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NADH-Ascorbate Free Radical and -Ferricyanide Reductase Activities Represent Different Levels of Plasma Membrane Electron Transport

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Plasma membranes isolated from rat liver by two-phase partition exhibited dehydrogenase activities for ascorbate free radical (AFR) and ferricyanide reduction in a ratio of specific activities of 1 : 40. NADH-AFR reductase could not be solubilized by detergents from plasma membrane fractions. NADH-AFR reductase was inhibited in both clathrin-depleted membrane and membranes incubated with anti-clathrin antiserum. This activity was reconstituted in plasma membranes in proportion to the amount of clathrin-enriched supernatant added. NADH ferricyanide reductase was unaffected by both clathrin-depletion and antibody incubation and was fully solubilized by detergents. Also, wheat germ agglutinin only inhibited NADH-AFR reductase. The findings suggest that NADH-AFR reductase and NADH-ferricyanide reductase activities of plasma membrane represent different levels of the electron transport chain. The inability of the NADH-AFR reductase to survive detergent solubilization might indicate the involvement of more than one protein in the electron transport from NADH to the AFR but not to ferricyanide.

KEY WORDS: Plasma membrane; liver; ascorbate; dehydrogenases; clathrin.

INTRODUCTION

Eukaryotic cells contain a variety of transplasma membrane redox activities, some of which have been related to control of cell growth stimulation, of certain special transport functions, and in defense against bacteria (Crane *et al.*, 1985). The different transplasma membrane redox components utilize a variety of electron donors and acceptors, exhibit different rates at which electron and proton movements occur, and may be spatially located in the membrane to produce differing orientations of electron and proton flow.

For example, ferricyanide and other iron-containing oxidants are reduced by the transplasma membrane electron-transport system and stimulate cell growth in serum-limiting media as well (Ellem and Kay, 1983; Landschulz *et al.*, 1984; Sun *et al.*, 1985). Also ascorbate free radical (AFR) is a putative natural electron acceptor that also stimulates cell growth (Alcaín *et al.*, 1990), but not necessarily via the same redox activities as the iron compounds. NADH-AFR reductase has been observed in plasma membranes (Goldenberg *et al.*, 1979; Goldenberg, 1982), and the integrity of glucan moieties of the cell surface glycoconjugates seems necessary for the optimal function of this activity (Navas *et al.*, 1988) but not for the bulk of ferricyanide reduction.

A component of ferricyanide reduction by NADH is resistant to fixation with glutaraldehyde. This component is characteristic of plasma membrane and mature Golgi apparatus elements in liver (Goldenberg *et al.*, 1979; Morré *et al.*, 1978; Navas *et al.*, 1988). Clathrin-coated membranes were much

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more reactive for this activity than were adjacent non-coated membranes. We suggest that this activity may be represented by the enzyme NADH-AFR reductase that may reside uniquely in those cellular membranes most often implicated in bulk translocations of membranes and in membrane movements (Sun *et al.*, 1983, 1984; Morré *et al.*, 1985).

As we show here, detergent solubilization suggests that more than a single protein may be involved in the NADH-AFR reductase of rat liver plasma membrane. Further, a dependence on clathrin of the plasma membrane electron flow from NADH to the AFR but not to ferricyanide is demonstrated. Our findings support the idea that these activities represent different levels of the whole electron transport chain in the plasma membrane.

MATERIALS AND METHODS

Cell Fractionation

Wistar rats (100–150 g) were provided with food and drinking water *ad libitum*. Animals were killed by decapitation and exsanguinated. Livers (ca. 25 g) were excised and homogenized in 50 ml (2 × vol./frozen wt.) of a medium containing 37 mM Tris maleate, pH 6.4, 0.5 M sucrose, 1% dextran (Sigma; average molecular weight 225 kD), and 5 mM mercaptoethanol. The homogenate was then centrifuged at 5000 *g* for 15 min. The middle 1/3 to 1/2 of the pellet was resuspended in 5 ml of 1 mM sodium bicarbonate, homogenized using a conical Teflon/glass tissue homogenizer, and centrifuged for 15 min at 5000 *g*. The supernatant was discarded and the light brown, top portion of the pellet was used in the aqueous two-phase partition as described (Navas *et al.*, 1989). This fraction was resuspended and combined with a mixture containing 6.4% (w/w) dextran T500 (Pharmacia, Uppsala, Sweden) and 6.4% (w/w) polyethylene glycol (PEG 3350, Fisher, USA) in 0.1 M sucrose and 5 mM potassium phosphate, pH 7.2. The mixture was shaken 40 times and centrifuged at 150 *g* for 5 min to separate the phases. The upper phase which contained the plasma membranes was diluted in 25 ml of 1 mM sodium bicarbonate and collected for analysis by centrifugation at 20,000 *g* for 30 min.

Marker Enzymes

K^+-activated, ouabain-sensitive pNPPase (EC 3.1.3.1), a specific partial activity of the sodium pump, was used as a marker enzyme for plasma membranes. The activity was assayed according to Kasiwamata *et al.* (1979). The mitochondrial marker, succinate dehydrogenase (EC 1.3.99.1), was measured as succinate-2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-phenyltetrazolium (INT) reductase (Pennington, 1961). Monoamine oxidase (EC 1.4.3.6) (Schnaitman, 1967) was employed as outer mitochondrial membrane marker, and the endoplasmic reticulum marker used was glucose-6-phosphatase (EC 3.1.3.9) (Noodlie and Arion, 1966). The Golgi apparatus marker was galactosyltransferase (UDP-galactose: *N*-acetylglucosamine galactosyl transferase) (EC 2.4.1.13) assayed as described (Palmiter, 1969).

NADH-ascorbate free radical reductase (EC 1.6.5.5) assays were in 50 mM Tris-HCl, pH 7.4, at a final volume of 2 ml. The NADH concentration was 0.15 mM. To this was added 3.3 mM of an equal mixture of ascorbate and dehydroascorbate, adjusted to pH 6.5 with 10 mM imidazole. Absorbance was measured at 340 nm.

NADH-ferricyanide reductase (EC 1.6.99.3) assays were in 25 mM Tris-HCl, 0.75 mM sodium phosphate, 150 mM NaCl and 5 mM KCl, pH 7.0, and ca. 35 μg protein in a final volume of 1.5 ml. The concentration of NADH was 0.1 mM. Absorbance was determined at 340 nm with reference at 500 nm. The extinction coefficient used for NADH was $6.21 \times 10^{-3}\ M^{-1}\ cm^{-1}$.

Solubilization of plasma membranes was carried out at different concentrations of CHAPS ranging from 0.01 to 3%. After incubation with the detergent for 1 h at 4°C, the solubilized fraction was separated by ultracentrifugation at 105,000 *g* for 1 h at 4°C.

Clathrin removal from plasma membrane was according to Schook *et al.* (1979). One volume of plasma membrane fraction was diluted with 4, 10, or 14 volumes of extraction buffer (20 mM Tris-HCl, pH 7.5, containing 1 mM 2-mercaptoethanol, 1 mM NaCl, and 1 mM EDTA) and then homogenized by 12–14 strokes in a Teflon/glass homogenizer and pelleted by centrifugation at 20,000 *g* for 30 min.

Plasma membrane reconstitution with clathrin-enriched fraction was also as described (Schook *et al.*, 1979). Depleted membranes were mixed with clathrin-enriched supernatants and dialyzed against 100 mM KCl, 75 mM $CaCl_2$, or 75 mM $MgCl_2$, at 4°C for at least 20 h with two changes of the dialysis medium. Reconstituted membranes were pelleted at 20,000 *g* for 30 min.

Table I. Marker Enzymes: Composition of Plasma Membranes Prepared from Rat Liver by Two-Phase Partition[a]

Marker	Specific activity		
	Total homogenate	Plasma membrane	Enrichment (PM/TH)
K^+-pNPPase (plasma membrane)	0.2 ± 0.1	10.9 ± 0.6	54
Monoamine oxidase (outer mitochondrial membrane)	6.2 ± 0.8	0.5 ± 0.08	0.08
Succ-INT-Rase (mitochondria)	5.0 ± 0.6	0.4 ± 0.06	0.08
Glucose-6-phosphatase (endoplasmic reticulum)	6.7 ± 0.3	0.05 ± 0.02	0.007
Galactosyl transferase (Golgi apparatus)	2.7 ± 0.5	1.5 ± 0.4	0.55

[a] Units of specific activity are nmol/h/mg protein for K^+-pNPPase, succinate-INT-reductase, and glucose-6-phosphatase, nmol/min/mg protein for monoamine oxidase, and nmol/h/mg protein for galactosyl transferase. Values were determined from three preparations ± SD.

SDS-gel Electrophoresis and Western Blotting

About 15 μg protein of depleted and reconstituted membranes were dissolved in SDS-dithiothreitol loading buffer, heated, and separated in 7.5% polyacrylamide gels. Proteins were then transferred to nitrocellulose sheets following routine blotting techniques. Blots were stained with antiserum specific for the heavy chain (180 kDa) of clathrin obtained from Sigma (USA) and used according to manufacturer's recommendations.

Protein was determined using the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

RESULTS

Plasma membrane fractions isolated from rat liver by two-phase partitioning consisted in about 90% of plasma membrane based on morphometry (Burón *et al.*, 1987). Contaminants were primarily mitochondria and endoplasmic reticulum. Marker enzyme activities of these fractions showed an enrichment of K^+-pNPPase, the plasma membrane marker, of about 50-fold relative to the total homogenate (Table I). Mitochondrial and endomembrane marker enzyme activities were decreased relative to total homogenate.

Plasma membranes from rat liver showed an activity of about 14 nmol of NADH oxidized/min/mg protein for the NADH-AFR reductase and of about 600 nmol of NADH oxidized/min/mg protein for the NADH-ferricyanide reductase (Table II).

NADH dehydrogenase activities of plasma membrane fractions were determined in supernatants and pellets obtained by ultracentrifugation after detergent (CHAPS) solubilization (Fig. 1). NADH-

Table II. Effect of Removal of Clathrin from Rat Liver Plasma Membranes on NADH Dehydrogenase Activities[a]

Treatments	NADH dehydrogenase activities			
	Plasma membranes		Clathrin-depleted membranes	
	AFR[b]	Ferricyanide[b]	AFR[b]	Ferricyanide[b]
None	14.5 ± 0.09	600 ± 26	—	—
1/14 dilution[c]	—	—	0.06 ± 0.01 (4)	578 ± 20 (96)
1/10 dilution[c]	—	—	2.4 ± 0.03 (16)	588 ± 29 (98)
1/4 dilution[c]	—	—	8.5 ± 0.03 (59)	594 ± 28 (99)

[a] Specific activities are expressed as nmol of NADH oxidized/min/mg protein. Brackets: % of nontreated. Values were determined from four preparations ± SD.
[b] Electron acceptors.
[c] Referred to as fraction/buffer solution (v : v).

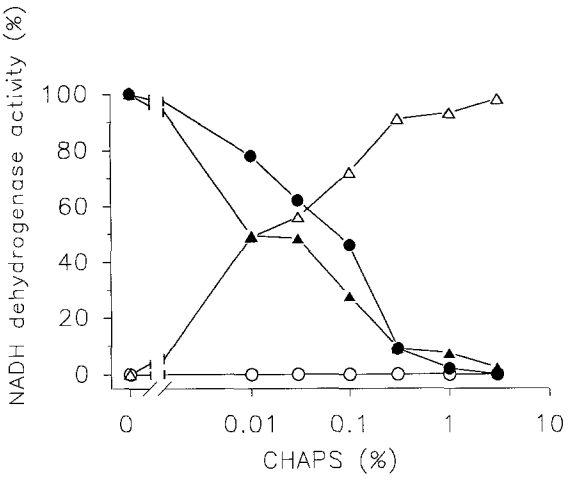

Fig. 1. Solubilization of NADH-AFR reductase (circles) and NADH-ferricyanide reductase (triangles) activities of rat liver plasma membranes. Open symbols: supernatants; solid symbols: pellets. $n = 3$, SD $\leq 8\%$.

AFR reductase activity was much reduced in membrane pellets after exposure to 0.1% CHAPS and was not detected in the supernatants containing the solubilized proteins. On the other hand, NADH ferricyanide reductase was solubilized from membranes, with up to 98% of the original activity being recovered in the supernatants. Addition of sonicated phospholipids to the solubilized fractions did not further recovered extra activities.

NADH-AFR reductase was significantly inhibited by anti-clathrin antiserum (Fig. 2). Inhibition was of 60% when the incubation was carried out at 4°C and increased to 80% in plasma membranes incubated at room temperature. These inhibitions were concentration-dependent and reached a maximum at 1/250 antibody dilution. NADH ferricyanide reductase was largely unaffected by anti-clathrin antiserum (Fig. 2) at either temperature.

Following removal of clathrin, NADH-AFR reductase was reduced in proportion to the degree of clathrin extraction and reached more than 95% inhibition at the highest buffer dilution (Table II). The bulk of the NADH-ferricyanide reductase was only slightly sensitive to clathrin removal from plasma membrane and retained 96% of the activity in clathrin-depleted membrane fractions. Clathrin-enriched supernatants showed neither NADH-AFR reductase nor NADH-ferricyanide reductase activities.

No significant NADH-AFR reductase activity of membranes extracted at 1/14 dilution was recovered after incubation with clathrin-enriched supernatants. However, clathrin-reconstituted plasma membranes derived from the mild extraction procedure (1/4 proportion, see Table II) showed a recovery of NADH-AFR reductase activity up to 80% with Ca^{++} and 78% with K^{+}, but only 63% in the presence of Mg^{++} of the nontreated plasma membranes (Fig. 3). Clathrin-reconstituted membranes did not show significant changes of the NADH ferricyanide reductase activity relative to either clathrin-depleted or untreated membranes.

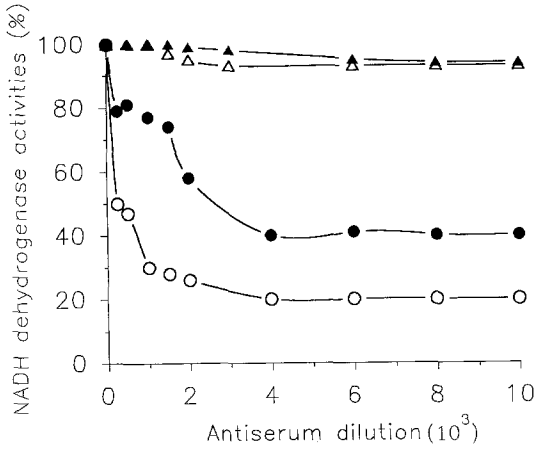

Fig. 2. Anti-clathrin antiserum inhibition of rat liver plasma membrane NADH-dehydrogenases. Incubations (5 min) were carried out at 4°C (solid symbols) or room temperature (open symbols). Circles: NADH-AFR reductase. Triangles: NADH-ferricyanide reductase. $n = 4$, SD $\leq 8\%$.

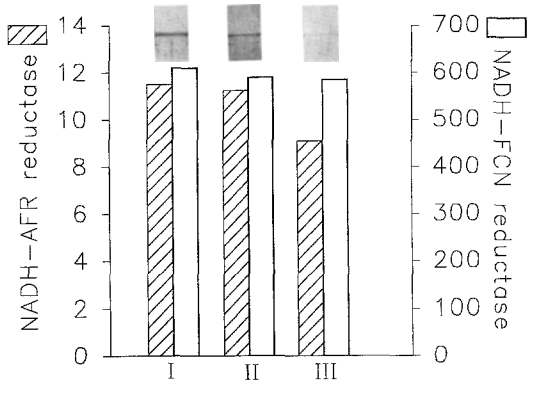

Fig. 3. Effect of reconstitution of membranes with clathrin-enriched supernatants on NADH dehydrogenases and immunostaining of the 180 kDA band of clathrin in membranes reconstituted at different conditions. Clathrin supernatants and depleted membranes were mixed and dialyzed against 75 mM Ca^{++}(I), 100 mM K^{+}(II), and 75 mM Mg^{++}(III). Dotted bars: NADH-AFR reductase. Open bars: NADH-ferricyanide reductase. $n = 4$, SD $\leq 10\%$.

Table III. Effects of Wheat Germ Agglutinin (WGA) Lectin on NADH Dehydrogenases of Rat Liver Plasma Membrane[a]

Dehydrogenase activity	Specific Activity (nmols/min/mg protein)	
	−WGA	+WGA
NADH-AFR reductase	14.3 ± 0.1	0.78 ± 0.06 (95%)
NADH-ferricyanide reductase	555 ± 49	524 ± 51 (6%)

[a] The final lectin concentration was 0.8 μg/ml. Brackets: percent of inhibition compared to no lectin. Values were determined from four preparations ± SD.

Clathrin reconstitution was monitored by immunostaining of the 180 kDa band of Western blots of the different membrane fractions with the anti-clathrin antiserum (Fig. 3).

The two dehydrogenases also were affected differently by WGA lectin. NADH-AFR reductase was inhibited up to 95%, but the NADH ferricyanide reductase was insensitive to incubation with the lectin (Table III).

DISCUSSION

Both NADH-AFR reductase (Burón *et al.*, 1987) and NADH-ferricyanide reductase (Goldenberg *et al.*, 1979) activities have been identified as activities intrinsic to the plasma membrane of eukaryotic cells. These plasma membrane dehydrogenases have been implicated, as well as components of the transplasma membrane redox system potentially involved in cell growth regulation (Crane *et al.*, 1985). Ferricyanide has been reported to reduce the serum requirements for growth of melanoma cells (Ellem and Kay, 1983), and to stimulate growth of Hela cells in serum-limiting media (Sun *et al.*, 1985). Ferricyanide also induced a change of the cytoplasmic redox potential of the cell (Navas *et al.*, 1986; Navas and Burón, 1989).

Ascorbate and its free radical, AFR, stimulated the growth of HL-60 cells in serum-limiting media (Alcaín *et al.*, 1990). Ascorbate and AFR also stimulate growth of other transformed cells *in vitro* (Park *et al.*, 1971; Park, 1985; Park and Kimler, 1991). Although the mechanism of action of ascorbate on growth regulation is unknown, a clear biological effect (Park *et al.*, 1971; Alcaín *et al.*, 1990, 1991) is seen, most probably based on the oxidoreductant properties of ascorbate. In face, HL-60 cells are able to regenerate ascorbate from its free radical through the transplasma membrane electron flow via the NADH-AFR reductase (Alcaín *et al.*, 1991). Ascorbate regeneration via transmembrane flow of electrons also would explain the reported stabilitation of ascorbate in culture media in the presence of cells (Alcaín *et al.*, 1990).

NADH-AFR reductase of rat liver plasma membrane is lost when the membranes are solubilized by CHAPS, and addition of phospholipids does not recover the activity. These data could support the interpretation that the electron flow from NADH to AFR appears to involve more than one protein (Goldenberg *et al.*, 1983; Coassin *et al.*, 1991) and the lack of activity in detergent-treated membranes is not due to delipidation of a single transmembrane protein. NADH-AFR reductase of the erythrocyte membrane was also completely deactivated at Triton concentrations above 0.04%, and the activity could not be recovered from the extract even after removal of detergent, indicating that membrane structure is necessary to maintain activity (Goldenberg *et al.*, 1983).

Although slow reduction of AFR in the cytoplasmic side of plasma membranes via the cytochrome b_5 was previously proposed by Schulze *et al.* (1970), they showed later that the NADH-AFR reductase is independent of both cytochrome b_5 and b_5 reductase, using an antibody against b_5 reductase and fractionation by zonal centrifugation (Schulze and Staudinger, 1971; Geiss and Schulze, 1975).

More recently, Kobayashi *et al.* (1991) have demonstrated, using pulse radiolysis techniques, that AFR can accept electrons from solubilized and purified b_5 reductase although at a rate about 30 times lower than that of the monodehydroascorbate reductase of cucumber (Hossain and Asada, 1985). The reaction of AFR with reduced cytochrome b_5 was even several orders of magnitude lower. The authors proposed that monodehydroascorbate reductase but not b_5 reductase has an active site structure that accepts the physiological substrate AFR. We conclude that the loss of NADH-AFR reductase activity observed after detergent treatment is unlikely to be due to the separation of cytochrome b_5 and the b_5

reductase but to the involvement of another enzyme. Furthermore, inhibition of b_5 reductase and cytochrome b_5 by lectins is not expected as these proteins are cytoplasmically oriented and hence not glycosylated.

Inhibition of NADH-AFR reductase by lectins was reported in our previous work (Navas *et al.*, 1988; Alcaín *et al.*, 1991) and interpreted as a requirement of the external domain of the membrane for this activity. A glycoprotein from the erythrocyte membrane (Wang and Alaupovic, 1978) has been reported to exhibit oxidoreductase activity. However, we have no evidence for glycoprotein involvement in the bulk ferricyanide reduction by rat liver plasma membranes.

In contrast to NADH-AFR, the NADH-ferricyanide reductase can be solubilized by CHAPS as previously determined for the NADH-ferricyanide oxidoreductase activities of plant (Luster and Buckhout, 1989) and yeast (Dancis *et al.*, 1990) plasma membranes. NADH-ferricyanide reductase of rat liver plasma membrane is not modified by WGA lectin.

Clathrin coats of membranes have been implicated in the mechanism to drive membrane translocations (Lin *et al.*, 1991). NADH-AFR reductase could be related to energy production via generation of a membrane potential, as has been shown for coated vesicles (Morré *et al.*, 1987) and plasma membrane of plants (González-Reyes *et al.*, 1992). Also, ascorbate increases the number of coated vesicles visible in the Golgi apparatus region of rat hepatocyte and hepatomas (Minnifield and Morré, 1984). Rat liver coated vesicles and Golgi apparatus have previously been shown to contain a NADH-AFR reductase that is enhanced in clathrin-incubated membranes (Morré *et al.*, 1985).

Extraction of clathrin from rat liver plasma membranes resulted in an inhibition of NADH-AFR, but not -ferricyanide, reductase activity. The inhibition was irreversible when the most drastic procedure (1/14 dilution) was used, but the mild extraction (1/4 dilution) allowed reconstitution of the redox activity. Chelating agents such as EDTA present in the extraction buffer are frequently used for removing soluble proteins adsorbed to membranes, but these reagents are sometimes able to denature protein, leading to irreversible loss of biological activity (Renswoude and Kempf, 1984). Chelator-to-protein ratio is likely more favorable in the mild extraction.

The findings show a clear difference between NADH-dependent AFR reductase and ferricyanide reductase activities, although the mechanisms implicated in the transplasma membrane electron flow remain uninvestigated. This conclusion is supported as well by the observations that the reduction of ascorbate by the intact cells is regulated by growth factors *in vitro* (Navas *et al.*, 1992). Further, CoQ has been recently propopsed as the intermediate step in the plasma membrane electron flow (Sun *et al.*, 1992), implying the existence of different components for the whole transmembrane system.

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