH oxidase by 30%. In contrast to the effects on NADH oxidase, G_{M3} and the mixed brain gangliosides were without effect on the activity of NADH–ferricyanide oxidoreductase. NADH oxidase was inhibited by long chain bases, compounds not characteristically considered as oxidase inhibitors. Both *N*-stearylamine (150 μM) and sphingosine (120 μM) inhibited NADH oxidase but had little effect on ferricyanide reduction (Table IV).

The growth factor-responsive NADH oxidase of the plasma membrane was distinguished from other oxidase activities by the response of the enzyme to inhibitors and detergents as well. Plasma membrane NADH oxidase differed from mitochondrial oxidase as shown by a lack of inhibition by cyanide and HOQNO and stimulation by several detergents (Tables V and

TABLE V

The effect of detergents on the NADH oxidases of plasma membrane and mitochondria

Detergent	Concn. (%)	NADH oxidase activity (nmol/min per mg protein)	
		plasma membrane	mitochondria
None		1.0	1.0
Sodium cholate	0.4	1.39	0.06
Triton X-100	0.1	1.56	0.35
CHAPS	0.3	1.17	0.18

TABLE VI

Response of the transferrin-stimulated NADH oxidase to inhibitors

Plasma membrane was isolated from rat liver by aqueous polymer two-phase partition. NADH oxidase activity was assayed at pH 7.0 in the presence of 1 mM KCN. Plasma membrane was incubated with inhibitors in the assay mixture for 5 min before addition of NADH to start the assay. Values are from triplicate determinations with two different membrane preparations ± standard deviation or from duplicate determinations (HOQNO and chloroquine), with consistent results. HOQNO = 2-heptyl-4-hydroxyquinoline-*N*-oxide.

Additions	Concn.	NADH oxidase (nmol/min per mg protein)
None		1.0 ± 0.1
Catalase	0.01%	1.13 ± 0.07
Azide	1 mM	1.29 ± 0.19
Antimycin A	2 μM	1.17 ± 0.35
HOQNO	1 μM	1.5
Chloroquine	500 μM	0.08

VI). For example, sodium cholate, at a concentration of 0.4%, stimulated the plasma membrane NADH oxidase 39% and Triton X-100, at a concentration of 0.1%, stimulated the plasma membrane NADH oxidase to 56% over the control. These activations were not time-dependent and served as a measure of the structure-linked latency of the oxidase. The CHAPS-extracted plasma membrane (0.3% CHAPS) fraction also showed only a 17% increase in the NADH oxidase specific activity over that in the plasma membranes.

In contrast to results with plasma membrane, the mitochondria NADH oxidase was inhibited 94% by sodium cholate and 65% by Triton X-100, measured under the same assay conditions as for the NADH oxidase of the plasma membrane. The CHAPS-extracted mitochondria showed an 82% decrease in the NADH oxidase activity relative to that of the mitochondria fraction (Table V). The plasma membrane NADH oxidase also was resistant to inhibition by sodium azide (1 mM), antimycin A (1 μM) and catalase (0.01%) (Table VI). However, NADH oxidase of rat liver plasma membrane was inhibited over 90% by the quinone analogue, chloroquine (0.5 mM).

To further investigate the possible interaction of the plasma membrane NADH oxidase with quinone components of the plasma membrane, several other quinone analogues were tested for effects on NADH oxidation. Pericidin at a concentration of 10^{-7} M was a potent inhibitor (85%) of both the basal and growth factor-stimulated activities of NADH oxidase (Table VII). HDMEQ (30 μg/ml) inhibited the enzyme completely. Addition of coenzyme Q_{10} (10 μM) or the quinone analogue DMNMQ stimulated the activity and coenzyme Q partially restored activity when added in the presence of pericidin.

The plasma membrane NADH oxidase was stimulated by lysophosphatidylcholine (lyso PC) and the free

TABLE VII

Effects of coenzyme Q analogues on NADH oxidase activity of rat liver plasma membrane and the stimulation of NADH oxidase by diferric transferrin (Fe_2TF)

Values are from duplicate determinations with consistent results. HDMEQ = 6-hexadecyl-2-methoxy-3-ethoxy-5-methyl-1,4-benzoquinone. DMNMQ = 6-naphthylmercapto-2,3-dimethoxy-1,4-benzoquinone.

Addition	Concn.	NADH oxidase (nmol/min per mg protein)	
		no Fe_2TF	+17 μM Fe_2TF
None		2.0	6.5
Piericidin A	0.1 μM	0.3	0.3
HDMEQ	30 μg/ml	0.0	0.0
DMNMQ	24 μg/ml	28.5	36.9
Coenzyme Q_{10}	10 μM	5.7	13.2
Piericidin A +coenzyme Q_{10}	0.1 μM + 10 μM	1.5	2.7

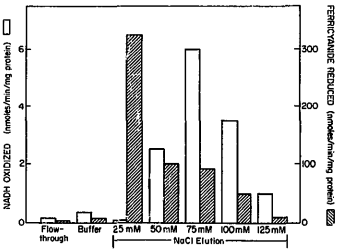

Fig. 5. Separation of NADH oxidase and NADH–ferricyanide oxidoreductase by ion exchange chromotography after solubilization from rat liver plasma membranes with CHAPS. The NADH oxidase (open bars) was retained at low salt elution steps and was released between 50 and 100 mM sodium chloride. In contrast, the bulk of NADH–ferricyanide oxidoreductase was eluted from the column with 25 mM sodium chloride.

fatty acid, linoleate, at 30 μM (Fig. 4). In the presence of 0.1% Triton X-100, the overall NADH oxidase activity was increased but the relative stimulations by the products of phospholipase A_2 were unaffected for lyso PC and increased for linoleate (Fig. 4B). Whereas, the activation by diferric transferrin (Fig. 3) and other growth factors occurred without perceptible lag, the activations by lyso PC and linoleate were time depen-

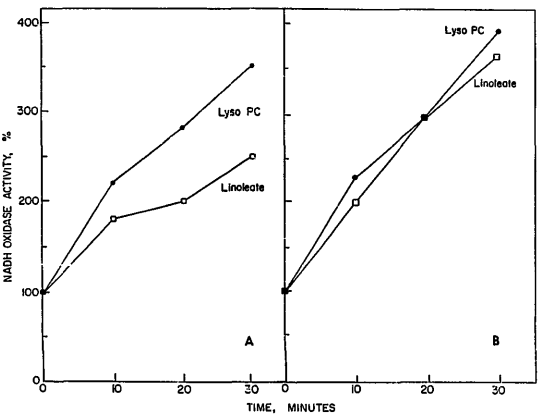

Fig. 4. Effect of linoleate and lysophosphatidylcholine (lyso PC) on the NADH oxidase activity of rat liver plasma membranes. (A) The activation is time dependent and results show relative activities averaged over the first, second and third 10 min of reaction respectively. After 30 min the activity tends to plateau [35]. The specific activity of the NADH oxidase prior to lipid addition was 2.35 ± 0.1 nmol/min per mg protein. (B) Activity measured in the presence of 0.1% Triton X-100. The NADH oxidase activity of the rat liver plasma membranes after solubilization by 0.1% Triton X-100 and before the addition of products of phospholipase A_2 action on phosphatidylcholine, free fatty acid (linoleate) and lysophosphatidylcholine (lyso PC) was 2.62 ± 0.11 nmol/min per mg protein.

dent. Activity increased over 30 min even in the presence of 0.1% Triton X-100 (Fig. 4).

Of the total proteins from the plasma membrane, 75% were extracted by CHAPS at a concentration of 2 mg/ml in buffer A over 2 h at 0°C. The specific activity in the extract of plasma membranes was increased about 1.5-fold over that of the starting plasma membrane preparation.

Proteins solubilized by CHAPS were bound to a DE-52 ion-exchange column and eluted stepwise with sodium chloride (Fig. 5). Most of the NADH oxidase activity was eluted with 50 to 100 mM sodium chloride. The highest specific activity of NADH oxidase was found in the fractions eluted by 75 mM sodium chloride, and was four times that in the CHAPS-extracted plasma membrane. Most of the ferricyanide reductase activity was eluted at 25 mM NaCl. The fractions eluting at 25 mM NaCl had little or no NADH oxidase activity.

Discussion

A growth factor-responsive NADH oxidase is described which may function as part of a redox system at the plasma membrane. Several lines of evidence support the existence of a redox system at the cell surface and a role of this plasma membrane redox system in the control of cell growth.

Impermeable oxidants act as growth stimulators. Studies by Ellem and Kay [16] showed that external ferricyanide could stimulate the growth of melanoma cells when growth factors present in serum were limiting. Ferric sulfate also stimulated the growth of 3T3 cells [17]. Stimulation of cell growth by oxidants was not limited to iron compounds. Growth of HeLa cells was stimulated by impermeant electron acceptors such as hexamine ruthenium III, a trivalent cation, and indigo tetrasulfonate [18]. The use of a series of impermeable indigo sulfonates with different standard redox potentials showed that external oxidants with redox potentials above −125 mV stimulated growth, while those with potentials below this value did not [18].

Tumors and transformed cells have modified NADH oxidase activities. The NADH–ferricyanide oxidoreductase in SV40 virus-transformed cells was reduced compared to untransformed (control) cells [19], although Powis et al. [20] showed that reduction of impermeable indigodisulfonate was more rapid with transformed AKR mouse embryo cells than with untransformed cells. Similarly, the activity of NADH oxidase (electron transfer to oxygen), which was stimulated by growth factor (diferric transferrin) in normal liver plasma membrane, was elevated and no longer responsive to growth factor in transformed tissue [8]. This finding first pointed to the NADH oxidase as a component of the plasma membrane redox system potentially important to growth control in mammalian cells and tissues.

The response of NADH oxidase to growth factors sets this enzyme of the plasma membrane apart from all other oxidoreductases and focuses attention on a potential role in growth control at the plasma membrane. For example, triiodothyronine (T_3), a growth factor of liver cells, was shown early to stimulate the NADH oxidation activity of rat liver plasma membranes [6]. In contrast, NADH dehydrogenase measured with indophenol and cytochrome *c* as acceptors was inhibited by T_3. This implied that the NADH oxidase and the dehydrogenase had at least one component that was different in responsiveness to T_3, and this component was responsible for electron transfer to oxygen. This finding also supported the concept that oxygen was the natural electron acceptor. In other studies, growth of animal cells was shown to require oxygen levels well above those required to support mitochondrial ATP-formation [21]. The NADH–acceptor oxidoreductase in plants has also been related to hormone response and growth control. It has long been recognized that the plant hormone auxin, which stimulates elongation of hypocotyls, causes an increase in oxygen consumption [22]. Furthermore, it was found that auxin stimulated the NADH oxidase in the plasma membrane, at levels that also stimulated growth [23]. The present work shows that the NADH oxidase activity of liver plasma membranes was stimulated by growth factors and hormones other than diferric transferrin including EGF and insulin. The response of NADH oxidase to a number of growth factors and hormones suggests a common mechanism of activation of the enzyme as well as a central role of the enzyme in growth regulation. The mechanism by which these molecules activate NADH oxidase has not been determined. However, some modification of protein structure may be involved. Growth factors caused an increase in the K_m (NADH) and the V_{max} of NADH oxidase. The significance of this alteration is difficult to evaluate since, with a two substrate enzyme, a change in the binding site for one substrate frequently will influence the binding affinity for the other substrate [24,25]. An opposite effect of the growth factors on the K_m for O_2 would be predicted but has thus far not been determined. The activation of NADH oxidase by growth factors was rapid and occurred without a perceptible lag after the addition of growth factor.

Other agents which are known to influence cell growth also influenced NADH oxidase activity of the plasma membrane. Compounds which caused cell differentiation such as retinoic acid and calcitriol, the hormonal form of vitamin D, inhibited NADH oxidase activity. Likewise, G_{M3}, which has been shown to be involved in the regulation of cell growth [26–28], inhib-

ited NADH oxidase. G_{M3}, which is located in the outer leaflet of plasma membrane, also inhibited the activity of a C-type protein kinase [29].

An important consideration of the present work was to establish the NADH oxidase of the plasma membrane as a unique enzymatic activity. The oxidase which catalyzes transfer of electrons from NADH to oxygen was shown to be distinct from the plasma membrane-associated dehydrogenase activity which transfers electrons from NADH to ferricyanide. The partially purified oxidase failed to reduce ferricyanide and the purified reductase exhibited no activity with oxygen as acceptor (Kim, C., Purdue University, unpublished results). Also, the transfer of electrons to ferricyanide was unresponsive to growth factors such as EGF and transferrin and insensitive to the inhibitory action of sphingosine, *N*-stearylamine and the ganglioside, G_{M3}.

NADH oxidase activity cannot be due to another known oxidase activity since the enzyme responded uniquely to a number of common oxidoreductase inhibitors. Insensitivity to cyanide at 1 mM sets NADH oxidase apart from heme-containing oxidases such as cytochrome c oxidase and peroxidases. Peroxidase activity was also ruled out by the lack of inhibition by catalase and the NADH to oxygen stoichiometry of 2.

The plasma membrane NADH oxidase was stimulated by three different detergents, in contrast with the results obtained with the NADH oxidase of mitochondria and the microsomal fractions. The inhibitory effect of detergents has been reported by other researchers on the mitochondrial NADH dehydrogenase [30]. Triton X-100 was also found to inhibit the NAD(P)H oxidase in the microsomal fraction at the same concentration that stimulated the plasma membrane NADH oxidase [31]. The opposite effect of detergents on the NADH oxidase from the plasma membrane and that from mitochondria and of the microsomal fraction, indicates that the NADH oxidase activity in the plasma membrane was not derived from the contamination by endoplasmic reticulum or by mitochondria, and that the plasma membrane NADH oxidase has properties different from those of the mitochondria and of the endoplasmic reticulum (microsomes). The small stimulatory effects of the detergents on the plasma membrane NADH oxidase might be due to the increased permeability of the plasma membrane to NADH and the corresponding increase in available binding sites for NADH.

The stimulation of NADH oxidase by coenzyme Q_{10} and the quinone analogue DMNMQ and the inhibition of activity by other quinone analogues, suggested the involvement of a quinone site. Partial recovery from inhibition by quinone analogues by addition of extra quinone further supported this notion. To what extent the NADH oxidase may function as part of an electron transport chain, coupled in some way to other redox constituents such as the NADH dehydrogenase, has not yet been determined.

Calcium ions had little effect on NADH oxidase activity except at millimolar concentration where inhibition occurred. The stimulatory effect of lyso PC and free fatty acid on the plasma membrane NADH oxidase was of interest in the context that fatty acids and lyso PC were natural products of phospholipase A_2 action. Phospholipase A_2 activities, widespread in animal cells, have been reported to be stimulated by growth factors [32] and to be coupled to GTP-binding proteins [33,34]. In a parallel series of studies, Brightman et al. [35] reported activation of a hormone stimulated NADH oxidase of the plasma membrane of plants by fatty acids and lysophospholipids.

Phospholipase A_2 and its products, lyso PC and free fatty acids modulate the ATPase of animal [36] and plant plasma membranes [37]. Lysophospholipids also stimulated protein kinase C activity from pig brain [38], and proton pumping and protein kinase activity in plants [39].

CHAPS extracted 75% of the enzyme activity from rat liver plasma membrane at a detergent to protein ratio of 2:1. NADH oxidase was separated from NADH–ferricyanide oxidoreductase activity by ion-exchange chromatography on a DE-52 column. At this step of purification, the active fraction contained a number of protein bands including two bands of molecular weight of 34 kDa and 72 kDa. These latter two bands correlated with two of the bands (36 and 72 kDa) associated with purified NADH oxidase of plasma membrane from plants [23]. The NADH oxidase of plants also was growth factor-responsive and has been related to growth control and hormone response of plants [40].

Although there is ample evidence for the involvement of the plasma membrane NADH oxidase in growth regulation, the basis for this action remains to be established. Several mechanisms have been proposed for growth control involving plasma electron transfer. These include an increase of cytoplasmic pH, mobilization of calcium ions, turnover of phosphatidyl inositol, alterations in the ratios of cyclic nucleotides, or changes in the redox state of pyridine nucleotides [3]. However, further work will be necessary to determine which, if any, of these different mechanisms may be involved.

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