Journal of Bioenergetics and Biomembranes, Vol. 32, No. 2, 2000

Interactions Between Ascorbyl Free Radical and Coenzyme Q at the Plasma Membrane[1]

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Received November 15, 1999; accepted February 21, 2000

A role for coenzyme Q in the stabilization of extracellular ascorbate by intact cells has been recently recognized. The aim of this work was to study the interactions between reduced ubiquinone in the plasma membrane and the ascorbyl free radical, as an approach to understand ubiquinone-mediated ascorbate stabilization at the cell surface. K-562 cells stabilized ascorbate and decreased the steady-state levels of the semiascorbyl radical. The ability of cells to reduce ascorbyl free radical was inhibited by the quinone analogs capsaicin and chloroquine and stimulated by supplementing cells with coenzyme Q_{10}. Purified plasma membranes also reduced ascorbyl free radical in the presence of NADH. Free-radical reduction was not observed in quinone-depleted plasma membranes, but restored after its reconstitution with coenzyme Q_{10}. Addition of reduced coenzyme Q_{10} to depleted membranes allowed them to reduce the signal of the ascorbyl free radical without NADH incubation and the addition of an extra amount of purified plasma membrane quinone reductase further stimulated this activity. Reduction was abolished by treatment with the reductase inhibitor *p*-hydroximercuribenzoate and by blocking surface glycoconjugates with the lectin wheat germ agglutinin, which supports the participation of transmembrane electron flow. The activity showed saturation kinetics by NADH and coenzyme Q, but not by the ascorbyl free radical in the range of concentrations used. Our results support that reduction of ascorbyl free radicals at the cell surface involves coenzyme Q reduction by NADH and the membrane-mediated reduction of ascorbyl free radical.

KEY WORDS: Plasma membrane; ascorbate regeneration; ascorbyl free radical; coenzyme Q

INTRODUCTION

Ascorbate is a compound that, in aqueous solutions in the presence of transition metals, is readily oxidized giving the relatively stable ascorbyl free radical (AFR) (Buettner and Jurkiewicz, 1996). However, in the absence of these catalysts, ascorbate autoxidation at physiological pH proceeds at a very slow rate (6×10^{-7} s^{-1}) (Buettner and Jurkiewicz, 1996). Enzymic oxidation of ascorbate can be also one-electron reactions that produce AFR as the catalysis product (Skot-

[1] Key to abbreviations: AFR, Ascorbate free radical; CHAPS, 3-[(cholamidopropyl)dimethyl-ammonio] 1-propanesulfonate; DHA, dehydroascorbate; DTT, dithiothreitol; EDTA, ethylendiaminotetraacetic acid; GSH, glutathione; PBS, Phosphate-buffered saline; *p*HMB, *p*-hydroxymercuribenzoate; PMSF, Phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CoQ, coenzyme Q, ubiquinone; $CoQH_2$, reduced coenzyme Q, ubiquinol; WGA, wheat germ agglutinin.

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land and Ljones, 1980). Although the reaction rate of AFR with oxygen is almost negligible, the rate of disproportionation, *i.e.*, the dismutation of two radicals to one ascorbate and one dehydroascorbate (DHA), is relatively fast ($3 \times 10^6\ M^{-1}\ s^{-1}$ at pH 7.0) and accounts significantly for the disappearance of ascorbate in solution. This disproportionation reaction is strongly pH dependent since it involves protons (Njus *et al.*, 1990).

Unless properly recycled to ascorbate, DHA will be irreversibly degraded to 2,3-diketo-1-gulonic acid (May *et al.*, 1998). Several mechanisms exist for maintenance of the reduced form, thus decreasing nutritional requirements in animals unable to synthesize this vitamin *de novo*. DHA can be rapidly incorporated into the cells by facilitated diffusion on the glucose transporter and once into the cell, DHA is readily reduced to ascorbate (May, 1999). Ascorbate regeneration from DHA has been considered to require GSH, either through enzymic or nonenzymic reactions (May *et al.*, 1996). The two-electron enzymic reduction of DHA has been attributed to thiol transferase and protein disulfide isomerase (Wells *et al.*, 1990). Thioredoxin reductase and 3α-hydroxysteroid dehydrogenase display NADPH-DHA reductase activity and, thus, have been also proposed to play a role in the maintenance of cytosolic ascorbate (Del Bello *et al.*, 1994; May *et al.*, 1997; Mendiratta *et al.*, 1998). However, a function for these enzymes in ascorbate regeneration *in vivo* is unlikely due to the high K_m values for the substrate DHA (Minetti *et al.*, 1992; May *et al.*, 1998). Furthermore, DHA reduction is not affected by depletion of intracellular GSH in HL-60 cells (Van Duijn *et al.*, 1998a), and it has been recently reported to occur independently of GSH, NADPH, or thioredoxin reductase in U-937 cells (May *et al.*, 1999).

The one-electron reduction of the AFR using NADH as the electron source appears to be the main way to regenerate ascorbate *in vivo* (Coassin *et al.*, 1991). The outer mitochondrial membrane contains NADH-AFR reductase activity composed of cytochrome b_5 reductase and outer mitochondrial membrane-specific cytochrome b_5 (Diliberto *et al.*, 1982; Lederer *et al.*, 1982; Shirabe *et al.*, 1995). Consistent with its important role in ascorbate maintenance, a mutation in the cytochrome b_5 reductase gene results in impaired NADH-dependent ascorbate regeneration in human cells isolated from a patient with type II methemoglobinemia (Shirabe *et al.*, 1995). On the other hand, it has been recently reported that thioredoxin reductase also displays NADPH-AFR reductase activity and thus, it might complement ascorbate regeneration from the free radical mediated by membrane-bound enzymes (May *et al.*, 1998),

Intact cells also stabilize extracellular ascorbate (Alcaín *et al.*, 1991; Navas *et al.*, 1992; Santos-Ocaña *et al.*, 1998a), which has been recognized as an important cellular function for the maintenance of an antioxidant system that protects the plasma membrane from oxidative damage (Villalba *et al.*, 1998). Most cells maintain a steep concentration gradient of ascorbate across their plasma membranes (Rose, 1988; Rose and Bode, 1993), which can not be explained by ascorbate release, but more likely by transmembrane ascorbate regeneration (May, 1999). Ascorbate stabilization by living cells requires coenzyme Q (CoQ, ubiquinone) for optimal functioning (Gómez-Díaz *et al.*, 1997; Santos-Ocaña *et al.*, 1998a). This activity has been interpreted on the basis of ascorbate regeneration through the reduction of either AFR (Rodríguez-Aguilera and Navas, 1994) or DHA (Schweinzer *et al.*, 1996) by the cells. In accordance with its proposed function in the stabilization of extracellular ascorbate, the plasma membrane AFR-reductase appears to be transmembrane (Navas *et al.*, 1988) and also requires CoQ (Villalba *et al.*, 1995; Santos-Ocaña *et al.*, 1998b), as demostrated for other transplasma membrane redox activities (Sun *et al.*, 1992). If stabilization is the result of CoQ-dependent ascorbate recycling from its free radical through transmembrane NADH-AFR reductase, the interaction between reduced CoQ ($CoQH_2$, ubiquinol) and the AFR is expected. The scavenging of ascorbate–quinone free radicals by Ehrlich ascites cells as a result of transmembrane reduction of the free radicals has been previously demonstrated (Pethig *et al.*, 1984, 1985) and HL-60 cells decrease steady-state levels of AFR (Van Duijn *et al.*, 1998a), although the possible mechanisms for free-radical reduction were not investigated.

In this work, we have studied the CoQ requirements for AFR reduction in both intact cells and isolated plasma membrane fractions. We used K-562 erythroleukemic cells, a cell line in which ascorbate stabilization can be clearly distinguished from ascorbate uptake (Schweinzer and Goldenberg, 1992), and CoQ dependence of both ascorbate stabilization and NADH-AFR reductase have been previously demonstrated (Gómez-Díaz *et al.*, 1997). We show here that K-562 cells decrease the steady-state concentrations of AFR generated by reaction of ascorbate with ascorbate oxidase. Reducing activity is mediated by $CoQH_2$ (or its semioxidized intermediate ubisemiquinone) and fits with reported properties of the plasma membrane NADH-AFR reductase. This supports that stabilization

of extracellular ascorbate is not merely the result of metal chelation by cell proteins (Schweinzer *et al.*, 1993), but is more likely related to ascorbate regeneration through transplasma membrane electron transport (Rodríguez-Aguilera and Navas, 1994). In addition, our results support that reduction of AFR at the plasma membrane is a two-step process involving the NADH-dependent transfer of reducing equivalents to the plasma membrane by CoQ-reductase, and the membrane-mediated reduction of AFR by $CoQH_2$, resulting in ascorbate regeneration.

MATERIAL AND METHODS

Cell Cultures

K-562 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Flow Laboratories), 100 units/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml amphotericin B (Sigma), at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were collected from cultures by centrifugation at $1000 \times g$ for 5 min, washed in serum-free RPMI medium and resuspended in phosphate-buffered saline (PBS).

Preparation of Plasma Membrane and Mitochondrial Fractions

Plasma membrane and mitochondria-enriched fractions were isolated from pig or rat liver. Plasma membranes were purified by the two-phase partition method from crude membrane fractions obtained from pig liver homogenates by differential centrifugation. The phase system was composed of 6.0% (w/w) Dextran T-500 (Pharmacia, Sweden), 6.0% polyethylene glycol 3350 (Fisher, USA), 0.25 M sucrose, and 15 mM Tris-H_2SO_4, pH 7.8 (Alcaín *et al.*, 1992). Plasma membranes were resuspended in 50 mM Tris-HCl, pH 7.6, containing 10% glycerol, 1 mM PMSF, and 1 mM EDTA and stored at −70°C until needed. Purity was checked by marker enzyme analysis (Navas *et al.*, 1994). A crude mitochondrial fraction was obtained from rat liver by differential centrifugation as described (Ozols, 1990).

Extraction and Restoration of CoQ

Both K-562 and pig liver cells contain CoQ_{10} as the natural ubiquinone homolog (Gómez-Díaz *et al.*, 1997; Arroyo *et al.*, 1998). CoQ_{10} was extracted with heptane from lyophilized plasma membranes as described (Norling *et al.*, 1974). Briefly, the lyophilized membranes (control membranes, 20 mg) were extracted with 15 ml of heptane for 6 h at 20°C in the dark. The solvent was then decanted and evaporated to obtain the extracted membranes. Reconstituted membranes were obtained by adding back CoQ_{10} in heptane to the extracted membranes, followed by evaporation of the solvent to allow for incorporation of the quinone into the dessicated membranes. CoQ_{10} was also added to control membranes to yield quinone-supplemented plasma membranes. Membranes were taken up in 50 mM Tris-HCl, pH 7.6 and used for assays of AFR reduction. This method results in quantitative incorporation of CoQ_{10} into the membranes (Sun *et al.*, 1992). Ethanolic CoQ_{10} was added directly to K-562 cells in assay buffer.

$CoQ_{10}H_2$ was prepared by adding 10 μl of sodium borohydride solution (10 mg/ml in water) to 1 ml of ethanolic CoQ_{10} (1 mM). To the resulting colorless solutions, 200 μL 1M NaCl and 200 μL hexane were added. Phases were separated by centrifugation and the hexane phase containing the hydroquinones was withdrawn and dried under N_2 stream. Dried samples were resuspended in ethanol and used immediately. $CoQH_2$ in ethanol was added to membranes in assay buffer and preincubated for 3 min at 37°C to allow for incorporation of the hydroquinone before AFR determinations.

For measuring CoQ contents, lipid extracts were dried and resuspended in 100 μL of ethanol. CoQ was then determined by reversed-phase HPLC. Chromatography was performed at 1 ml/min with an Ultrasphere C-18 5 μm precolumn (0.46 × 5 cm) fitted at the top of a C-18 analytical column (0.46 × 25 cm, Beckman, USA). The mobile phase was 70% ethanol–30% methanol and eluates were monitored at 275 nm. The CoQ_{10} peak was identified by its retention time and by automatic recording of absorption spectra of substances eluted from the column. CoQ_{10} was quantified by integration of peaks and comparison with external standards (Sigma, Spain).

AFR Determinations and NADH-AFR Reductase

All assays were performed at 37°C with constant gentle stirring in a total volume of 1 ml. Steady-state levels of AFR were determined with an uv-vis spectrophotometer (DU-650, Beckman, USA) by direct spec-

trophotometric reading at 360 nm, a method that can be used as an alternative to electron spin resonance (EPR, ESR) (Skotland and Ljones, 1980; Bielski, 1982; Kobayashi *et al.*, 1991; May *et al.*, 1998). The assay mixture contained 1 mM ascorbate in PBS, pH 7.4. NaCl at 150 mM was included into the buffer to counteract the cell-surface charge, thus favoring the access of anionic AFR to the cell surface (Pethig *et al.*, 1984). Samples were monitored at 360 nm for a minute before addition of ascorbate oxidase. AFR levels were measured from the absorbance change observed after addition of 100 units/ml ascorbate oxidase into the reaction mixture. Steady-state concentrations of AFR (recorded during the first minute of reaction) were calculated using an extinction coefficient of 5 mM^{-1} cm^{-1} (Skotland and Ljones, 1980). AFR concentrations measured in sample-free medium were compared with those obtained in the presence of 0.05–0.25 mg of plasma membrane or mitochondria proteins, or up to 10^6 K-562 cells/ml. Spectrophotometric readings were corrected for background due to cells, membranes, and/or NADH. In all assays, background-corrected readings were five- to ten-fold higher than the limit of detection by the instrument. NADH-AFR reductase was assayed by measuring the oxidation rate of NADH at 340 nm upon addition of 100 units of ascorbate oxidase to a reaction medium containing 0.2 mM NADH, 1 mM sodium ascorbate, and 0.07 mg of mitochondria or 0.55 mg of plasma membrane protein in PBS, pH 7.4.

To test the effect of a blockade of sulfydryl groups or glycoproteins, samples were preincubated for 2 min at 37°C in the presence of the thiol reagent *p*-hydroxymercuribenzoate (*p*HMB, up to 100 μM) or the lectin wheat germ agglutinin (WGA, up to 10 μg/ml) respectively. Excess *p*HMB or WGA was removed by centrifugation.

Purification of the CoQ Reductase from Isolated Plasma Membranes

Pig liver plasma membranes were treated with 0.5 M KCl in 25 mM Tris-HCl, pH 7.6 containing 1 mM EDTA, 1 mM PMSF, 0.1 mM DTT, and 10% glycerol to remove peripheral proteins and, then, integral proteins were extracted by solubilization with 2.5% 3-[(cholamidopropyl)dimethyl-ammonio] 1-propanesulfonate (CHAPS). The protein extract was separated from the membrane residue by centrifugation at 105,000 × *g* for 1 h at 4°C. The CoQ-reductase was then 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 (Villalba *et al.*, 1995). DTT was removed from purified samples by dialysis to avoid interference with reducing assays. Purification was monitored by SDS–PAGE. Protein determinations were carried out by the dye-binding method described for membrane samples (Stoscheck, 1990). Bovine γ-globulin was used as standard.

RESULTS

Addition of ascorbate oxidase to a reaction mixture containing ascorbate resulted in the detection of a signal at 360 nm (Fig. 1A), reported to be due to AFR (Skotland and Ljones, 1980). The absorbance increase was directly proportional to the amount of ascorbate oxidase added to the assay and no signal was observed in controls carried out without ascorbate or by adding heat-inactivated enzyme to buffer containing ascorbate (not shown). AFR concentrations were calculated to be in the micromolar range using an extinction coefficient of 5 mM^{-1} cm^{-1}. When ascorbate oxidase was used at 100 mU/ml, the steady-state concentrations of AFR were maximal during the first minute of reaction, reaching values of about 3 to 4 μM, a value that agrees with reported concentrations of AFR generated by pulse radiolysis (Kobayashi *et al.*, 1991). Absorbance at 360 nm then tended to decay as ascorbate oxidation ceased when all the oxygen had been used up (Skotland and Ljones, 1980).

We have taken advantage of the strong pH dependency of the AFR life-time (Njus *et al.*, 1990) to confirm that absorbance changes at 360 nm indeed

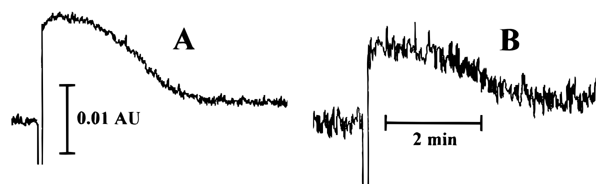

Fig. 1. Detection of AFR by direct measuring at 360 nm and reduction of steady-state concentrations of the free radicals by K-562 cells. Ascorbate oxidase (100 mU/ml) was added at point represented by large deflections in absorbance recordings. (A) Control assay without cells; (B) assay in the presence of 10^6 K-562 cells/ml. Higher noise levels in (B) are due to an increase of turbidity of the reaction mixture-containing cells.

measured AFR concentrations, by correlating our absorbance recordings with AFR levels determined by EPR. Absorbance increases at 360 nm were measured after addition of the same activity units of ascorbate oxidase to different assay mixtures made with buffers of pH, ranging from about 4 to 10. Volumes of ascorbate oxidase stock solution added to each assay were corrected to achieve the same rates of ascorbate oxidation at any pH, measured by absorbance decrease at 265 nm. As shown in Fig. 2, steady-state concentrations of AFR determined from signal at 360 nm were minimal in low pH buffers but considerably increased at pH above 8.

When K-562 cells were present in the assay, the increase of absorbance at 360 nm upon addition of ascorbate oxidase was significantly lower (Fig. 1B). Cells did not change the pH of the assay reaction, thus the lowering in the AFR signal was not attributable to this effect. In addition, to discard the inhibition of ascorbate oxidase by cells, the enzyme was incubated in the absence and presence of cells and aliquots were taken at 0 and 5 min and tested for oxidation of ascorbate (125 μM). No inhibition of ascorbate oxidase by cells was observed during the time frame used in our experiments (not shown).

Quantification of AFR from the absorbance change showed that steady-state concentrations of the free radical decreased linearly with the amount of cells added to the assay and about 1 μM AFR was scavenged by 10^6 K-562 cells/ml (Fig. 3A). A transplasma membrane, CoQ-dependent AFR-reductase has been proposed to be involved in ascorbate regeneration by K-562 cells (Gómez-Díaz *et al.*, 1997). If such a system is responsible for the lowering of AFR concentrations shown here, the scavenging of the free radical by the cells should also display a CoQ dependency. As reported in Table I, addition of CoQ_{10} to the cells stimulated their ability to scavenge AFR. CoQ_{10} at 50 μM produced a threefold stimulation (Table I), but adding higher amounts of the quinone did not result in a further increase of scavenging activity (not shown). CoQ_{10} (oxidized and reduced) did not affect AFR signal when added in the absence of cells.

Consistent with the involvement of CoQ in AFR reduction by the cells, the activity was sensitive to various quinone antagonists such as chloroquine and capsaicin. The requirement for the integrity of cell surface glycoconjugates to maintain optimal levels of plasma membrane NADH-AFR reductase and ascor-

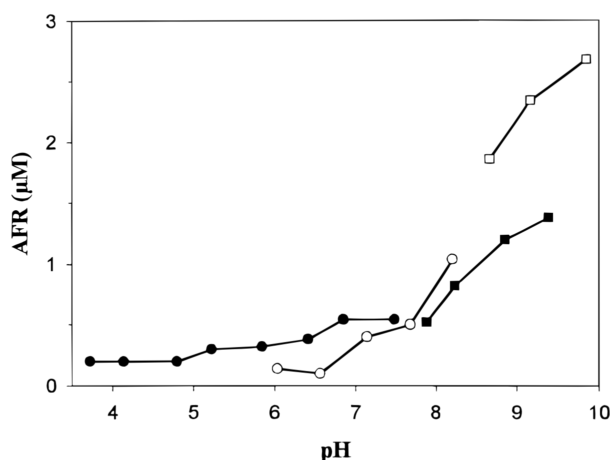

Fig. 2. Effect of pH on steady-state concentrations of AFR measured from the absorbance increase at 360 nm. Ascorbate was prepared at 0.15 mM in different buffers and AFR was then generated by the addition of 22 mU ascorbate oxidase. The volume of stock solution of ascorbate oxidase added was adjusted to correct for different activities of the enzyme at different pH, in order to achieve the same rates of ascorbate oxidation. The followed buffers used were: 20 mM citrate-phosphate (●), 20 mM phosphate buffer (○), 20 mM Tris-HCl (■), and 20 mM glycine-NaOH (□). Results shown are representative of two separate experiments.

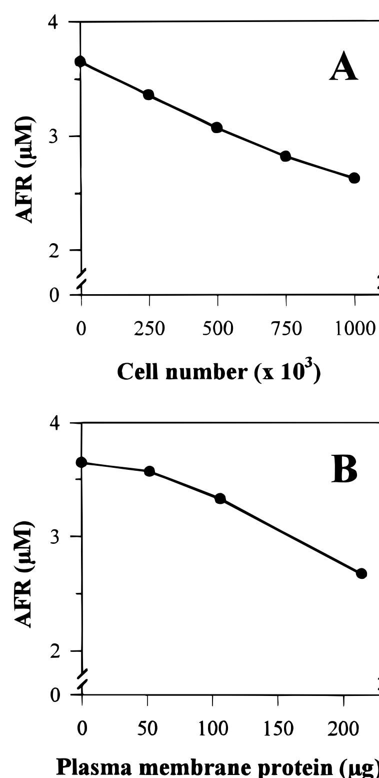

Fig. 3. Decrease of steady-state concentrations of AFR in the presence of increasing amounts of K-562 cells (A) or isolated plasma membranes plus 100 μM NADH (B). Results shown are representative of three separate experiments.

Table I. Effect of Different Compounds on AFR Scavenging by K-562 Cells[a]

Addition	Concentration	AFR scavenging (μM/10^6 cells)	Effect (%)
No cells	—	—	—
K-562 cells	—	1.0 ± 0.08	—
K-562 cells + CoQ_{10}	10 μM	1.2 ± 0.07	+20
K-562 cells + CoQ_{10}	50 μM	3.6 ± 0.1[d]	+260
K-562 cells + capsaicin	150 μM	0.7 ± 0.05[b]	−30
K-562 cells + Chloroquine	0.5 mM	0.6 ± 0.03[c]	−40
Chloroquine + WGA	0.5 μg/ml	0.4 ± 0.05[d]	−60

[a] Listed compounds were added to K-562 cells (250,000–500,000 cells/ml) in assay buffer and preincubated for 3 min prior to assay for AFR reduction. Absorbance changes at 360 nm upon addition of 100 mU ascorbate oxidase were then recorded; AFR concentrations were calculated using an extinction coefficient of 5 mM^{-1} cm^{-1}. Scavenging activity was calculated from the difference in steady-state levels of AFR in the absence and the presence of cells, and referred to 10^6 cells. The *effect* column represents variations in scavenging activity due to the compounds tested (in percentage). Data with (+) mean activation, whereas data with (−) mean inhibition of AFR scavenging. Data represent mean ± S.D. ($n = 3$).
[b] $p < 0.05$.
[c] $p < 0.01$.
[d] $p < 0.001$ versus cells alone.

bate stabilization by whole cells has been previously demonstrated and a significant inhibition of both activities by the lectin WGA has been reported (Navas *et al.*, 1988; Alcaín *et al.*, 1991). Incubating the cells in the presence of this lectin also produced a significant inhibition on the ability of cells to scavenge AFR (Table I). The activity of ascorbate oxidase used to generate AFR was not affected by the compounds tested because no significant effects on steady-state levels of AFR in the absence of cells were observed (not shown).

Isolated plasma membranes lacked AFR reducing activity by themselves. However, plasma membranes displayed scavenging activity when assays were carried out in the presence of 100 μM NADH. The activity was proportional to the amount of plasma membrane added and about 1 μM AFR was scavenged in assays containing 0.2 mg/ml plasma membrane protein (Fig. 3B). The scavenging activity measured with plasma membranes plus NADH was inhibited by the quinone antagonists chloroquine and capsaicin and inhibition was partially reversed by CoQ_{10} (Table II). It has been reported that the cytochrome b_5 reductase can act as the primary reductase for delivering electrons to CoQ and drive a transplasma membrane electron transport (Villalba *et al.*, 1995; Navarro *et al.*, 1998). Consistent with the participation of this enzyme in AFR reduction by isolated plasma membranes plus NADH, the activity was sensitive to the reductase inhibitor *p*HMB. Only a slight inhibition of about 10% was observed at 1 μM *p*HMB, but the bulk of the scavenging activity was inhibited at 100 μM *p*HMB (Table II).

According to the different mechanisms suggested by May (1999) for electron transport in erythrocyte plasma membranes, our results obtained with liver plasma membranes could be interpreted on the basis of either a direct reduction of AFR by the cytochrome b_5/cytochrome b_5 reductase on the cytoplasmic side of the plasma membrane, or the transmembrane reduction of the free radical. To distinguish between *cis* and *trans*-oriented reduction of AFR, we have compared the sensitivity to the lectin WGA of scavenging activities of plasma membranes and mitochondria, because it has been previously demostrated that mitochondria carries out AFR reduction through the cytochrome b_5/cytochrome b_5 reductase system (Diliberto *et al.*, 1982; Lederer *et al.*, 1982; Shirabe *et al.*, 1995). Pretreating plasma membranes with the lectin produced a strong inhibition of the scavenging activity. Interestingly, WGA inhibited both the basal and the CoQ_{10}-stimulated activity of isolated plasma membranes (Table II), which indicate that both activities may require the integrity of glycan moieties exposed to the external surface for optimal functioning and supports a transmembrane orientation. Liver mitochondria also displayed substantial AFR scavenging activity in the presence of NADH. Although steady-state levels of AFR could not be accurately measured because the free radical was very rapidly consumed, no effect was observed by including 10 μg/ml WGA in the assays

Table II. Effect of Various Compounds on the Scavenging Activity of Plasma Membranes in the Presence of NADH[a]

Addition	Concentration	AFR scavenging (μM/mg protein)	Effect (%)
None	—	5.1 ± 0.6	—
CoQ_{10}	50 μM	6.2 ± 0.5	+21
Chloroquine	500 μM	2.0 ± 0.4[c]	−60
Capsaicin	150 μM	2.2 ± 0.3[c]	−56
CoQ_{10} + capsaicin	50 μM/150 μM	3.9 ± 0.5[b]	−24
*p*HMB	1 μM	4.6 ± 0.6	−10
*p*HMB	100 μM	0.1 ± 0.07[d]	−98
WGA	1 μg/ml	0.9 ± 0.1[d]	−82
CoQ_{10} + WGA	50 μM/1 μg/ml	0.9 ± 0.2[d]	−82

[a] All assays contained plasma membranes (0.2 mg/ml) and NADH (100 μM) in assay buffer at a final volume of 1 ml. Membranes were preincubated with the listed compounds for 3 min and then, 100 mU ascorbate oxidase were added and the absorbance change at 360 nm recorded. Amounts of AFR scavenged by plasma membranes are listed. Data were calculated from steady-state concentration of AFR measured in the absence and the presence of plasma membranes plus NADH and referred to in milligrams protein. The *effect* column represents variations of scavenging activity relative to that obtained with plasma membranes plus NADH (in percentage). Data with (+) mean activation, whereas data with (−) mean inhibition. Data represent mean ± S.D. ($n = 3$).

[b] $p < 0.05$ versus capsaicin addition.

[c] $p < 0.01$.

[d] $p < 0.001$ versus no addition.

(not shown), which is consistent with a *cis*-oriented activity through the cytochrome b_5/cytochrome b_5 reductase. These results agree with those obtained for NADH-AFR reductase. As previously published (Navas *et al.*, 1988; Villalba *et al.*, 1993), the activity in plasma membranes was strongly inhibited by WGA (3.49 ± 1.58 nmol mg^{-1} min^{-1} in the absence and 0.11 ± 0.17 nmol mg^{-1} min^{-1} in the presence of WGA at 10 μg/ml). NADH-AFR reductase of liver mitochondria was one order of magnitude higher than in plasma membrane, although it probably represented a different level of electron transport because the activity was not inhibited by the lectin (35.53 ± 5.42 nmol mg^{-1} min^{-1} in the absence, and 49.53 ± 9.63 nmol mg^{-1} min^{-1} in the presence of WGA at 10 μg/ml).

To further provide evidence of the role played by CoQ_{10} and the NADH-CoQ_{10} reductase in the scavenging of AFR by the cells, we extracted lyophilized plasma membranes with heptane to remove the quinone. CoQ_{10} was then added to extracted and also to unextracted membranes to obtain reconstituted and supplemented samples, respectively. Extraction of the quinone from plasma membranes was confirmed by HPLC analysis (not shown). None of these membranes displayed significant AFR reducing activity in the absence of NADH, but preincubation with the pyridine nucleotide restored the activity in control, reconstituted, and supplemented membranes. Consistent with a requirement for CoQ, heptane-extracted membranes displayed very little activity even in the presence of NADH (Table III). Interestingly, addition of 50 nmol of the reduced ubiquinol ($CoQ_{10}H_2$) to the extracted membranes enabled them to reduce the signal of AFR

Table III. CoQ_{10} and CoQ_{10} Reductase Requirements for the AFR Reduction by Isolated Plasma Membranes[a]

Sample	Addition	AFR scavenging (μM/mg protein)
C	None	0.2 ± 0.1
E	None	0.1 ± 0.06
R	None	0.2 ± 0.1
S	None	0.2 ± 0.1
C	100 μM NADH	5.2 ± 0.7[c]
E	100 μM NADH	0.4 ± 0.05[b]
R	100 μM NADH	3.4 ± 0.3[c]
S	100 μM NADH	6.2 ± 0.8[c]
E	50 μM $CoQ_{10}H_2$	4.5 ± 0.6[c,d]
S	100 μM NADH + 0.5 μg CoQ reductase	7.3 ± 0.9

[a] Plasma membranes were lyophilized (control, C) and extracted with heptane for 6 h at 20°C in the dark (extracted, E). CoQ_{10} (50 nmol) was added to extracted (reconstituted, R) and also to unextracted membranes (supplemented, S). AFR-reduction activity was calculated from the amount of AFR scavenged (μM) and was referred to in milligram protein. Data represent mean ± S.D. ($n = 3$).

[b] $p < 0.01$.

[c] $p < 0.001$ versus their respective controls without further addition.

[d] $p < 0.001$ versus extracted membranes plus NADH.

in the absence of NADH. AFR scavenging by $CoQ_{10}H_2$ required the membrane because $CoQ_{10}H_2$ alone had no effect on the steady-state levels of AFR and 50 nmol of $CoQ_{10}H_2$ incorporated into 0.2 mg sonicated egg-yolk phospholipids did not produce significant reducing activity by these liposomes (data not shown). Cytochrome b_5 reductase did not show significant NADH-dependent scavenging of AFR. However, the activity tended to act like a stimulant after incorporation of an extra amount of purified enzyme [confirmed by western blot immunostaining (Gómez-Díaz *et al.*, 1997)] into CoQ_{10}-supplemented plasma membranes (Table III).

We have studied the kinetics of AFR scavenging by cells and plasma membranes with respect to NADH and steady-state concentrations of AFR. Reduction of AFR by isolated plasma membranes showed a saturation response with respect to NADH (Fig. 4). An apparent K_m value for the pyridine nucleotide of 5 μM and a V_{max} of 5.9 μM AFR scavenged/mg protein were calculated from reciprocal plots. To calculate enzyme kinetics with respect to AFR, different volumes of ascorbate oxidase stock solution were added to the assay buffer containing ascorbate to achieve different steady-state concentrations of AFR. Reducing activity of both K-562 cells (Fig. 5A) and isolated plasma membranes (Fig. 5B) increased linearly with the amount of AFR generated in the assay and no saturation effect was observed for AFR concentrations in the range of 0 to 4 μM.

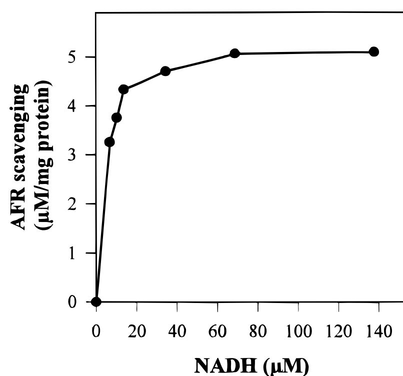

Fig. 4. Kinetics of AFR scavenging by plasma membranes (0.2 mg/ml). Specific scavenging activity referred to in milligrams protein is represented. Assays were carried out in the presence of increasing concentrations of NADH. AFR concentration in all assays was about 3 μM. Results shown are representative of three separate experiments.

DISCUSSION

AFR is a rather stable free radical produced during the metal or enzyme-catalyzed oxidation of ascorbate. In the absence of a reactant for AFR, the free radicals spontaneously disproportionate giving one ascorbate and one DHA (Bielski, 1982). If not properly recycled to the fully reduced form, DHA will be irreversible degraded to 2,3-diketo-1-gulonic acid, resulting in the loss of this antioxidant vitamin (May *et al.*, 1998). DHA can be rapidly incorporated into the cell and then reduced to ascorbate, although the exact mechanism for DHA reduction has not been fully established (May *et al.*, 1999). On the other hand, regeneration of intracellular ascorbate from AFR has been demonstrated at the genetic level to be mediated by the outer mitochondreal membrane-specific cytochrome b_5/cytochrome b_5 reductase (Shirabe *et al.*, 1995). Ascorbate can be also maintained in its reduced state outside the cell (Rose, 1988; Alcaín *et al.*, 1991; Rose and Bode, 1993). A transmembrane AFR reductase has been proposed to mediate regeneration of extracellular ascorbate (Villalba *et al.*, 1995; Gómez-Díaz *et al.*, 1997; Santos-Ocaña *et al.*, 1998a, b). However, some criticism concerning the enzymic nature of ascorbate stabilization by whole cells has also arisen and results have been explained merely on the basis of metal chelation by cell proteins, resulting in a decrease of the rate of transition metal-catalyzed oxidation of ascorbate (Schweinzer *et al.*, 1993). The aim of this work was to study the interactions between AFR and the plasma membrane as an approach to understanding ascorbate stabilization by whole cells and its relationship with transmembrane AFR-reductase.

Because of the unusual stability of the free-radical intermediate, low steady-state concentrations of AFR are maintained during oxidation of ascorbate and can be detected by EPR techniques (Laroff *et al.*, 1972; Sharma and Buettner, 1993). The AFR also exhibits distinctive spectral properties that allow for quantification of free radical concentrations by direct spectrophotometric reading at 360 nm (Skotland and Ljones, 1980; Bielski, 1982; Kobayashi *et al.*, 1991; May *et al.*, 1998). We have made use of the strong pH dependency of the AFR life-time to validate this latter method for AFR quantification. We measured steady-state levels of the free radical at a different pH from the absorbance change at 360 nm upon addition of ascorbate oxidase, we then compared our results with those obtained by EPR by other authors. The effect of

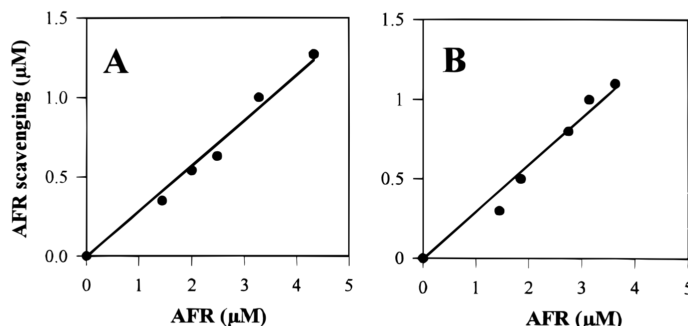

Fig. 5. Scavenging activity of K-562 cells (250,000–500,000 cells/ml) (A) and isolated plasma membranes (0.2 mg/ml) plus 100 μM NADH (B) at increasing concentrations of AFR. Different concentrations of AFR in the assay mixture were achieved by addition of appropriate volumes of ascorbate oxidase stock solution. To calculate specific scavenging activity, initial concentrations of AFR were also determined upon addition of the same volume of ascorbate oxidase stock solution to the reaction buffer without K-562 cells or plasma membranes. Results shown are representative of three separate experiments.

pH on steady-state concentrations of AFR deduced from absorbance increases at 360 nm (Fig. 2) was identical to that obtained by measuring AFR by EPR (Buettner and Jurkiewicz, 1996). Thus, the absorbance change at 360 nm upon addition of ascorbate oxidase is a reliable method to determine AFR concentrations in our experiments.

When K-562 cells were present in the assay, AFR concentrations were significantly lower. A similar reduction of AFR levels (measured by EPR) has been reported by Van Duijn *et al.* (1998) to occur in the presence of HL-60 cells, although these authors did not investigate the mechanisms for AFR scavenging. It has been previously reported that the presence of K-562 cells in ascorbate-containing buffer decreases the oxidation rate of this vitamin (Schweinzer *et al.*, 1993; Gómez-Díaz *et al.*, 1997), which agrees with results obtained with many cell types including HL-60 (Alcaín *et al.*, 1991), neuroblastoma (Medina *et al.*, 1992), retinoblastoma (Medina and Schweigerer, 1993), and yeasts (Santos-Ocaña *et al.*, 1995, 1998a). Ehrlich ascites cells also decrease steady-state concentrations of the free radicals produced by ascorbate–quinone mixtures, increasing the rate of free radical consumption in a process that is controlled by sulfydryl groups and the cell-surface charge (Pethig *et al.*, 1983, 1984). Since AFR was generated in our experiments by an enzyme-driven assay and not by transition metal-catalyzed oxidation of ascorbate, the lowering of AFR concentrations observed in the presence of cells can not be interpreted on the basis of a decrease in ascorbate oxidation rates due to chelation of transition metal ions by cell proteins (Schweinzer *et al.*, 1993). Rather, our results are compatible with ascorbate regeneration through a transmembrane reduction of AFR, which results in a decrease of steady-state levels of the free radical.

Which redox system is responsible for AFR reduction by the cells? At least two different redox systems have been identified in animal plasma membranes. It is apparent that ascorbate is the major electron donor for a transmembrane reductase (May *et al.*, 1996 , May, 1999; May and Qu, 1999; Van Duijn *et al.*, 1998a), although a different system is also present which relies on NADH and CoQ (Sun *et al.*, 1992; Van Duijn *et al.*, 1998a; Villalba *et al.*, 1995, 1996; May, 1999). Exposure of Ehrlich ascites tumor cells to AFR–semiquinone free radicals results in a depletion of NAD(P)H in cells (Pethig *et al.*, 1985). Ascorbate stabilization by whole cells and the plasma membrane NADH-AFR reductase both require CoQ for optimal functioning (Villalba *et al.*, 1995; Gómez-Díaz *et al.*, 1997) and this dependence has been demonstrated at the genetic level using yeast mutants unable to synthesize CoQ_6 (Santos-Ocaña *et al.*, 1995, 1998a). Consistent with the participation of the quinone in the scavenging activity, AFR reduction by whole cells was stimulated by CoQ_{10} and inhibited by CoQ antagonists. Inhibition by the lectin WGA supports the notion that the integrity of the cell surface carbohydrates is also

required, as demonstrated for the plasma membrane NADH-AFR reductase (Navas *et al.*, 1988) and ascorbate stabilization by K-562 cells (Gómez-Díaz *et al.*, 1997). On the other hand, ascorbate itself could be also a donor for transmembrane reduction of extracellular AFR if coupled to an energy-generating step such an H^+-ATPase, as occurs in the chromaffin granule system (Njus *et al.*, 1990; May, 1999). However, reduction potentials of ascorbate and CoQ do not support such a mechanism (May, 1999) and, furthermore, ascorbate has been reported to promote the oxidation of CoQ_1H_2 to CoQ_1, involving superoxide radicals in a reaction that is strongly affected by phase separation of the reactants (Roginsky *et al.*, 1996).

Although isolated plasma membranes lacked significant scavenging activity, it was restored by NADH. Inhibition by *p*HMB supports the participation of the cytochrome b_5 reductase as a primary reductase delivering electrons for the scavenging activity (Navarro *et al.*, 1995; Villalba *et al.*, 1995). AFR reduction by whole cells and by plasma membranes plus NADH might not represent the same level of activity. As suggested by May (1999), reduction of AFR by plasma membranes could be interpreted as a direct reduction of the free radical on the cytoplasmic side of the plasma membrane, either by the cytochrome b_5 reductase (Kobayashi *et al.*, 1991), or by the cytochrome b_5/cytochrome b_5 reductase, this latter system being similar to the NADH-AFR reductase from outer mitochondrial membranes (Diliberto *et al.*, 1982; Lederer *et al.*, 1982). However, several lines of evidence argue against this interpretation. First, purified cytochrome b_5 reductase lacked significant NADH-dependent scavenging activity, but had an stimulatory effect on CoQ-supplemented plasma membranes. Although the direct reduction of AFR by the cytochrome b_5 reductase has been demonstrated, it is apparently a nonspecific process that occurs at rates 30 times lower than AFR reductase (Kobayashi *et al.*, 1991). Second, reduction of AFR by cytochrome b_5/cytochrome b_5 reductase requires an isoform of cytochrome b_5, which is specific from outer mitochondrial membranes (Diliberto *et al.*, 1982; Lederer *et al.*, 1982). Third, the activity measured in plasma membranes required CoQ and was inhibited by WGA, which is not expected for electron transfer between cytochrome b_5 reductase and cytochrome b_5. Accordingly, neither AFR-scavenging nor NADH-AFR reductase were inhibited by WGA in mitochodrial fractions. Ehrlich ascites cells homogenates also lose their ability to scavenge free radicals produced from ascorbate–quinone mixtures, and this is restored by NAD(P)H. Strikingly, the dominant free radical-reducing activity of these cell homogenates was not found associated with mitochondria (Pethig *et al.*, 1985).

Thus, AFR reduction by both whole cells and plasma membranes plus NADH appear to represent the same phenomenon, likely involving transmembrane flux of electrons by CoQ-dependent NADH-AFR reductase. CoQ mediates some plasma membrane-associated redox activities (Sun *et al.*, 1992) and CoQ-dependence distinguishes *trans*-oriented redox activity from other activities related to *cis*-electron transport (Villalba *et al.*, 1995). Since the quinone moiety of CoQ is freely movable within the lipid bilayers (Lenaz *et al.*, 1995), it could transfer reducing equivalents across the plasma membrane in a redox cycle of CoQ oxidation-reduction (Villalba *et al.*, 1996). CoQ reduction would take place at the cytosolic side of the plasma membrane by cytochrome b_5 reductase. K_m values for NADH of both the reductase (Villalba *et al.*, 1995) and NADH-dependent reduction of AFR are in the same range and, also, both activities are similarly inhibited by thiol reagents and CoQ antagonists. AFR reduction would take place at the external side of the plasma membrane. Topography studies of the plasma membrane NADH-AFR reductase have indicated an inner NADH- and an outer AFR-binding site (Goldenberg *et al.*, 1983; Villalba *et al.*, 1996). It is important to note that $CoQH_2$ alone or in liposomes had little effect on steady-state concentrations of AFR, but the addition of this compound to plasma membranes allowed them to reduce AFR without NADH. Since both basal and CoQ-stimulated activities were inhibited by WGA, it is apparent that some component of the glycocalix may favor the access of AFR to the bilayer surface, where it could be reduced by $CoQH_2$. Alternatively, transmembrane reduction of AFR might still require other components in addition to CoQ. For instance, it has been shown that transmembrane reduction of ferricyanide by erythrocyte membranes requires α-tocopherol (May *et al.*, 1996), although this dependence has not been confirmed in nucleated HL-60 cells (Van Duijn *et al.*, 1998a). Whether or not ferricyanide and AFR accept electrons from the same membrane component remains to be established (May *et al.*, 1999) but α-tocopherol could facilitate AFR reduction by a CoQ-dependent oxidoreductase (May *et al.*, 1999) because $CoQH_2$ can donate electrons to reduce α-tocopheroxyl radicals and regenerate α-tocopherol (Beyer, 1994). This is in accordance with data presented previously by Constantinescu *et al.*

(1993, 1994), who reported the participation of the cytochrome b_5 reductase system in NADH-driven enzymic recycling of α-tocopherol in erythrocyte membranes.

AFR reduction by both whole cells and isolated plasma membranes did not follow saturation kinetics with respect to the steady-state concentrations of AFR. An AFR reductase has been reported in erythrocyte membranes to display a high affinity for the AFR, with an apparent K_m in the low picomolar range (Schweinzer and Goldenberg, 1992). However, these authors did not directly measure AFR concentrations in their assays, but estimated AFR concentrations from the equilibrium constant of ascorbate–DHA mixtures. It has been recently shown that the equilibrium constant is not a reliable method to calculate AFR concentrations, but the free radical must be directly determined (Van Duijn *et al.*, 1998b; Van der Zee and Van den Broek, 1998). In addition, the direct reduction of AFR by cytochrome b_5 reductase (although at rates 30 times lower than AFR reductase) is not saturated with respect to the steady-state concentration of AFR in the range of 6 μM (Kobayashi *et al.*, 1991).

In conclusion, CoQ and CoQ-reductase play key roles in the maintenance of antioxidant molecules whose participation is required at the plasma membrane. CoQ-reductase is responsible for the maintenance of $CoQH_2$, that avoids membrane lipid peroxidation by either mediating the regeneration of tocopherols or by direct radical scavenging in compartments devoid of tocopherols (Beyer *et al.*, 1996; Landi *et al.*, 1997; Navarro *et al.*, 1998). In addition, $CoQH_2$ can provide electrons for the regeneration of ascorbate from AFR to enhance antioxidant protection and to maintain extracellular stores of this vitamin.

ACKNOWLEDGMENTS

Supported by Grants No. PB98-0329-CO2-01 and PB98-0329-CO2-02 of the Spanish Ministerio de Educación y Cultura. FLC and AA were granted by the Spanish Ministerio de Educación y Cultura. FN and CGD were granted by the Universidad de Córdoba.

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