Uncoupled Redox Systems in the Lumen of the Endoplasmic Reticulum

PYRIDINE NUCLEOTIDES STAY REDUCED IN AN OXIDATIVE ENVIRONMENT*

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The redox state of the intraluminal pyridine nucleotide pool was investigated in rat liver microsomal vesicles. The vesicles showed cortisol reductase activity in the absence of added reductants, which was dependent on the integrity of the membrane. The intraluminal pyridine nucleotide pool could be oxidized by the addition of cortisol or metyrapone but not of glutathione. On the other hand, intraluminal pyridine nucleotides were slightly reduced by cortisol or glucose 6-phosphate, although glutathione was completely ineffective. Redox state of microsomal protein thiols/disulfides was not altered either by manipulations affecting the redox state of pyridine nucleotides or by the addition of NAD(P)+ or NAD(P)H. The uncoupling of the thiol/disulphide and NAD(P)+/NAD(P)H redox couples was not due to their subcompartmentation, because enzymes responsible for the intraluminal oxidoreduction of pyridine nucleotides were distributed equally in smooth and rough microsomal subfractions. Instead, the phenomenon can be explained by the negligible representation of glutathione reductase in the endoplasmic reticulum lumen. The results demonstrated the separate existence of two redox systems in the endoplasmic reticulum lumen, which explains the contemporary functioning of oxidative folding and of powerful reductive reactions.

The lumen of the endoplasmic reticulum (ER)3 is a separate metabolic compartment of the eukaryotic cell (1). Because of the limited and selective permeability of the ER membrane and because of special intraluminal reactions, it differs from the cytosol in numerous parameters. One of the most characteristic differences is that luminal thiols (including protein thiols and glutathione) are present in a more oxidized state than the cytosolic ones. Although the ratio between reduced and oxidized glutathione is 100:1 in the cytosol, this value is around 1–2:1 in the ER lumen (2). The redox potential calculated from these values is approximately −0.24 and −0.18 V, respectively. Recent observations suggest an even larger difference between the redox potentials of the two compartments (3).

The typical redox potential of a given compartment is a major determinant of rate and direction of redox reactions. The oxidizing environment in the ER lumen is both a prerequisite and a consequence of the oxidative folding of secretory and membrane proteins (4–6). By generalizing the observations related to the redox state of thiols found in the ER of cells engaged in protein secretion, it is now supposed that the redox conditions in the ER lumen are uniformly oxidizing. However, several intraluminal reactions have been described, which require reducing equivalents. Such reactions involve the isomerization of disulfides (7), the steps of the vitamin K cycle (8), the biotransformation of several endogenous ketones and aldehydes (9), and the reactivation of steroids. One of the main sources of intraluminal reducing equivalents is hexose-6-phosphate dehydrogenase (H6PDH), which generates NADPH at the expense of the oxidation of glucose 6-phosphate (10). This phoshoester, produced in the cytosol, can reach the luminal compartment by the mediation of its specific transporter glucose 6-phosphate transporter (11).

11β-Hydroxysteroid dehydrogenase type 1 (11βHSD1) is an NADP(H)-dependent oxidoreductase of the ER lumen (12, 13), which may have an important role in the pathogenesis of metabolic syndrome (14–16). Although the enzyme can catalyze both the reduction of cortisol and the oxidation of cortisol in vitro, it appears to act exclusively as a reductase in vivo (17). This circumstance suggests that pyridine nucleotides are predominantly present in the reduced form in the ER lumen, which implies a lack of electron transfer between (reduced) pyridine nucleotides and (oxidized) thiols. This would be in sharp contradiction with the situation present in the cytosol, where oxidized glutathione is reduced back at the expense of the reducing equivalents supplied by NADPH (18). Therefore, the aim of the present study was to directly prove the redox state of pyridine nucleotides and to explain the separate existence of the two redox systems in the ER lumen. Here we present evidence for the presence of a pyridine nucleotide pool in the ER lumen, which is indeed prevalently reduced and does not communicate with thiols/disulfides because of the absence of glutathione reductase.

MATERIALS AND METHODS

Preparation of Microsomal Vesicles and Subcellular Fractions from Rat Liver—Microsomes were prepared from livers of overnight fasted male Sprague-Dawley rats (180–230 g), using fractional centrifugation (19). The vesicles were washed and resuspended in MOPS-KCl buffer (100 mM KCl, 20 mM NaCl, 1 mM MgCl2, 20 mM MOPS, pH 7.2), then

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3 The abbreviations used are: ER, endoplasmic reticulum; 11βHSD1, 11β-hydroxysteroid dehydrogenase type 1; H6PDH, hexose-6-phosphate dehydrogenase; MOPS, 4-morpholinopropanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.
immediately frozen in liquid nitrogen, and kept in liquid nitrogen until use (within 6 months). The protein concentration in microsomal samples was determined using the method of Lowry et al. (20) with bovine serum albumin as a standard. The integrity of the microsomal membranes was assessed by using the mannose-6-phosphate assay (21) and by measuring p-nitrophenol glucuronidation (22, 23), which showed a latency less than 95%.

To evaluate the possibility that cytosolic pyridine nucleotides are entrapped within the microvesicular vesicles during the transient disruption of the ER membrane, the whole fractionation procedure was also performed in the presence of radiolabeled NAD⁺. Therefore, 0.76 μCi of [¹⁴C]NAD⁺ (specific activity 55 Ci/mol) was added to 30 ml of the homogenization mixture containing 3 g of liver tissue, before or after the homogenization step. The radioactivity associated with the microsomal fraction (derived from 3 g of liver) was 2060 ± 100 and 2110 ± 150 (dpm ± S.D., n = 3) with the addition of [¹⁴C]NAD⁺ before and after the homogenization step, respectively. In both instances, the microsomal fraction contained about 0.12% of the radioactivity present in the homogenate, which corresponded to about 0.35 pmol/mg of microsomal protein of the added radiolabeled NAD⁺. This result excludes the possibility that cytosolic pyridine nucleotides would have been entrapped in the vesicles generated by the fragmentation of the ER network during the homogenization step.

In some experiments, mitochondrial, cytosolic, and microsomal fractions were obtained by standard differential centrifugation from rat liver homogenate (24) and were maintained in liquid N₂ until used. The subcellular fractions were characterized by measuring marker enzyme activities (25). The activity of cytochrome c oxidase, glucose-6-phosphatase, and 5'-nucleotidase in the microsomal fraction was 1.2 ± 0.3, 30.1 ± 5.0, and 6.0 ± 1.1, respectively (expressed as percent value of the corresponding activity measured in the whole homogenate; means ± S.D., n = 3). Rough and smooth microsomal vesicles were prepared and characterized as described previously in detail (25).

Fluorimetric Detection of Reduced Pyridine Nucleotides—Reduced pyridine nucleotides were detected based on their characteristic fluorescence spectrum in microsomes. Fluorescence was monitored at a 350-nm excitation and a 460-nm emission wavelength by using a Cary Eclipse fluorescence spectrophotometer (Varian).

Transport Measurements by Light Scattering Technique—The permeability of the microsomal membranes toward various compounds was measured by the continuous detection of the osmotically induced changes in size and shape of microsomal vesicles (19). Briefly, microsomal vesicles (50 μg of protein/ml) were equilibrated for 2 h in a hypotonic medium (5 mM PIPES potassium salt, pH 7.0) at 22 °C. Light scattering of the microsomes was then monitored at 550 nm, using a Cary Eclipse fluorescence spectrophotometer (Varian), equipped with a temperature-controlled cuvette holder (22 °C) and magnetic stirrer. Investigated compounds were added as a small volume (<5% of the total incubation volume) of a concentrated osmolyte solution (0.5 M in the case of NAD⁺, NADPH, and glucose 6-phosphate and 2 M in the case of sucrose). Upon the addition of osmolytes an increase in the light scattering signal can be observed, which is due to vesicle shrinking. In case of a nonpermeant compound, a sustained signal can be seen, although the influx of a permeant compound into the vesicles results in vesicular swelling and in the decrease of the signal.

Transport Measurements by Rapid Sedimentation Method—Microsomes (10 mg of protein/ml) were incubated in the presence of 0.2 mM NAD⁺ in the MOPS-KCl buffer at 37 °C. At the indicated times 0.5-ml samples were withdrawn and mixed with 0.5 ml of ice-cold buffer containing 10% polyethylene glycol. Polyethylene glycol-aggregated microvesicular swellings and in the decrease of the signal.
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FIGURE 1. The integrity of the membrane is required for cortisone reduction in rat liver microsomes. Microsomes (1 mg/ml protein) were incubated at 22 °C in MOPS-KCl buffer containing 10 μM cortisone and in the presence (open symbols) or absence (filled symbols) of alamethicin (0.1 mg/mg protein). Cortisol production was measured as reported under “Materials and Methods.” Data are means ± S.D. of four experiments.

FIGURE 2. The microsomal membrane is poorly permeable toward pyridine nucleotides. A–D, osmotically induced changes in size and shape of microsomal vesicles (50 μg protein/ml) were detected by the light scattering technique. Where indicated (arrows), a concentrated solution of the different osmolytes was added. Light scattering traces obtained with 20 mM NADP+ (A) or with 20 mM NADPH (B) are shown. For comparison, the characteristic traces of a nonpermeant (sucrose, 75 mM, C) and a permeant (glucose 6-phosphate, G6P; 25 mM, D) compound are also shown. Arrowheads indicate the addition of alamethicin (0.1 mg/mg protein). Representative traces from four experiments are shown. E, the uptake of 0.2 mM NADP+ has been measured by rapid sedimentation of microsomal vesicles preincubated with NADP+. Intravesicular NADP+ was measured enzymatically as reported under “Materials and Methods.” Data are means ± S.E. of four experiments.

FIGURE 3. Intraluminal NADPH is not accessible for the extraluminal microsomal NADPH oxidase activity. Microsomes (2 mg of protein/ml) were incubated in a fluorimeter cuvette, and the level of reduced pyridine nucleotides was monitored as reported under “Materials and Methods.” A, release of intraluminal reduced pyridine nucleotides by the pore-forming agent alamethicin makes them accessible to oxidation. Alamethicin (Alam, arrow) was added to trace 2, a.u., arbitrary units. B, fluorescence emission spectra (350-nm excitation wavelength) of microsomal suspensions recorded in samples withdrawn are indicated by arrowheads in A as samples a and b. Inset shows the difference of the two spectra. C, liver microsomes rapidly oxidize exogenously added NADPH or NADH; the arrows indicate the addition of NADPH (1, 1 μM; 2, 2 μM; 5, 5 μM) or NADH (3, 1 μM; 4, 2 μM; 5, 1 μM; 7, 5 μM). Representative traces from 4 to 6 experiments are shown.

from PerkinElmer Life Sciences. All other reagents were of analytical grade.

RESULTS

Rat Liver Microsomes Contain an Overwhelmingly Reduced Pyridine Nucleotide Pool—11βHSD1, an intraluminal enzyme of the hepatic ER, which uses pyridine nucleotides as cofactor, catalyzes the oxidoreduction of cortisol and cortisone. In vivo, the preferential direction of the reaction is the reduction of cortisone to cortisol (17), suggesting that the luminal pyridine nucleotides are at least partially in reduced form. In accordance with this assumption, addition of cortisone to native rat liver microsomes resulted in a rapid formation of cortisol, which reached a maximum within few minutes (Fig. 1). The permeabilization of microsomal vesicles by the pore-forming agent alamethicin almost completely abolished cortisone reduction (Fig. 1). A possible interpretation of these results is that some NADPH required for the reduction of cortisone by 11βHSD1 is present in the lumen of native microsomal vesicles. Upon permeabilization, the luminal NADPH is released into the incubation medium, and its concentration likely becomes too low for allowing the reduction of cortisone. Indeed, the intravesicular water space of liver microsomes is ~3–4 μl per mg of protein (30), and 1 mg of microsomal protein was present in 1 ml of the incubation system in the experiments of Fig. 1. Moreover, the outer microsomal surface appeared to possess an NAD(P)H oxidase activity, which was likely responsible for the oxidation of NADPH released from the microsomal lumen to the incubation medium (see below).

The results strongly suggested that rat liver microsomes maintained a luminal pyridine nucleotide pool during the separation procedures, i.e. the ER membrane is not permeable toward NAD(P)+ or NAD(P)H. Accordingly, a light scattering assay of the permeability of microsomal vesicles toward both NADP+ and NADPH (20 mM each, see Fig. 2, A and B) showed that a sustained elevation of the signal (i.e. a sustained
osmotic shrinkage of vesicles) was observed upon their addition. This indicates that the microsomal membrane is hardly permeable for these compounds. As demonstrated in Fig. 2C, the shape of these traces was similar to that revealed upon the addition of sucrose, a prototypic non-permeant compound (19). Similar results were obtained with NAD(H) (data not shown). As a control, the addition of the permeant compound glucose 6-phosphate (19) resulted in an increase in light scattering, which was followed by a recovery of the signal (i.e. by a swelling phase reflecting the permeation of the exose-phosphate; see Fig. 2D). In all the experiments (Fig. 2, A–D), the complete permeabilization of microsomal vesicles by alamethicin resulted in a maximal swelling.

The microsomal permeability for lower physiological concentrations of pyridine nucleotides was investigated by using a rapid sedimentation method (26). In the presence of 200 μM NADP⁺, no uptake of the nucleotide was detectable at incubation times shorter than 15 min. At longer incubation times, a time-dependent uptake was observed (Fig. 2E). However, this uptake was not accounted for by the complete equilibration of the extravesicular and intravesicular NADP⁺ concentrations, even after 1 h of incubation. At that time, the calculated intravesicular NADP⁺ concentration was only one-third of the extravesicular one, assuming that the intravesicular microsomal water space is 3.6 μl/mg protein, as measured previously in similar microsomal preparations (19).

To investigate the redox state of intraluminal pyridine nucleotides, microsomes were incubated in a fluorimeter cuvette, and reduced pyridine nucleotide content was monitored as reported under “Materials and Methods.” The change in the fluorescence signal caused by permeabilization of the microsomal membrane is hardly permeable for these compounds. As demonstrated in Fig. 2C, the shape of these traces was similar to that revealed upon the addition of sucrose, a prototypic non-permeant compound (19). Similar results were obtained with NAD(H) (data not shown). As a control, the addition of the permeant compound glucose 6-phosphate (19) resulted in an increase in light scattering, which was followed by a recovery of the signal (i.e. by a swelling phase reflecting the permeation of the exose-phosphate; see Fig. 2D). In all the experiments (Fig. 2, A–D), the complete permeabilization of microsomal vesicles by alamethicin resulted in a maximal swelling.

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To investigate the redox state of intraluminal pyridine nucleotides, microsomes were incubated in a fluorimeter cuvette, and reduced pyridine nucleotides were detected on the basis of their characteristic fluorescence (350-nm excitation and 460-nm emission wavelengths). Without any additions, a sustained signal was observed (see Fig. 3A and Fig. 4A), indicating that in the absence of exogenous substrates, but in the presence of oxygen, microsomal oxidoreductions did not affect the redox state of luminal pyridine nucleotides. This signal was reduced by the permeabilization of the microsomal membrane as well as by the addition of substrates that are expected to oxidize NADPH. These observations indicate that a measurable contribution to the fluorescence signal is accounted for by intraluminal reduced pyridine nucleotides.

Microsomal membrane permeabilization upon the addition of alamethicin resulted in a decrease of the fluorescence signal (Fig. 3A, trace b). The fluorescence spectra before and after addition of alamethicin are shown in Fig. 3B, and the difference between the spectra (Fig. 3B, inset) corresponds to the fluorescence spectrum of reduced pyridine nucleotides. The loss in the signal caused by permeabilization of the microsomal membrane should reflect their contribution to the fluorescence spectrum of native microsomes. Indeed, once the intraluminal NAD(P)H is released, it should be rapidly oxidized by NAD(P)H oxidases present on the microsomal outer surface, as demonstrated by the fact that both NADH and NADPH exogenously added to intact microsomes are oxidized (Fig. 3C). By taking into account the increase in the fluorescence upon the addition of known amounts of NADPH (Fig. 3C), the decrease upon alamethicin permeabilization (Fig. 3A), and a microsomal luminal water space of 3.6 μl/mg protein, an ~0.4 mM intravesicular NAD(P)H concentration can be calculated.

The addition of cortisone, a substrate that is reduced by the intraluminal 11βHSD1, also resulted in a concentration-dependent decrease of the signal (Fig. 4A). The difference between the fluorescence spectra of microsomes before and after the addition of cortisone also corresponded to the spectrum of NADPH (Fig. 4B). Metyrapone, another substrate of 11βHSD1 (9), produced an effect similar to that of cortisone (Fig. 4C). The maximal decrease in the fluorescence caused by cortisone addition was 1.17 ± 0.11 (n = 5) (arbitrary units measured at 2 mg of microsomal protein per ml, means ± S.E.), and in the case of alamethicin permeabilization the decrease was 1.28 ± 0.18 (n = 4). These data indicate that the dominant intraluminal reduced pyridine nucleotide is NADPH, because 11βHSD1 activity is specific for NADP(H).
In additional experiments, we observed that reducing agents caused only a moderate increase in the signal. Glucose 6-phosphate, which can reduce \( \text{NAD}^+ \) by the action of the intraluminal H6PDH, caused a slight increase in the signal (Fig. 5A). However, glucose 6-phosphate could revert the effect of cortisone, and this activity was abolished by the specific inhibitor, S3483 (31), of the ER transporter of the phosphoester (Fig. 5B). Addition of cortisol also resulted in a minor increase of the fluorescence signal (Fig. 5C). However, the addition of a high concentration of cortisol (10 \( \mu \text{M} \)) could revert the effect of 1 \( \mu \text{M} \) cortisone (Fig. 5D).

Glutathione Does Not Affect the Redox State of Intraluminal Pyridine Nucleotides—To investigate the cross-talk between thiol/disulfide and \( \text{NAD(P)}^+ / \text{NAD(P)}^- \) redox systems, additional experiments were performed. We have shown previously that the most abundant cytosolic reducing agent GSH can enter liver microsomal vesicles, although GSSG is poorly permeant (32). We also showed that GSH, once entered the microsomal lumen, is oxidized to GSSG likely at the expenses of protein disulfides (32). Despite its ability of crossing microsomal membranes, GSH did not alter the redox state of intraluminal pyridine nucleotides (Fig. 6). Moreover, GSH did not affect the ability of cortisone to oxidize intraluminal NADPH, as well as the ability of glucose 6-phosphate to revert the oxidizing effect of cortisone (Fig. 6, compare traces a and b). The oxidizing effect of cortisone was concentration-dependent either in the presence or the absence of GSH (compare A and B). As expected, because of its poor permeability, GSSG was also ineffective (data not shown).

Pyridine Nucleotides Do Not Influence the Intraluminal Redox State of Protein Thiols—The effect of pyridine nucleotides on the redox state of microsomal protein thiols/disulfides was evaluated in order to explore further a possible connection between the two redox systems. Several redox compounds of low molecular weight can cross the ER membrane and can modify the redox state of intravesicular protein thiols. Such an effect has been reported in the case of dehydroascorbic acid (33) and FAD (34). On the contrary, pyridine nucleotides either in reduced or in oxidized forms did not affect the protein thiol redox state (Table 1). Because this negative result could simply be due to their limited access to intravesicular targets, the experiment was repeated in permeabilized vesicles. Pyridine nucleotides were ineffective even in this case (data not shown).

The Separation of the Two Redox Systems Is Not because of a Different Spatial Distribution—The synthesis, post-translational modification, and oxidative folding of secretory and membrane proteins take place in the rough ER, although the metabolism of steroids and other reactions of bio-transformation are usually linked to the smooth ER. Therefore, it can be supposed that pyridine nucleotides are primarily present in the latter, whereas thiols/disulfides in the former subfraction of the ER. If this is true, the separation of the two redox systems can be simply explained by a spatial disconnection. To ascertain this possibility, the effect of cortisone on intraluminal NADPH was evaluated in smooth and rough microsomal fractions. In both the microsomal subfractions, cortisone caused a similar...
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TABLE 1
Effect of various oxidizing and reducing agents on the protein thiol content of rat liver microsomal vesicles

| Addition          | Microsomal protein thiol content (%) | % control |
|-------------------|--------------------------------------|-----------|
| None              | 100.0 ± 9.2                          |           |
| NAD+ (500 μM)     | 97.2 ± 5.0                           |           |
| NADP+ (500 μM)    | 91.9 ± 8.1                           |           |
| NADH (500 μM)     | 95.3 ± 9.2                           |           |
| NADPH (500 μM)    | 94.6 ± 6.8                           |           |
| Dithiothreitol (500 μM) | 116.9 ± 3.1*                       |           |
| FAD (500 μM)      | 76.3 ± 14.2*                         |           |
| Cortisone (10 μM) | 98.8 ± 1.4                           |           |
| Metyrapone (10 μM) | 99.4 ± 0.8                          |           |
| Cortisol (10 μM)  | 101.9 ± 2.2                          |           |

* Values were significantly different from control (p < 0.05).

decrease in the fluorescence signal, which was reverted by glucose 6-phosphate (data not shown). Moreover, no significant difference was found in 11βHSD1 activity of rough and smooth (permeabilized) microsomes (data not shown). Furthermore, Western blot analysis of H6PDH, glucose 6-phosphate transporter, and 11βHSD1 proteins revealed a similar expression in the two microsomal subfractions (data not shown).

The Separation of the Two Redox Systems Is because of the Low Representation of Glutathione Reductase in the ER Lumen—Glutathione is maintained in the reduced form in the cytosol and the mitochondrial matrix by the action of glutathione reductase (35). The enzyme transfers electrons from NADPH to glutathione disulfide. Therefore, it can be hypothesized that the absence of this enzyme in the ER lumen underlies the phenomena described above. The presence of glutathione reductase in the subcellular fractions of rat liver was evaluated by Western blot. As expected, the glutathione reductase protein was well represented in the cytosolic and mitochondrial fractions. On the contrary, a very faint band could be observed in the microsomal fraction (Fig. 7). Glutathione reductase activity was also measured in the cytosolic and microsomal fractions. Negligible activity was found in microsomal vesicles, which was slightly increased upon permeabilization (Table 2). Even in the latter case, the microsomal glutathione reductase activity was less than 5% of the cytosolic value.

DISCUSSION

Pyridine nucleotides (either in reduced or oxidized forms) are unable to permeate cellular membranes by simple diffusion. Although neither pyridine nucleotide transporters nor intraluminal synthetic pathways have been described in the ER, previous evidence for the existence of an intraluminal pyridine nucleotide pool has been forwarded (36–38). The intraluminal pool of these compounds. (iv) A variety of studies indicates that microsomal vesicles still contain ER luminal components as follows: e.g. proteins such as protein-disulfide isomerase (46), calreticulin (47), and BiP (48) or even small molecules such as glutathione (3).

Because the lumen of the ER is regarded as a compartment of oxidizing environment, and especially in secretory organs where it is equipped with potent oxidizing apparatus, one can suppose that pyridine nucleotides are also present in the oxidized form. In this study, we found just the opposite. Microsomes were able to supply cortisone reductase with reducing equivalents (Fig. 1). Moreover, the redox state of the intraluminal pyridine nucleotide pool could be shifted more easily toward oxidized than reduced direction (Fig. 5).

These reduced pyridine nucleotides must be bona fide intraluminal because extraluminal NAD(P)H would be promptly a victim for the NADPH oxidase activity (Fig. 3). Our observations are in agreement with the findings of Hino and Minakami (37), who have reported a dormant NADPH-generating H6PDH activity in the lumen, which can be activated by oxidants, i.e. by oxidizing intraluminal NADPH. On the basis of the present results, an intraluminal concentration of reduced pyridine nucleotides of about 0.4 mM can be calculated (see “Results”). Assuming that glucose 6-phosphate or cortisol can fully reduce the microsomal pyridine nucleotide pool (Fig. 5, A and C), a concentration of about 0.05 mM can be also calculated for oxidized pyridine nucleotides. Therefore, in native microsomes the intraluminal pool of pyridine nucleotides appears to be prevalently reduced.

However, the redox state of intraluminal pyridine nucleotides is not mirrored by the redox state of microsomal protein thiols. Neither the manipulations affecting the redox state of intraluminal pyridine nucleotides nor the addition of NAD(P)+/NAD(P)+ altered the redox state of microsomal protein thiols/disulfides (Table 1). These results are in agreement with previous findings that NAD+ or NADP+ cannot promote disulfide formation in influenza hemagglutinin (49).

The absence of electron transfer from thiols to NADP+ is not surprising,
taking into account the much lower redox potential of pyridine nucleotides. However, the electron flow in the opposite direction is thermodynamically feasible, and the reaction can be catalyzed by glutathione and thioredoxin reductases. Both NADPH and glutathione are prevalently reduced in the cytosol, where the connection is established by glutathione reductase (35). In an environment where thiols are overwhelmingly oxidized to disulfides, one would expect an absent/negligible pyridine nucleotide pool or a mainly oxidized status of the pyridine nucleotides. Therefore, the finding that thiol/disulfide and NAD(P)^+ /NAD(P)H redox systems are uncoupled in the ER lumen is surprising. A plausible explanation for the uncoupling might be that the two redox systems are physically sub-compartmentalized. Indeed, the machinery of oxidative folding should be present in rough ER, whereas steroid metabolism is regarded as a typical function of smooth ER. However, we did not find significant differences between rough and smooth vesicles with respect to 11βHSD1 and H6PDH activities. The abundance of the two enzyme proteins was also similar in the two microsomal fractions, as revealed by Western blot analysis. Therefore, a spatial separation does not explain the findings. We are aware that our results do not exclude a more refined, "raft-like" subcompartmentation of the two redox systems in the ER.

Glutathione reductase and thioredoxin reductases are key enzymes for the catalysis of electron transfer between reduced pyridine nucleotides and glutathione disulfide. Thioredoxin reductases have been reported to be absent in the microsomal fraction of rat liver (50). Glutathione reductase is well represented in the cytosol and in the mitochondrial compartment; however, there are only sporadic reports on its occurrence in the ER. Hino et al. (27) have reported a glutathione reductase activity in rat liver microsomal vesicles, which is 10-fold lower than the activity measured in the cytosol. We have measured an even lower microsomal activity of glutathione reductase (Table 2), and we have observed a little immunoreactivity for the enzyme protein in Western blot analysis (Fig. 7). The observed weak immunoreactivity may be due to cytosolic or mitochondrial contamination of the microsomal fraction, which is in agreement with the presence of a minor cytochrome c oxidase activity in the microsomal fraction itself (see "Materials and Methods").

In conclusion, the results demonstrate a functional separation of the thiol/disulfide and the NAD(P)H/NAD(P) redox systems in the ER lumen, which is likely due to a very low representation of glutathione reductase in the intraluminal compartment. The findings explain the contemporary functioning of the oxidative folding machinery and of powerful reductive reactions in the ER compartment.

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