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Monoascorbate free radical-dependent oxidation-reduction reactions of liver Golgi apparatus membranes

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Abstract Golgi apparatus from rat liver contain an ascorbate free radical oxidoreducatse that oxidizes NADH at neutral pH with monodehydroascorbate as acceptor to generate a membrane potential. At pH 5.0, the reverse reaction occurs from NAD^+. The electron spin resonance signal of the ascorbate-free radical and its disappearance upon the addition of NADH (pH 7) or NAD^+ (pH 5.0) confirms monodehydroascorbate involvement. Location of monodehydroascorbate both external to and within Golgi apparatus compartments is suggested from energization provided by inward or outward flux of electrons across the Golgi apparatus membranes. The isolated membranes are sealed, oriented cytoplasmic side out and impermeable to NAD^+ and ascorbate. NAD^+ derived through the action of Golgi apparatus β-NADP phosphohydrolase is simultaneously reduced to NADH with monodehydroascorbate present. The response of the NADH- (NAD^+-) ascorbate free radical oxidoreductase system to pH in Golgi apparatus provides a simple regulatory mechanism to control vesicle acidification.

Keywords Golgi apparatus · Acidification · NADH ascorbate · β-NADP phosphorylase · Ascorbate free radical oxidoreductase · Rat liver

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Introduction

Vesicular acidification is a process central to mechanisms of vesicular trafficking (Marshansky 2007). The important roles of endosomal receptor-ligand dissociation (Forgac 1992) and in activation of lysosomal hydrolytic enzymes (Van Dyke 1996) are well established. Less well established are the molecular mechanisms whereby luminal pH is controlled at the level of the cisternae of the Golgi apparatus.

Ascorbate has been reported as a constituent present in clathrin-coated vesicles (Sun et al. 1984; Morré et al. 1985), Golgi apparatus (Sun et al. 1984), chromaffin granules (Njus et al. 1983; Srivastava et al. 1984) and secretory vesicles (Van Zastrow et al. 1984) and implicated as important to the secretory process (Morré et al. 1985; Njus et al. 1983; Harris 1996; Sun et al. 1983). In this report, we demonstrate a monodehydroascorbate reductase of Golgi apparatus, which, depending upon pH, can result in the oxidation or reduction of pyridine nucleotides at the expense of intravesicular ascorbate free radical with the resultant generation of a membrane potential. The reaction would alter the relative proportions of ascorbate and dehydroascorbate within the vesicles. Additionally, when NADP is hydrolyzed by an NADP phosphatase activity of

Golgi apparatus at an optimal pH of about 5.0 (Navas et al. 1986), a large proportion of the NAD^+ formed is recovered as NADH. Thus, NADH-monodehydroascorbate oxidoreducatse may serve as one mechanism to regulate vesicle acidification by Golgi apparatus and/or, because of the membrane potential generated, to energize membranes to facilitate vesicle displacements and transport.

Materials and methods

Intact Golgi apparatus were prepared using the rapid procedure of Morré et al. (1972) from male Holtzman rats (ca. 250 g), provided a standard diet and drinking water ad libitum. Protein determinations were according to Lowry et al. (1951) with bovine serum albumin as standard. The medium to measure NADH-monodehydroascorbate oxidoreducatse (EC 1.6.5.4) contained 50 mM Tris-HC1, pH 7.4 or sodium or potassium acetate, pH 5.0, and a final volume of 2.8 ml. The final concentration of NADH or NAD^+ was 2.0 μM. 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, with reference at 500 nm.

The disappearance of ascorbate free radical in Golgi apparatus upon addition of NADH or NAD^+ was demonstrated by the disappearance of its characteristic electron spin resonance (ESR) signal measured using a Varian E-109-ESR spectrophotometer at 9.4 GHZ microwave frequency and in a cell compartment controlled thermostatically at 30°C.

To test for the permeability of the Golgi apparatus membranes to NAD^+, 0.3 ml of the Golgi apparatus fraction (about 0.5 mg protein) resuspended in 0.25 M sucrose was added to 0.3 ml of a medium containing 50 mM sodium or potassium acetate, pH 5.0, 0.25 M sucrose and [^{3}H]NAD^+ (50 μCi: specific activity of 30 μCi/mmol, ICN Radiochemicals) at a final concentration of 2 μM. The uptake was stopped by rapid centrifugation of the Golgi apparatus through a layer of silicone oil (0.2 ml of dimethyldiphenylpolysiloxane, density of 1.05 g/ml) into 0.6 ml of 10% $HC10_4$ (density 1.07 g/ml) (Neuburger et al. 1985). The uptake was determined as a function of time at 25°C.

To determine the sidedness of the isolated Golgi apparatus, membranes were fixed on 2.5% glutaraldehyde in cacodylate buffer, pH 7.2, for 1 h at 4°C. After the fixation, fractions were rinsed in the buffer and a portion was sonicated four times each for 15 s to break the fixed vesicles. Both the sonicated and the unsonicated samples were incubated with 2 μg/ml peroxidase-labeled concanavalin A (con A) in cacodylate buffer, pH 7.2 for 1 h at room temperature. The samples were then washed in the same buffer and the dimainobenzidine reaction to detect the peroxidase-labeled con A was carried out according to Graham and Karnovsky (1966).

Pyridine nucleotide concentrations were determined in Golgi apparatus after incubation with 500 μM $NADP^+$ for 10 min in the presence of different amounts of monodehydroascorbate. The cycling assay procedure of Matsumura and Miyachi (1980) was used for determinations of pyridine nucleotide. The pyridine nucleotide concentrations of controls in which the reaction mixture was incubated with boiled Golgi apparatus or without Golgi apparatus fractions were subtracted.

The Golgi apparatus marker, UDP-galactose: N-acetylglucosamine galactosyltransferase (galactosyltransferase) was assayed according to Palmiter (1969). Mitochondrial markers, succinate dehydrogenase (Pennington 1961) and cytochrome oxidase (Sun and Crane 1969) were assayed as referenced.

Results

The oxidation of NADH or reduction of NAD^+ by NADH-monodehydroascorbate oxidoreducatse of Golgi apparatus of rat liver occurs in a manner proportional to time and amount of protein added (Fig. 1) and is dependent upon pH (Fig. 1) and the concentration of reactants (Fig. 2). The oxidation of NADH occurs optimally at pH 7.5 (Fig. 1c). However, at pH 5.0, the reverse reaction, reduction of NAD^+ to form NADH, also may occur (Fig. 1d–f), but only over a very narrow range of pH (Fig. 1f). Both reactions are dependent upon added monodehydroascorbate with nearly identical Kms of 1.66 mM (Table 1). For NAD^+ reduction at pH 5.0, the Km for NAD^+ was also about 1 μM (Table 1).

The Golgi apparatus preparations used in this study were contaminated less than 1% by mitochondrial membranes as determined from analyses of marker enzymes succinate dehydrogenase and cytochrome oxidase (Table 2) and less than 2% by mitochondrial fragments as determined by electron microscopy of preparations negatively stained with 1% phosphotungstic acid and confirmed from analyses of thin sections.

NAD^+ reduction was not inhibited by an argon atmosphere and the uncouplers, valinomycin, CCCP and oligomycin, as well as the mitochondrial inhibitors, KCN and HOQNO, were without effect. Nigericin and monensin, both of which interchange monovalent cations for protons, stimulated NAD^+ reduction by 40 and 60% at concentrations of 2.5 and 30 μM, respectively.

Approximately 50% of the total ascorbate of liver homogenate was sedimented with membrane-enclosed cell components in rat liver including Golgi apparatus and coated vesicles (Sun et al. 1984). Some of the ascorbate

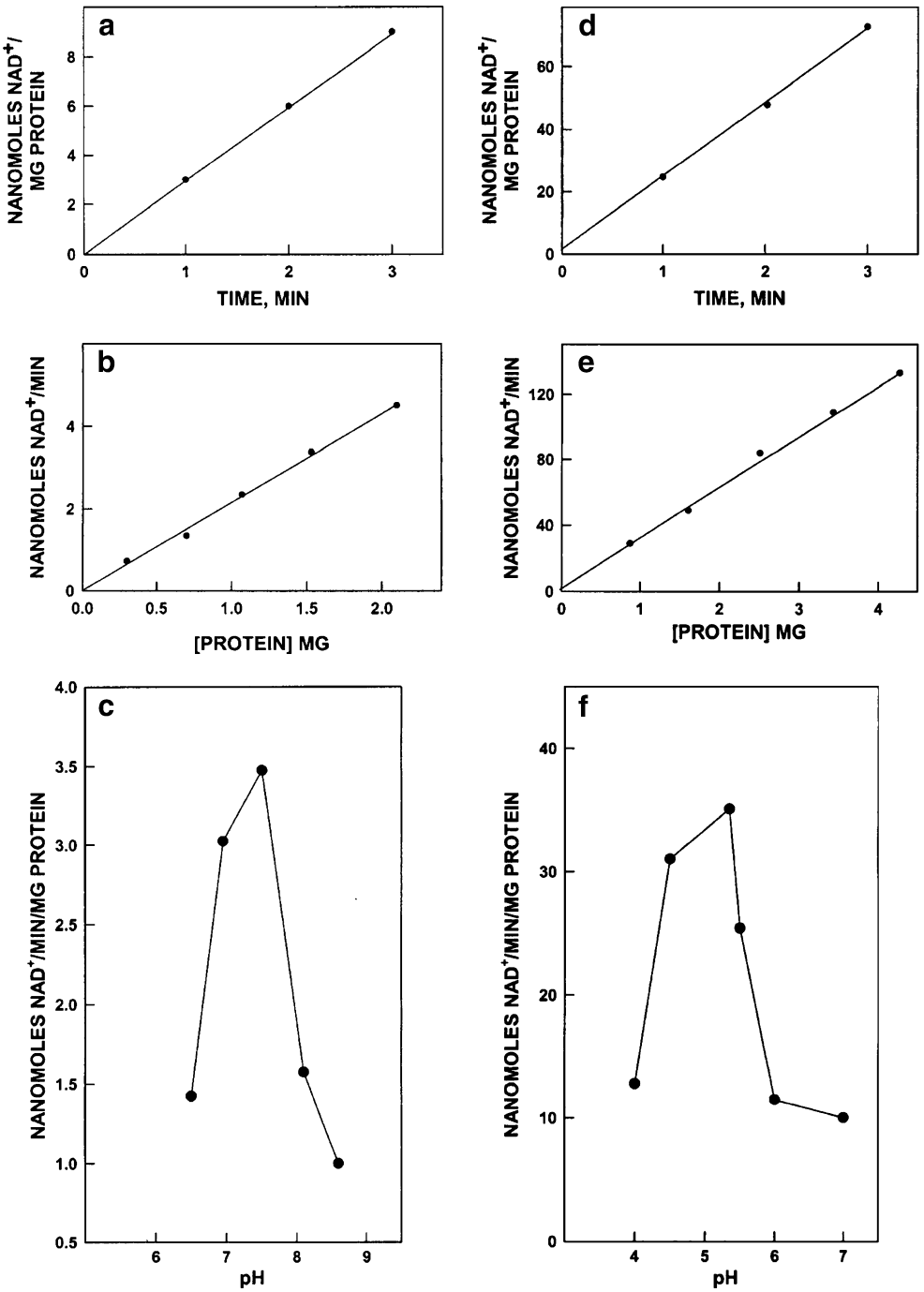

Fig. 1 Characteristics of NADH-ascorbate free radical oxidoreductase of Golgi apparatus from rat liver. **a**–**c** NADH or NAD^+ as a function of: **a** Time of incubation at 37° C. **b** Protein concentration. **c** pH. **d**-**f**. NAD^+ or NADH as a function of: **a** Time of incubation at 37°C. **e** Protein concentration. **f** pH

was present as the active free radical in these cell components, as evidenced from the appearance of an ESR signal (Fig. 3). The latter was eliminated upon addition of NADH at pH 7.5 (Fig. 3). The endogenous signal was increased when isolated Golgi apparatus were supplied with exogenous monodehydroascorbate (Fig. 4a). When NAD^+ was added to the preparations at pH 5.0, the monodehydroascorbate signal again was decreased (Fig. 4b).

To show that the Golgi apparatus membranes were both sealed and oriented cytoplasmic side out as well as impermeable to NAD^+, a series of control experiments were conducted using [^{3}H]NAD^+. Uptake over 20 min at 25°C was determined to be less than 10^{-3} picomoles/mg protein. That the vesicles were sealed and with the cytoplasmic side out was demonstrated by the lack of penetration and reactivity of peroxidase-labeled con A with membrane-associated glycoconjugates (Fig. 5a). In contrast, if the cisternae or vesicles were broken by sonication (after fixation) to allow entry of reactants, then reactivity of peroxidase labeled con A with glycoconjugates at the cisternal or vesicle interiors was observed (Fig. 5b, arrows).

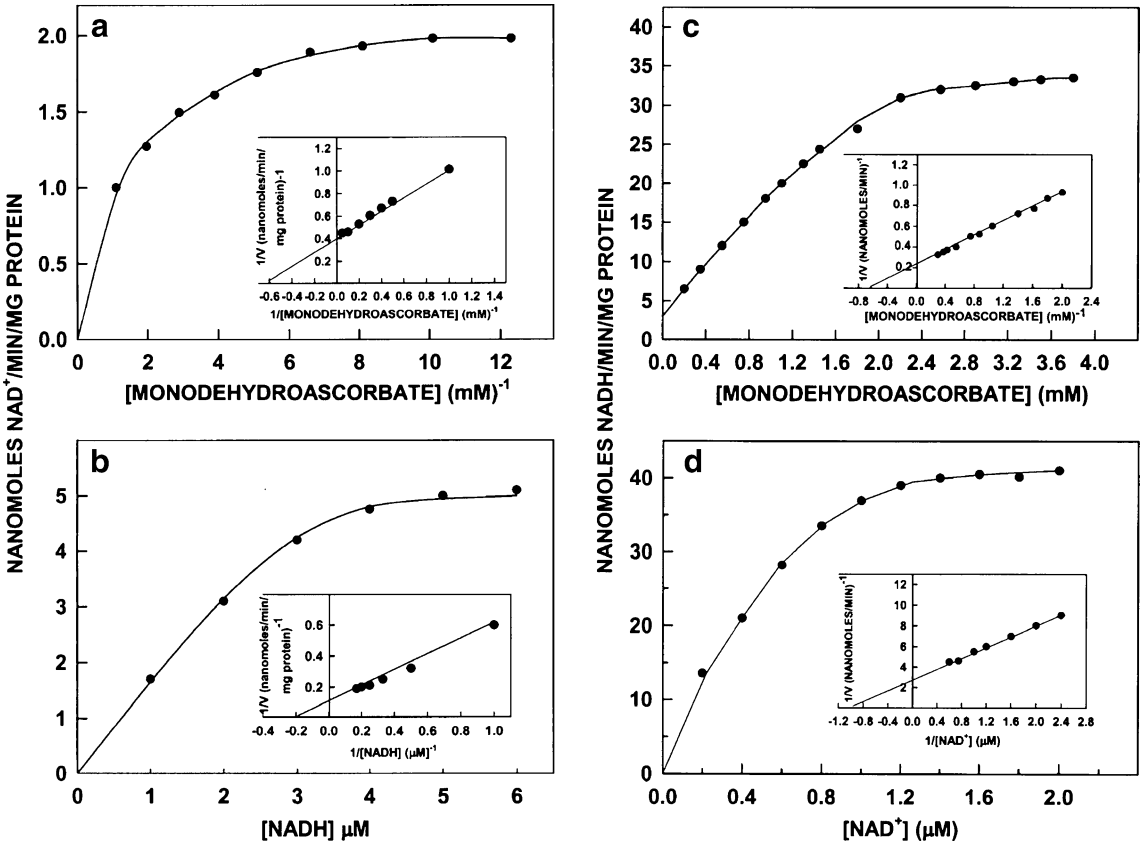

Fig. 2 Kinetics of NADH/NAD$^+$ oxidation/reduction by Golgi apparatus of rat liver. **a** and **b** Specific activity of NADH oxidation as a function of: **a** Amount of monodehydroascorbate (equal mixture of ascorbate + dehydrohydroascorbate). **b** NADH concentration. **c** and **d** Specific activities of NAD$^+$ reduction as a function of: **c** Amount of monodehydroascorbate. **d** NAD$^+$ concentration. The kinetic constants determined by replotting the data as recriprocals are summarized in Table 1

At both pH 7.0 with NADH and with NADH and monodehydroascorbate present and at pH 5.0 with NAD$^+$ and monodehydroascorbate present, a membrane potential was generated in the sealed Golgi apparatus membranes as measured by carbocyanine dye fluorescence (Table 3). Either component alone resulted in a small increase in potential but the greatest effect was from the monodehydroascorbate plus pyridine nucleotide in combination.

Through the action of endogenous β-NADPase, incubation of Golgi apparatus with NADP at pH 5.0 produced NAD$^+$, 60% of which was reduced to NADH (Table 4). If Golgi apparatus were incubated under the same conditions, but with the addition of different amounts of monodehydroascorbate, the hydrolysis of NADP was accelerated and the ratio of reduced NADH relative to the oxidized form increased proportionately to an optimum at 0.2 mM

Table 1 Kinetic constants of NADH-ascorbate free-ascorbate free radical oxidoreductase of rat liver Golgi apparatus

Kinetic constant	NADH → NAD$^+$	NAD$^+$ → NADH
Km, Ascorbate + Dehydroascorbate	1.7 mM	1.7 mM
Vmax, Ascorbate + Dehydroascorbate	2.5 nanomoles/min/mg protein	50 nanomoles/min/mg protein
Km, NADH/NAD$^+$	1 µM	1 µM
	10 nmoles/min/mg protein	0.34 nanomoles/min/mg protein
pH optimum	7.5	5.0

Table 2 Comparison of Golgi apparatus and mitochondria marker enzymes in the Golgi apparatus fractions isolated from rat liver

Fraction	Marker enzymes and specific activity[a]		
	Golgi apparatus	Mitochondria	
	Galactosyltransferase	Succinate-INT reductase	Cytochrome oxidase
Total homogenate	2.9±0.05	3.2±1.1	64.05±2.70
Golgi apparatus	210.5±1.70	0.11±0.02	1.42±0.12

[a] Units of specific activity are nanomoles of acceptor-dependent disappearance of UDP-galactose/h/mg protein for galactosyltransferase; micromoles of INT reduced/h/mg protein for succinate-INT reductase, and nanomoles oxygen consumed/min/mg protein for cytochrome oxidase

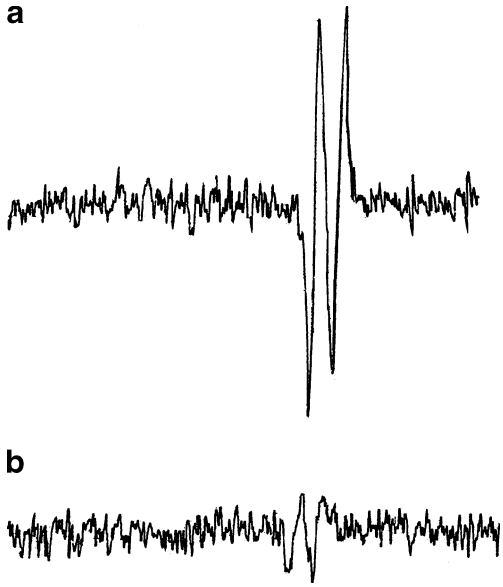

Fig. 3 Ascorbate free radical ESR signal in the g = 2.5 region of Golgi apparatus of rat liver. Golgi apparatus fractions (0.5 mg/ml) were incubated at pH 7.5, in the cell compartment of a Varian E-109-ESR spectrophotometer, before (**a**) and (**b**) after the addition of 45 μM NADH

monodehydroascorbate (Fig. 6). With higher concentrations of monodehydroascorbate, no additional NADP was cleaved and the oxidized form, NAD^+, again was favored.

Discussion

Ascorbic acid is found as a natural constituent present in the interiors of coated vesicles (Sun et al. 1984; Morré et al. 1985), Golgi apparatus (Sun et al. 1984) and in chromaffin (Njus et al. 1983; Srivastava et al. 1984) and secretory

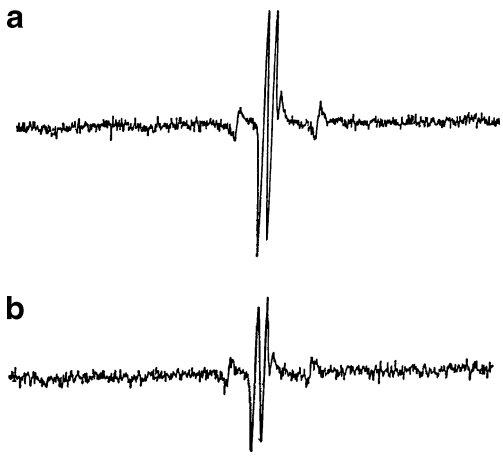

Fig. 4 Ascorbate free radical ESR signal in the g=2.5 region of Golgi apparatus (0.5 mg/ml) were incubated with 3.3 mM monodehydro-ascorbate, pH 5.0, in the cell compartment of a Varian E-109-ESR spectrophotometer, before (**a**) and after (**b**) the addition of 0.35 mM NAD^+

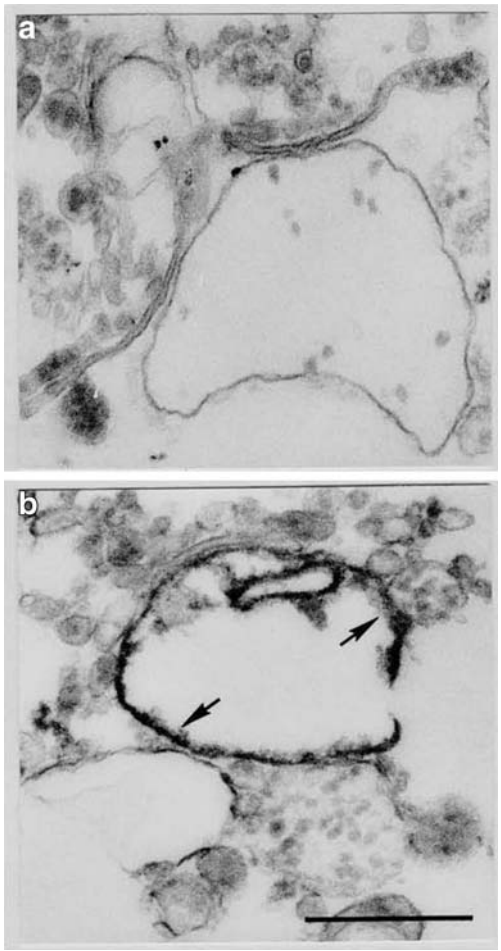

Fig. 5 Peroxidase-labeled concanavalin A binding to isolated Golgi apparatus vesicles from rat liver. The reaction was carried out with fixed Golgi apparatus in the presence of 200 mg/ml peroxidase-concanavalin A for 1 h at room temperature; **a** control. **b** fixed samples sonicated 4× 15 s before the incubation with the lectin to rupture the vesicles. *Arrows* show peroxidase-concanavalin A deposits in the interior of opened vesicles. Unsonicated preparations lack peroxidase-concanavalin A deposits showing that the vesicles are sealed. Scale marker = 0.5 μM

granules (Van Zastrow et al. 1984). It is a bivalent oxidation-reduction molecule that acts via a free radical intermediate, known as monodehydroascorbate. The latter serves both as a one-electron oxidant and as a one-electron reductant (Iyanagi et al. 1985). The ascorbate radical has been described as an electron acceptor for NADH oxidation in plasma membranes (Orringer and Roer 1979; Goldenberg 1980; Goldenberg et al. 1983), microsomal membranes (Staudinger et al. 1961; Lumper et al. 1967; Geiss and Schulze 1975), Golgi apparatus (Sun et al. 1984), and coated vesicles (Sun et al. 1984; Morré et al. 1985). Additionally, ascorbic acid acts as a transmembrane electron donor to reduce cytochrome c or ferricyanide in pituitary neurosecretory vesicles (Russell et al. 1985). During this process the ascorbate induces an inside positive membrane potential. Also, NADH ascorbate free radical oxidoreducatse activity has been associated with

Table 3 Changes in potential charge across Golgi apparatus membranes measured by carbocyanine dye fluorescence

Additions	Arbitrary units/min/mg protein
$NADH \rightarrow NAD^+$, pH 7.5	
None	0
5 mM monodehydroascorbate[a]	−0.6
45 μM NADH	−0.6
5 mM monodehydroascorbate[a] + 45 μM NADH	−1.1
$NAD^+ \rightarrow NADH$, pH 5.0	
None	0
3.3 mM monodehydroascorbate[a]	−0.8
2 μM NADH	−0.5
3.3 mM monodehydroascorbate[a] + 2 μM NADH	−2.7

[a] Equal mixture of ascorbate + dehydroascorbate

generation of a membrane potential in chromaffin granules (Njus et al. 1983).

A role of ascorbic acid as a potential electron acceptor from NADH for a membrane-located energy transduction mechanism in the Golgi apparatus of rat liver or in secretory granules has been suggested (Sun et al. 1984; Morré et al. 1985; Njus et al. 1983). The ascorbate free radical is involved in these redox reactions as well (Sun et al. 1984).

In addition to the usual oxidation of NADH, ascorbate free radical may reduce NAD^+ via NADH-ascorbate free radical oxidoreducatse at low pH. This is predicted from the pH dependency to the Nerntz equation where, at pH 5, the potential of NAD^+ is such that reduction by the ascorbate/semi-dehydroascorbate redox couple will occur. The latter has an optimum rate of NADH production of 30–40 nmoles/min/mg protein with isolated Golgi apparatus. Since the Km for monodehydroascorbate in NAD^+ reduction is the same as the Km for monodehydroascorbate in NADH oxidation (Sun et al. 1984), we suppose that the same enzyme, NADH, ascorbate free radical oxidoreducatse, is involved both in

Table 4 Pyridine nucleotide content of Golgi apparatus after incubation with 500 μM $NADP^+$ at pH 5.0

Pyridine nucleotide	Picomoles/mg protein[a]	
	0 min	10 min
$NADP^+$	68±2	41±2
NADPH	0	0
NAD^+	5±0.5	17±1
NADH	12±1	29±1

[a] The data were corrected by the results obtained after the incubation of boiled Golgi apparatus with 500 μM $NADP^+$

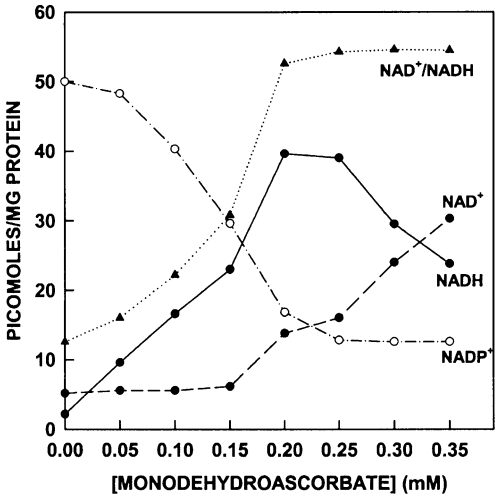

Fig. 6 Pyridine nucleotide concentration in Golgi apparatus of rat liver after their incubation with 500 μM $NADP^+$ in the presence of different amounts of monodehydroascorbate (equal mixture of ascorbate and dehydroascorbate) as determined by the cycling assay of Matsumura and Miyachi (1980)

NAD^+ reduction and NADH oxidation with ascorbate free radical as electron donor or acceptor. The Kms of NADH-ascorbate free radical-oxidoreducatse, NADH and NAD^+ are similar to the Km for NADH reported previously for ascorbate free radical oxidoreducatse of human erythrocyte membranes (Goldenberg et al. 1983).

The disappearance of the ascorbate free radical ESR signal in the Golgi apparatus was seen after NADH oxidation or NAD^+ reduction, respectively. The cycling assay shows that NAD^+ produced by hydrolysis of NADP also is reduced in presence of low concentrations of ascorbate free radical.

Based on electron microscopy, more than 95% of the vesicles were sealed as evidenced by the failure of peroxidase-labeled concanavalin A to enter the vesicles. In contrast, if the vesicles first were broken by sonication after fixation but before addition of the peroxidase-labeled concanavalin A, then reactivity was observed at the interior membrane surfaces of the broken vesicles. This demonstrated, as well, that the cytoplasmic side out orientation of the vesicles was maintained as concanavalin A receptors in situ are located at the cisternal and vesicle interiors. Finally, the vesicles appear essentially impermeable to NAD^+ (less than 10^{-3} picomoles taken up/mg protein over 20 min). Others have shown previously that hepatocytes (Smith et al. 1981) and mitochondrial inner membranes (DePierre and Ernster 1977) are impermeable to NAD^+. These findings point to the likelihood that the NAD^+ reduction by Golgi apparatus occurs at the cytoplasmic surface of the membranes. Increasing evidence suggests that NAD (including NAD^+ and NADH) and NADP (including $NADP^+$ and NADPH) are both common and important mediators of various biological processes not limited to energy metabolism, mitochondrial

functions and oxidative stress but also directly involved in gene expression, immunological functions, aging and cell death (Ying 2008).

Because a membrane potential was generated, electron and/or proton transport across the membrane is assumed with the external ascorbate acting as a redox pool for the internal ascorbate (Fig. 6). A shift in the internal ascorbate/dehydroascorbate ratio in the direction of ascorbate may help explain how, because of its low pH optimum, the NADP phosphatase activity of Golgi apparatus could become more active to account for accelerated conversion of $NADP^+$ to NAD^+ with increasing amounts of added external ascorbate free radical (Fig. 6). Any NAD^+ produced at the low pH would be converted to NADH and the NADH thus generated would support additional vesicle acidification (Barr et al. 1984). Thus, depending on pH, the response of internal ascorbate to external pyridine nucleotides may differ. Presumably, as shown by $NADP^+$ hydrolysis, a similar response to internal pH might be expected such that the coupled system of reactions might serve as a regulatory mechanism for vesicle acidification. At neutral pH, electron and proton flow would be inward to facilitate acidification. However, as the pH approaches 5.0, the reaction would reverse to induce an outward flow of both protons and electrons. The net result would provide a simple mechanism for homeostatic pH control by Golgi apparatus cisternae and vesicles as well as potentially important generation of cytosolic NADH at the level of the trans Golgi apparatus network.

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