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Antioxidant Ascorbate Is Stabilized by NADH-Coenzyme Q_{10} Reductase in the Plasma Membrane

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Plasma membranes isolated from K562 cells contain an NADH-ascorbate free radical reductase activity and intact cells show the capacity to reduce the rate of chemical oxidation of ascorbate leading to its stabilization at the extracellular space. Both activities are stimulated by CoQ_{10} and inhibited by capsaicin and dicumarol. A 34-kDa protein (p34) isolated from pig liver plasma membrane, displaying NADH-CoQ_{10} reductase activity and its internal sequence being identical to cytochrome b_5 reductase, increases the NADH-ascorbate free radical reductase activity of K562 cells plasma membranes. Also, the incorporation of this protein into K562 cells by p34-reconstituted liposomes also increased the stabilization of ascorbate by these cells. TPA-induced differentiation of K562 cells increases ascorbate stabilization by whole cells and both NADH-ascorbate free radical reductase and CoQ_{10} content in isolated plasma membranes. We show here the role of CoQ_{10} and its NADH-dependent reductase in both plasma membrane NADH-ascorbate free radical reductase and ascorbate stabilization by K562 cells. These data support the idea that besides intracellular cytochrome b_5-dependent ascorbate regeneration, the extracellular stabilization of ascorbate is mediated by CoQ_{10} and its NADH-dependent reductase.

KEY WORDS: Plasma membrane; ascorbate stabilization; coenzyme Q; cytochrome b_5 reductase.

INTRODUCTION

Ascorbate is a first-order antioxidant that protects cellular components from free radical-induced damage, either by a direct quenching of various soluble free radicals or by scavenging lipid peroxidation-initiating radicals (Frei, 1994). Membrane-bound tocopheroxyl radicals can be reduced to tocopherol by ascorbate thus preventing oxidative damage in membrane lipids (Frei, 1994; Briviba and Sies, 1994). Antioxidant action of ascorbate leads to a two-step oxidation through the ascorbate free radical (AFR) and producing dehydroascorbate. Although there is evidence for a cytoplasmic dehydroascorbate reductase, this is not the sole pathway for maintenance of ascorbate, this process being also carried out by the NADH-AFR reductase (Meister, 1994; Coassin *el al.*, 1991)

As different animals and humans cannot synthesize this compound, the mechanisms to stabilize ascorbate available in the diet are of extreme importance. In fact, ascorbate in tissues is maintained primarily in the reduced state (Rose and Bode, 1993), clearly depending on the presence of cells (Minetti, *et al.*, 1992). Ascorbate is also maintained in its reduced state by cells in culture (Minetti *et al.*, 1992). Stabilization of ascorbate at the cell surface is a mechanism to maintain the antioxidant property of this vitamin and appears to be mediated by an enzyme system. Quenching of ascorbyl radical at the cell surface (Pethig *et al.*, 1985) shows similar properties to the ascorbate stabilization activity also observed *in vitro* (Rodríguez-Aguilera and Navas, 1994). This activity is modulated

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by growth factors (Navas *et al.*, 1992), cAMP (Rodríguez-Aguilera *et al.*, 1993), and N-myc expression (Medina *et al.*, 1992), but the nature of the enzymatic components responsible for this stabilization has to be elucidated.

We have recently purified a protein of 34 kDa from the plasma membrane of pig liver displaying a phospholipid-dependent NADH-CoQ_{10} reductase activity (Villalba *et al.*, 1995; Navarro *et al.*, 1995). CoQ_{10} is a lipid-soluble antioxidant that mediates the electron transport in the plasma membrane (Sun *et al.*, 1992), including also AFR as oxidant (Villalba *et al.*, 1995). Interaction of both ascorbate and CoQ_{10} with α-tocopherol as a mechanism of membrane lipids protection is well established (Buettner, 1993), but there is no evidence of direct interaction between ascorbate and CoQ_{10} in membranes (Beyer, 1994). We show here a CoQ_{10} requirement for both NADH-AFR reductase of isolated plasma membrane and ascorbate stabilization at the cell surface in K562 cells. These two activities are also stimulated by the addition of purified NADH-CoQ_{10} reductase to plasma membrane vesicles or whole cells respectively. The relationship between ascorbate and CoQ_{10} in the plasma membrane interphase is discussed as an integrated mechanism to maintain the antioxidant property of ascorbate using cytoplasmic NADH as unique electron source.

MATERIALS AND METHODS

Culture Conditions

K 562 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Flow Laboratories), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μ/ml amphotericin B (Sigma) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were concentrated from stock cultures by centrifugation at 1000 *g* for 5 min and washed in serum-free RPMI 1640 medium.

Plasma Membrane Preparation

Microsomes were obtained from K562 cell homogenates. Plasma membrane vesicles were then isolated by the two-phase partition method (Navas *et al.*, 1989) and purity was checked by marker enzyme analysis. Membranes were resuspended in 50 mM Tris/HCl, pH 7.6, containing 10% glycerol, 1 mM PMSF, 1 mM EDTA, and 0.1 mM DTT, and stored either under liquid nitrogen or at −86°C.

CoQ_{10} Quantification

Plasma membranes (12 mg) were first disrupted with 1% SDS in a final volume of 1 ml, and 2 ml of 95% ethanol–5% isopropanol was added. CoQ_{10} was then recovered from SDS-alcoholic solution by extraction with 5 ml hexane. Extraction was repeated once and both hexane phases were combined. After evaporation under vacuum, extracts were resuspended in 100 μl ethanol. CoQ_{10} determination was carried out by HPLC separation with UV monitoring at 275 nm. The procedure described here recovered more than 98% of CoQ_{10}.

Extraction and Restoration of CoQ_{10}

CoQ_{10} was extracted with heptane from lyophilized plasma membranes as described (Norling *et al.*, 1974). CoQ_{10} in heptane was added to both extracted and unextracted membranes and the solvent was evaporated to allow for incorporation of the quinone into the desiccated membranes. Membranes were then taken up in 50 mM Tris/HCl, pH 7.6, for assays.

Protein Purification

The NADH-CoQ_{10} reductase was extracted from purified pig liver plasma membranes (Villalba *et al.*, 1995) with the nondenaturing zwitterionic detergent 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) at the concentration of 2.5%. Before detergent extraction, extrinsic proteins were removed from the membranes by treating them with 0.5 M KCl in 50 mM Tris/HCl, pH 7.6, containing 1 mM EDTA, 1 mM PMSF, 0.1 mM DTT, and 10% glycerol. The protein extract was separated from the membrane residue by centrifugation at 105,000 *g* for 1 h at 4°C. Then, the NADH-CoQ_{10} reductase was purified by size exclusion chromatography on Sephacryl S-300 HR, ion exchange chromatography on DEAE-Sepharose 6B CL, and affinity chromatography on 5′ADP agarose followed by elution with NADH.

Reconstitution into Liposomes

Purified phospholipids were separated from a crude mixture of egg yolk phospholipids (Sigma) by acetone precipitation of the ether-soluble fraction. The purified phospholipids were then resuspended in 20 mM Tris/HCl, pH 7.6, containing 1 mM EDTA. The oxygen in the phospholipid suspension was eliminated by sparging with a stream of nitrogen. The resulting turbid phospholipid suspension (10 mg/ml) was clarified by sonication under a nitrogen atmosphere. CoQ_{10} in ethanol was added to the phospholipid liposomes to a final quinone concentration of 50 μM. The mixture was then incubated for 3 min at 37°C to allow for incorporation of quinones. CHAPS was removed from the purified enzyme preparations by dialysis, and then the protein (3 μg) was reconstituted into phospholipid liposomes by freeze–thaw–sonication.

Ascorbate Oxidation

Short-term ascorbate oxidation was folowed by a direct reading at 265 nm in 0.1 M Tris/HCl buffer, pH 7.4, for 10 min at 37°C either in the presence or absence of cells as described (Winkler, 1987). Specific activity of the ascorbate stabilization was calculated from the difference between the rates of ascorbate oxidation with and without cells (Rodríguez-Aguilera, *et al.,* 1993). An extinction coefficient of 11.2 mM^{-1} cm^{-1} was used in calculations of specific activities.

Enzyme Activities

NADH-AFR reductase (EC 1.6.5.5) was assayed by measuring NADH oxidation at 340 nm upon addition of 66 mU ascorbate oxidase to a reaction mixture containing 0.4 mM ascorbate. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used. Marker enzyme activities were: K^+-stimulated, ouabain-inhibited, *p*-nitrophenylphosphatase (K^+-pNPPase) (EC 3.1.3.1) for plasma membrane assayed according to Kashiwamata *et al.* (1979). Succinate dehydrogenase (EC 1.3.99.1) measured as INT reductase (Pennington, 1961) and cytochrome *c* oxidase (EC 1.9.3.1) (Storrie and Madden, 1990) were assayed as markers for mitochondria. NADPH-cytochrome *c* reductase (EC 1.6.2.3) (Mahler, 1955) was used as endoplasmic reticulum marker, and UDP-galactose: *N*-acetylglucosamine galactosyltransferase (galactosyltransferase) (EC 2.4.1.13) as Golgi apparatus marker (Palmiter, 1969). Protein determinations were carried out by the dye-binding method modified for membrane samples (Stoscheck, 1990), with bovine γ-globulin as standard.

Induction of Differentiation

Differentiation was induced by 2 ng/ml of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) for 24 h. Viable cells were above 90% as determined by the trypan blue exclusion technique. The phenotypic expression of erythroid differentiation was monitored by the benzidine dihydrochloride test for hemoglobin as described (Fibach *et al.,* 1983).

RESULTS

Ascorbate undergoes autoxidation in aqueous solution and the presence of cells inhibits its oxidation rate (Rodríguez-Aguilera and Navas, 1994). The difference between the oxidation rates in the absence and presence of cells is considered here as ascorbate stabilization specific activity. This activity was about 1.4 nmol/min/10^6 cells for the K562 cell line, and was increased by the addition of CoQ_{10}, the difference being significant above 20 μM (Fig. 1).

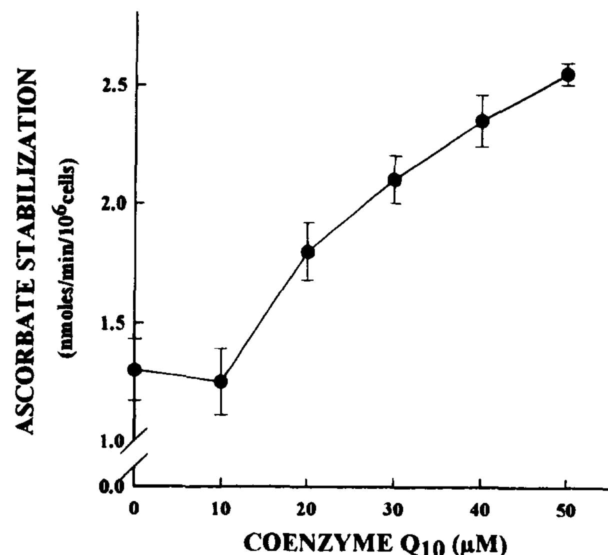

Fig. 1. Role of CoQ_{10} integration on the extracellular ascorbate stabilization in K562 cells. CoQ_{10} was dissolved in ethanol and cells were incubated for 5 min at 37°C. Cells were then recovered by centrifugation and resuspended in 0.1 M Tris/HCl buffer for assays.

Plasma membrane fractions isolated from K562 cells by two-phase partition showed a very high level of purity, showing a significant increase of about 30-fold of the plasma membrane marker K^+-pNPPase compared to total homogenate (Table I). On the other hand, both succinate-INT reductase and cytochrome *c* oxidase as measurement of mitochondrial contamination and endoplasmic reticulum NADPH-cytochrome *c* reductase were about 10-fold lower in the plasma membrane compared to those of starting homogenates. Further, Golgi apparatus marker was also 0.3 times reduced in plasma membrane compared to total homogenate.

These plasma membranes contained a basal NADH-AFR reductase activity of about 6.6 nmol/min/mg protein that was significantly increased by the incorporation of 50 μM CoQ_{10} (supplemented membranes, Table II). Extraction of CoQ_{10} with heptane reduced NADH-AFR reductase by 20%. Addition of extra CoQ_{10} to these extracted membranes restored the original activities (Table II). Further, NADH-AFR reductase of these fractions isolated from K562 cells was inhibited about 25% by 10 μM capsaicin and 35% by 50 μM dicumarol. Similarly, the stabilization of extracellular ascorbate was inhibited about 36% by 10 μM capsaicin and 40% by 50 μM dicumarol.

A 34-kDa protein (p34) showing NADH-CoQ_{10} reductase activity was purified from pig liver plasma membrane, being apparently responsible for NADH-AFR reductase in these membranes (Villalba *et al.*, 1995). Purified p34 added to plasma membranes from K562 cells induced a significant increase of NADH-AFR reductase activity (Fig. 2). This increase was abolished when p34 was either boiled or preincubated with a polyclonal antibody against p34, which specifically binds to this protein although does not inhibit its NADH-CoQ_{10} reductase activity, most likely because it binds to a site different from the active site, impairing integration of p34 into the plasma membrane (Navarro *et al.*, 1995). The degree of incorporation into these membranes was monitored by western blot immuno-

Table I. Marker Enzyme Characteristics of Plasma Membrane Fractions Isolated from K562 cells by Two-Phase Partition[a]

Marker enzyme	Specific activity: Total homogenate	Specific activity: Plasma membrane	Ratio
K^+-pNPPase	0.23 ± 0.01	6.9 ± 0.2	29.8
Succinate-INT reductase	2.4 ± 0.6	0.25 ± 0.08	0.1
Cytochrome *c* oxidase	5.1 ± 0.7	0.48 ± 0.13	0.09
NADPH-cyt *c* reductase	16.2 ± 1.3	1.3 ± 0.2	0.08
Galactosyltransferase	6.6 ± 2.5	2.1 ± 0.9	0.3

[a] Specific activities are expressed as μmol/h/mg protein for K^+-pNPPase and succinate-INT reductase; nmol/h/mg protein for NADPH-cytochrome *c* reductase and galactosyltransferase, and nmol/min/mg protein for cytochrome *c* oxidase. Values were determined from four experiments ± SD.

Table II. Role of CoQ_{10} on NADH-AFR Reductase of Plasma Membrane Isolated from K562 Cells[a]

Sample type	NADH-AFR reductase	Variation (%)
Control	6.6 ± 0.3	—
Extracted	5.3 ± 0.3[2]	− 20%
Reconstituted	7.2 ± 0.4[2,3]	+ 10%
Supplemented	9.1 ± 0.4[1]	+ 38%

[a] Lyophilized membranes (control) were extracted with heptane for 6 h at 20°C in the dark (extracted). CoQ_{10} in heptane was added to both extracted membranes (reconstituted) and control membranes (supplemented) at a final concentration of 50 μM. Variations were calculated from the difference relative to control. Negative value means inhibition and positive variation means activation. Data were obtained from four separate experiments ± SD.
[1] $p<0.01$ vs. control.
[2] $p<0.05$ vs. control.
[3] $p<0.01$ vs. extracted.

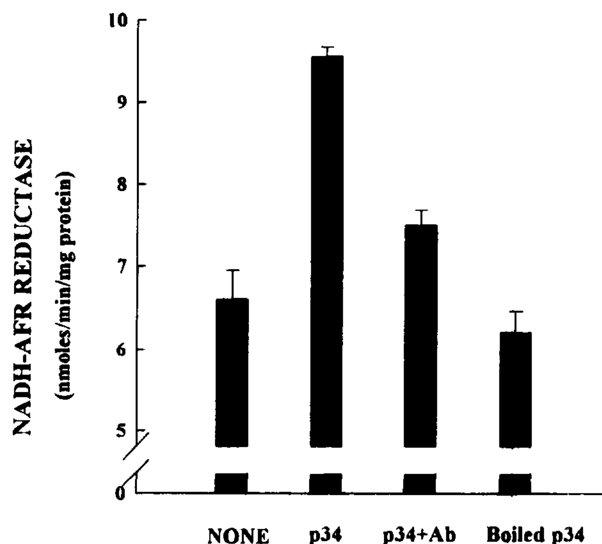

Fig. 2. Stimulation of NADH-AFR reductase by NADH-CoQ_{10} reductase. p34 (NADH-CoQ_{10} reductase) isolated from pig liver plasma membrane was added to plasma membranes isolated from K562 cells by two-phase partition for 20 min at 37°C. Controls were carried out either boiling the protein or preincubating it with polyclonal antibody against p34 before the addition to plasma membranes.

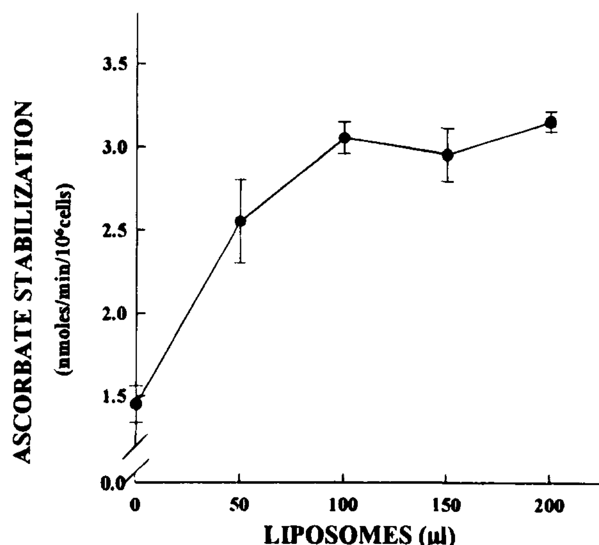

Fig. 3. Stimulation of ascorbate stabilization by $NADH-CoQ_{10}$ reductase. Ascorbate stabilization activity was measured in K562 cells after their incubation with p34-reconstituted liposomes. 5×10^5 cells were incubated for 30 min at 37°C to allow for the fusion of liposomes to the plasma membrane.

staining, confirming that native p34 was well incorporated (data not shown). Further, this protein was also reconstituted in liposomes, and K562 cells were incubated with different amounts of these liposomes and the ascorbate stabilization activity was then measured. As is shown in Fig. 3, the stabilization of ascorbate was significantly increased in liposome-treated cells, reaching a plateau at about 100 µl of liposomes per 5×10^5 cells. Neither ascorbate stabilization nor NADH-AFR reductase activities were detected in these liposomes.

Treatment of K562 cells with TPA induced their erythroid differentiation and, after 24 h, about 24% of cells were benzidine positive, a marker for hemoglobin. Table III shows the redox properties of these TPA-induced K562 cells. The ascorbate stabilization activity was double in differentiated compared to nondifferentiated cells. Plasma membrane fractions isolated from differentiated cells showed an NADH-AFR reductase activity 1.5 times higher than those isolated from nondifferentiated cells. Also, the former membranes contained almost 2 times more CoQ_{10} than the latter ones, although both fractions contained similar levels of the mitochondrial marker cytochrome *c* oxidase.

DISCUSSION

It is essential for cells to maintain antioxidants in order to avoid cellular damages induced by oxidative stress. Ascorbate is a first-order antioxidant whose stabilization is a basic objective of cells, specially those lacking its biosynthesis. Although a glutathione-dependent reduction of dehydroascorbate is apparently important, it is not the only pathway for maintenance of ascorbate. In fact, the one-electron reduction of ascorbate free radical catalyzed by the NADH-AFR reductase functions in the conservation of ascorbate (Meister, 1994; Coassin *et al.*, 1991).

Highly purified plasma membrane fractions contain NADH-AFR reductase activity (Villalba *et al.*, 1993) that could be responsible for the stabilization of extracellular ascorbate (Minetti *et al.*, 1992; Alcaín *et al.*, 1991). We show here the requirement for CoQ_{10} in both plasma membrane NADH-AFR reductase and extracellular stabilization of ascorbate by whole K562 cells. Also, CoQ_{10}-mediated electron transport antagonists inhibited both activities. CoQ_{10} has been shown to participate in the bulk of plasma membrane electron transport (Sun *et al.*, 1992) and also to mediate AFR reduction in purified pig liver plasma membrane (Villalba *et al.*, 1995). The efficiency of CoQ_{10} to stimulate NADH-AFR reductase in isolated membrane vesicles

Table III. Effect of TPA-Induced Differentiation on Redox Properties and CoQ_{10} Content of K562 Cell Plasma Membranes[a]

Parameter	Nondifferentiated	Differentiated	Ratio D/ND
Ascorbate stabilization	1.4 ± 0.01	2.7 ± 0.03[b]	1.9
NADH-AFR reductase	6.0 ± 0.1	8.9 ± 0.3[b]	1.5
Coenzyme Q_{10}	138 ± 13	236 ± 27[b]	1.7
Cytochrome *c* oxidase	0.48 ± 0.14	0.43 ± 0.31	0.9

[a] Differentiation was induced by 2 ng/ml TPA for 24 h. Specific activities are expressed as nmol/min/mg protein for NADH-AFR reductase and cytochrome *c* oxidase, nmol/min/10^6 cells for ascorbate stabilization, and CoQ_{10} content as pmol/mg protein. Values were determined from four experiments ± SD.

[b] $p \leq 0.01$ vs. nondifferentiated.

is lower than the stimulation of ascorbate stabilization by CoQ_{10} in the whole cell. If some cytoplasmic factors would be necessary for the optimal activity in intact cells as it has been considered (Navas *et al.*, 1992; Rodríguez-Aguilera *et al.*, 1993; Medina *et al.*, 1992), the procedure used for the plasma membrane isolation could be responsible for the loss of these factors. Similarly, it has been shown that the stimulation of NADH-linked mitochondrial dehydrogenases by CoQ_{10} is limited by the miscibility of this compound in the membrane phospholipids (Lenaz *et al.*, 1995).

TPA-induced differentiation produces an increase of transplasma membrane electron transport during the first 48 h in HL-60 cells (Burón *et al.*, 1993). K562 cells also show a TPA-induced increase of plasma membrane electron transport in parallel to a higher content of CoQ_{10} in the plasma membrane, because no extra mitochondrial contamination was observed. Thus, induced differentiation offers another approach to correlate both CoQ_{10} and AFR reduction at the plasma membrane.

Evidence has accumulated that extracellular ascorbate stabilization is an enzymatic process and, although the enzymes involved are not yet identified, the NADH-AFR reductase appears as the more appropriate candidate (Pethig *et al.*, 1985; Rodríguez-Aguilera and Navas, 1994). Liver plasma membrane contains an integrated 34-kDa protein, showing NADH-CoQ_{10} reductase activity and whose internal sequence is identical to that of cytochrome b_5 reductase (Villalba *et al.*, 1995; Navarro *et al.*, 1995), which mediates the NADH-dependent AFR reduction in these membranes. In K562 cells this protein is able to increase both NADH-AFR reductase of plasma membrane and ascorbate stabilization in the whole cell. Thus, plasma membrane cytochrome b_5 reductase is able to mediate the regeneration of ascorbate in a manner apparently independent of cytochrome b_5. AFR reacts with reduced cytochrome b_5 reductase but no reaction was observed with cytochrome b_5 measured by pulse radiolysis (Kobayashi *et al.*, 1991). The rate of AFR-induced NADH oxidation by cytochrome b_5 reductase was about 30 times lower than that of NADH-AFR reductase (Hossain and Asada, 1985). Thus, this latter activity must have an active site structure that accepts the physiological substrate AFR, and is probably deficient in cytochrome b_5 reductase (Kobayashi *et al.*, 1991). These components are likely dissociated during detergent solubilization of membranes (Coassin *et al.*, 1991; Villalba *et al.*, 1993) and in the case of plasma membrane would involve an electron carrier as CoQ_{10}, and the integrity of the glycocalix (Alcaín *et al.*, 1991; Pethig *et al.*, 1985; Rodríguez-Aguilera *et al.*, 1993). It has been demonstrated that CoQ_{10} hydroquinone decreases steady-state concentrations of AFR in a similar manner as oxidized CoQ_{10} plus NADH. This effect required the quinone to be integrated into the plasma membrane, but was not observed in phospholipid liposomes reconstituted with the reduced CoQ_{10} (Villalba, J. M., submitted).

Residual AFR-reductase activity remained after gentle extraction of quinones with heptane. In addition to the slow reaction of AFR with cytochrome b_5 reductase (Hossain and Asada, 1985), CoQ_{10} deeply buried in the lipid bilayer could mediate the observed reductase activity. Similar residual activities remaining after gentle extraction with heptane have been observed for ferricyanide- and AFR-reductases and NADH-oxidase of plasma membranes from several sources (Sun *et al.*, 1992; Villalba *et al.*, 1995).

Cytochrome b_5 reductase is also responsible for the mitochondrial outer membrane NADH-AFR reductase (Ito *et al.*, 1981; Shirabe *et al.*, 1995), which uses an outer membrane-specific cytochrome b_5 as substrate (Lederer *et al.*, 1983). The content of cytochrome b_5 in plasma membrane is apparently very variable and always very low compared with other membranes (Remacle, 1980; Crane *et al.*, 1985; D'Arrigo *et al.*, 1993). Although NADH-cytochrome *c* reductase in plasma membrane must use cytochrome b_5 (Kant and Steck, 1972), less than 40% of cytochrome b_5 bound to plasma membrane can be reduced by the NADH-cytochrome b_5 reductase (Remacle, 1980), and CoQ_{10} does not modulate cytochrome *c* reductase (Villalba *et al.*, 1995). NADH-AFR reductase of plasma membrane was formerly considered independent of both cytochrome b_5 and b_5 reductase, using an antibody against the b_5 reductase and fractionation by zonal centrifugation (Schulze and Staudinger, 1971; Geiss and Schulze, 1975), but we clearly demonstrate here the role of cytochrome b_5 reductase on plasma membrane-dependent NADH-AFR reductase and ascorbate stabilization in K562 cells. Further, cytochrome b_5 reductase-dependent AFR reduction by the plasma membrane is mediated by CoQ_{10}, which connects two antioxidants required for protecting membrane lipids against peroxidation (Buettner, 1993), a relationship that has not been previously considered.

Cytochrome b_5 reductase is an ubiquitous flavoprotein that is involved in different electron transport-mediated functions, each one depending on specific electron carriers available (Shirabe *et al.*, 1995). Cyto-

chrome b_5 reductase is connected with AFR reduction at two cellular locations, one at the outer membrane of mitochondria that uses a specific b_5 as electron carrier (Ito *et al.,* 1981; Shirabe *et al.,* 1995), and another at the cell surface (Alcaín *et al.,* 1991; Pethig *et al.,* 1985) that it is mediated by the lipophilic antioxidant CoQ_{10}.

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REFERENCES

Alcaín, F. J., Burón, M. I., Villalba, J. M., and Navas, P. (1991). *Biochim. Biophys. Acta* **1073**, 380–385.
Beyer, R. E. (1994). *J. Bioenerg. Biomembr* **26**, 349–358.
Briviba, K., and Sies, H. (1994). In *Natural Antioxidants in Human Health and Disease* (Frei, B., ed), Academic Press, London, pp. 107–128.
Buettner, G. R. (1993). *Arch. Biochem. Biophys.* **300**, 535–543.
Burón, M. I., Rodríguez-Aguilera, J. C., Alcain, F. J., and Navas, P. (1993). *Biochem. Biophys. Res. Commun.* **192**, 439–445.
Coassin, M., Tomasi, A., Vannini, V., and Ursini, F. (1991). *Arch. Biochem. Biophys.* **290**, 458–462.
Crane, F. L., Löw, H., and Clark, M. G. (1985). In *The Enzymes of Biological Membranes,* Vol. 4 (Martonosi, A. N., ed), Plenum Press, New York, pp. 465–510.
D'Arrigo, A., Manera, E., Longhi, R., and Borgese, N. (1993). *J. Biol. Chem.* **268**, 2802–2808.
Fibach, E., Treves, A., and Rachmilewitz, E. A. (1983). *Cancer Res.* **43**, 4136–4141.
Frei, B.(1994). *Am J. Med.* **97**, 5S–13S.
Geiss, D., and Schulze, H. U. (1975). *FEBS Lett.* **60**, 374–379.
Hossain, M. H., and Asada, K. (1985). *J. Biol. Chem.* **260**, 12920–12926.
Ito, A., Hayashi, S.-I., and Yoshida, T. (1981). *Biochem Biophys. Res. Commun.* **101**, 591–598.
Kant, J. A., and Steck, T. L. (1972). *Nature* **240**, 26–28.
Kashiwamata, S., Goto, S., Semba, R. K., and Suzuki, F. N. (1979). *J. Biol. Chem.* **254**, 4577–4584.
Kobayashi, K., Harada, Y., and Hayashi, K. (1991). *Biochemistry* **30**, 8310–8315.
Lederer, F., Chrir, R., Guiard, B., Cortial, B., and Ito, A. (1983). *Eur. J. Biochem.* **132**, 95–102.
Lenaz, G., Bovina, C., Castellucio, C., Cavazzoni, M., Estornell, E., Huertas, J. R., Pich, M. M., Pallotti, F., Castelli, G. P., and Rauchova, H. (1995). *Protoplasma* **184**, 50–62.
Mahler, H. R. (1955). *Methods Enzymol.* **2**, 688–693.
Medina, M. A. del Castillo-Olivares, A., and Schweigerer, L. (1992). *FEBS Lett.* **311**, 99–101.
Meister, A. (1994). *J. Biol. Chem.* **269**, 9397–9400.
Minetti, M., Forte, T., Sotiani, M., Quaresima, V., Menditto, A., and Ferrari, M. (1992). *Biochem. J.* **282**, 459–465.
Navarro, F., Villalba, J. M., Crane, F. L., MacKellar, W. C., and Navas, P. (1995). *Biochem. Biophys. Res. Commun.* **212**, 138–143.
Navas, P., Nowack, D. D., and Morré, D. J. (1989). *Cancer Res.* **49**, 2147–2156.
Navas, P., Alcaín, F. J., Burón, M. I., Rodríguez-Aguilera, J. C., Villalba, J. M., Morré, D. M., and Morré, D. J. (1992). *FEBS Lett.* **299**, 223–226.
Norling, B., Glazek, E., Nelson, B. D., and Ernster, L. (1974). *Eur. J. Biochem.* **47**, 475–482.
Palmiter, R. D. (1969). *Biochim. Biophys. Acta* **178**, 35–46.
Pennington, R. J. (1961). *Biochem. J.* **80**, 649–654.
Pethig, R., Gascoyne, P. R. C., McLaughlin, J. A., and Szent-Györgyi, A. (1985). *Proc. Natl. Acad. Sci. USA* **82**, 1439–1442.
Remacle, J. (1980). *Biochim. Biophys. Acta* **597**, 564–576.
Rodríguez-Aguilera, J. C., and Navas, P. (1994). *J. Bioenerg. Biomembr.* **26**, 379–384.
Rodríguez-Aguilera, J. C., Navarro, F., Arroyo, A., Villalba, J. M., and Navas, P. (1993). *J. Biol. Chem.* **268**, 26346–26349.
Rose, R. C., and Bode, A. M. (1993). *FASEB J.* **7**, 1135–1142.
Schulze, H. U., and Staudinger. H.(1971). *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 1659–1674.
Shirabe, K., Landi, M. T., Takeshita, M., Uziel, G., Fedrizzi, E., and Borgese, N. (1995). *Am. J. Hum. Genet.* **57**, 302–310.
Storrie, B., and Madden, E. A. (1990). *Methods Enzymol.* **182**, 203–235.
Stoscheck, C. M. (1990). *Methods Enzymol.* **180**, 50–68.
Sun, I. L., Sun, E. E., Crane, F. L., Morré, D. J., Lindgren, A., and Löw, H. (1992). *Proc. Natl. Acad. Sci. USA* **89**, 11126–11130.
Villalba, J. M., Canalejo, A., Rodriguez-Aguilera, J. C., Burón, M. I., Morré, D. J., and Navas, P. (1993). *J. Bioenerg. Biomembr.* **25**, 411–417.
Villalba, J. M., Navarro, F., Córdoba, F., Serrano, A., Arroyo, A., Crane, F. L., and Navas, P. (1995). *Proc. Natl. Acad. Sci. USA* **92**, 4887–4891.
Winkler, B. S. (1987). *Biochim. Biophys. Acta* **925**, 258–264.