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FAD Transport and FAD-dependent Protein Thiol Oxidation in Rat Liver Microsomes*

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The transport of FAD and its effect on disulfide bond formation was investigated in rat liver microsomal vesicles. By measuring the intravesicular FAD-accessible space, we observed that FAD permeates across the microsomal membrane and accumulates in the lumen. Rapid filtration experiments also demonstrated the uptake and efflux of the compound, which could be inhibited by atractyloside and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid. FAD entering the lumen promoted the oxidation of protein thiols and increased the intraluminal oxidation of glucose-6-phosphate. These findings support the notion that, similar to yeast, free FAD may have a decisive role in the mechanism of oxidative protein folding in the endoplasmic reticulum lumen of mammalian cells.

Secreted proteins contain a significant amount of disulfide bonds, which are required for their proper folding, stability, and functioning. Disulfide formation occurs in the lumen of the endoplasmic reticulum (ER)[1] of eukaryotic cells. The oxidation of cysteine thiols requires a continuous flux of electrons toward the final electron acceptor(s). This flux is mediated by an electron transfer chain, which is composed of proteins and redox compounds of small molecular weight (1–5).

Although key proteins of the machinery have already been identified, the chemical nature of small molecules as possible cofactors is still disputed. Several redox-active compounds have been postulated as participants in the electron transfer chain, *e.g.* vitamin K (6), vitamin E (7), glutathione disulfide (8), and dehydroascorbate (9). Recent findings with yeast suggest that FAD has a crucial role in the process. ER-associated FAD-dependent oxidases (Ero1p (10–12), Erv2p (13–15), Fmo1p (16, 17)) are proposed to be components of the oxidative folding machinery. Moreover, it has been demonstrated that the activity of Ero1p is dependent on the presence of free FAD, and free cytosolic FAD is able to cross the yeast ER membrane (18).

The mechanism of oxidative folding is less explored in mammalian cells. The mammalian analogues of Ero1p, Ero1-Ls, have been described in the human ER (19, 20), and their role in the electron transfer has been proved (21, 22). However, the further route of the electrons from Ero1-L is unknown. Based on the observations in yeast, it can be supposed that free FAD plays an indispensable role in the promotion of oxidative folding also in mammalian cells. Indeed, riboflavin deficiency causes intracellular accumulation of interleukin-2 with increased expression of genes of unfolded protein response in Jurkat cells (23). However, neither the transport of FAD through the ER membrane nor its oxidative effect on microsomal protein thiols has been described in mammalian cells. The aim of the present study was to investigate these processes in rat liver microsomal vesicles. The results demonstrate that FAD transport and subsequent disulfide bond formation are present in the hepatic ER.

EXPERIMENTAL PROCEDURES

Preparation of Rat Liver Microsomes—Liver microsomes were prepared from fed male Sprague-Dawley rats (180–230 g of body weight; Charles River Hungary) as reported earlier (24). Microsomal fractions were resuspended in buffer A containing: 100 mM KCl; 20 mM NaCl; 1 mM $MgCl_2$; 20 mM Mops; pH 7.2. The suspensions were rapidly frozen and maintained under liquid N_2 until used. Intactness of microsomal vesicles was controlled by measuring the latency of mannose-6-phosphatase activity (25). The intactness/latency was greater than 90% in all microsomal preparations employed. Protein concentration of microsomes was determined using Bio-Rad protein assay solution with bovine serum albumin as a standard according to the manufacturer's instructions.

Measurement of Intravesicular FAD Space—Liver microsomes (10 mg of protein/ml) were resuspended in buffer A. The microsomal suspension was incubated in the presence of 1 mM FAD or [^{3}H]water (0.2 μCi/ml) or [^{3}H]inulin (0.17 μCi/ml) for 30 min at room temperature. To measure isotope spaces, microsomes were centrifuged (100,000 × *g*, 1 h), and the radioactivity associated with the pellets was measured to enable calculation of extra- and intravesicular isotope spaces. To measure the intravesicular FAD spaces, the 100,000 × *g* pellet was resuspended, and its FAD content was measured fluorimetrically by a Cary Eclipse fluorescence spectrophotometer (Varian) at 450-nm excitation and 530-nm emission wavelengths. Background microsomal fluorescence was measured in all experiments, and all data were corrected to this value. Parallel samples were incubated in the presence of FAD plus the pore-forming compound alamethicin (0.05 mg/mg of protein; Ref. 26). Alamethicin was also added to some pellets containing FAD, and then they were resuspended and centrifuged again (100,000 × *g*, 1 h).

Rapid Filtration Experiments—For the measurement of glucose-6-phosphate uptake and accumulation, liver microsomes (2 mg of protein/ml) were incubated in buffer A containing 10 μM glucose-6-phosphate

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[1] The abbreviations used are: ER, endoplasmic reticulum; Mops, 4-morpholinepropanesulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid.

TABLE I
Intravesicular apparent space of FAD in rat liver microsomes

Liver microsomes were resuspended (10 mg of protein/ml) in buffer A. The microsomal suspension was incubated in the presence of 1 mM FAD or [^{3}H]water (0.2 μCi/ml) or [^{3}H]inulin (0.17 μCi/ml) for 30 min at room temperature. To measure isotope spaces, microsomes were centrifuged (100,000 $\times$ *g*, 1 h), and the radioactivity associated with the pellets was measured to enable calculation of extra- and intravesicular isotope spaces. To measure intravesicular FAD space, the 100,000 $\times$ *g* pellet was resuspended, and its FAD content was measured fluorimetrically at 450-nm excitation and 530-nm emission wavelengths.

	Apparent space	
	Total	Intravesicular
	μl/mg of protein	
[^{3}H]Inulin	9.41	
[^{3}H]Water	13.77	4.36
FAD	15.86	6.43

plus [^{14}C]glucose-6-phosphate (8–10 mCi/ml) at 22 °C. In a series of incubations, the pore-forming antibiotic alamethicin (0.1 mg/ml) was added to distinguish the intravesicular and the bound radioactivity (27, 28). The alamethicin-releasable portion of radioactivity was regarded as intravesicular. At the indicated time intervals, samples (0.1 ml) were rapidly filtered through cellulose acetate/nitrate filter membranes (pore size, 0.22 μm) and were washed with ice-cold buffer A containing 1 mM DIDS. The radioactivity associated with microsomes retained by filters was measured by liquid scintillation counting.

For the measurement of FAD uptake and accumulation, the experimental conditions were the same except that the concentration of microsomal proteins was 1 mg/ml. Vesicles retained by the filters were solubilized with 2% Triton X-100, and FAD was measured fluorimetrically (see above). Candidate inhibitors of the transport were added 5 min before FAD. FAD efflux was measured after the loading of vesicles (10 mg of protein/ml) by incubating them in the presence of 1 mM FAD for 30 min at room temperature. Efflux was initiated by the 10-fold dilution of the medium with FAD-free buffer A.

Measurement of Microsomal Protein Thiol Oxidation—Microsomes (2.5 mg/ml), diluted in Tris-HCl buffer (50 mM Tris, pH 7.2), were incubated in the presence or absence of free FAD at 37 °C. After 20 min of incubation under atmospheric conditions, the protein fraction was precipitated by 10% trichloroacetic acid, washed three times by 70% acetone, and resuspended in a buffer containing 50 mM Tris, 8 M urea, and 2% sodium dodecyl sulfate (pH 7.0). Thiol content of the resuspended protein fraction was measured by the Ellman method using a Hitachi U-1500 spectrophotometer.

Materials—Alamethicin, glucose-6-phosphate (dipotassium salt), FAD, DIDS, flufenamic acid were from Sigma. [^{3}H]Water (1 mCi/g) and [^{3}H]inulin (500 mCi/g) were from PerkinElmer Life Sciences. D-[^{14}C-(U)]Glucose-6-phosphate (0.1 mCi/ml; 300 mCi/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO). Cellulose acetate/nitrate filter membranes (pore size, 0.22 μm) were from Millipore. All other chemicals were of analytical grade.

RESULTS

Intravesicular FAD-accessible Space in Rat Liver Microsomal Vesicles—In a first set of experiments, the permeability of FAD through the microsomal membrane was investigated by measuring the FAD-accessible intravesicular space of the vesicles. The intra- and extravesicular apparent spaces of vesicles were detected by using [^{3}H]water as fully membrane-permeant and [^{3}H]inulin as non-permeant compounds, respectively.

FAD occupied a greater intravesicular space than water (Table I), indicating that this compound easily permeates the microsomal membrane. The accumulation was due to a real uptake since in the presence of the pore-forming compound alamethicin, the intravesicular FAD accumulation could not be observed, and FAD binding to proteins and membranes was responsible for less than 15% of the total association (data not shown). Therefore, FAD accumulation might be caused by its uptake and possible intravesicular metabolism.

FAD Transport in Rat Liver Microsomal Vesicles—The above experiments already indicated the transport of FAD into/from microsomal vesicles. FAD uptake could be directly monitored by the conventional rapid filtration-based transport assay. After a fast initial phase, FAD uptake reached a maximum value at around 10 min and occupied ~5 μl/mg of protein intravesicular space (Fig. 1*A*). The majority of microsome-associated FAD could be released by the pore-forming agent alamethicin, indicating that FAD was present intravesicularly. The transport was bidirectional; a fast efflux was observed from FAD-loaded vesicles upon dilution (Fig. 1*B*).

We have tested the effect of some commonly used anion transport inhibitors and also the mitochondrial FAD transport inhibitor atractyloside (29) on microsomal FAD uptake. Preincubation of microsomes in the presence of DIDS and atractyloside inhibited the uptake of FAD, whereas *N*-ethylmaleimide was poorly effective, and flufenamic acid was ineffective (Figs. 1 and 2). The inhibitory effect of DIDS and atractyloside was concentration-dependent (Fig. 3). The FAD efflux was also inhibitable by DIDS (Fig. 1*B*). Therefore, the compound was consistently used to block the efflux during the washing procedure of rapid filtration. As can be seen in Fig. 1*A*, the absence of DIDS in the washing buffer resulted in a systematic underestimation of FAD uptake due to its efflux during sample processing.

Free FAD Supports Intraluminal Oxidative Processes in Rat Liver Microsomal Vesicles—If FAD is transported across the microsomal membrane, it should promote intraluminal oxidative processes. To prove this assumption, two intraluminal oxidative processes, glucose-6-phosphate dehydrogenation and protein thiol oxidation, were investigated. It has been reported that the intravesicular oxidation of glucose-6-phosphate by hexose-6-phosphate dehydrogenase, an enzyme present in the lumen of the endoplasmic reticulum (30), can be stimulated by allowing the reoxidation of intraluminal NAD(P)H to NAD(P)$^+$. Metyrapone, a membrane-permeant substrate for a reductase present in the endoplasmic reticulum (31), was used to convert back NAD(P)H (32). This compound caused a dose-dependent increase in the accumulation of radioactivity in microsomes incubated with [^{14}C]glucose-6-phosphate. The accumulated compound was 6-phosphogluconate, the product of hexose-6-phosphate dehydrogenase (32). On this basis, we supposed that other NAD(P)H oxidizing agents, including FAD, are also able to increase in the accumulation of radioactivity in microsomes incubated with [^{14}C]glucose-6-phosphate. In fact, microsomes preincubated in the presence of 1 mM FAD showed a 1.6 times higher accumulation of radioactivity at the steady-state phase upon [^{14}C]glucose-6-phosphate addition (Fig. 4).

In another set of experiments, the effect of FAD was examined on disulfide bond formation in microsomal vesicles. The concentration of protein thiols in rat liver microsomes incubated at 37 °C under atmospheric conditions did not change significantly with time. Addition of FAD resulted in a marked consumption of protein thiols. Maximal effect was reached at 0.2 mM FAD concentration, which caused the oxidation of about one-third of the total amount of microsomal thiol groups (Fig. 5). The effect was concentration-dependent, and 10 μM FAD already was effective, but FAD concentrations higher than 200 μM or incubations longer than 20 min did not result in a further significant increase in thiol oxidation. Inhibition of FAD uptake by atractyloside prevented the oxidative effect of FAD on microsomal protein thiols (Table II). Atractyloside in itself did not influence the redox state of microsomal protein thiols in the applied concentration.

DISCUSSION

Disulfide bond formation in secretory proteins requires an electron transfer chain from protein thiols to molecular oxygen in the endoplasmic reticulum of eukaryotic cells. The current model of the system, established in yeast, describes an essential protein relay involving protein disulfide isomerase and the

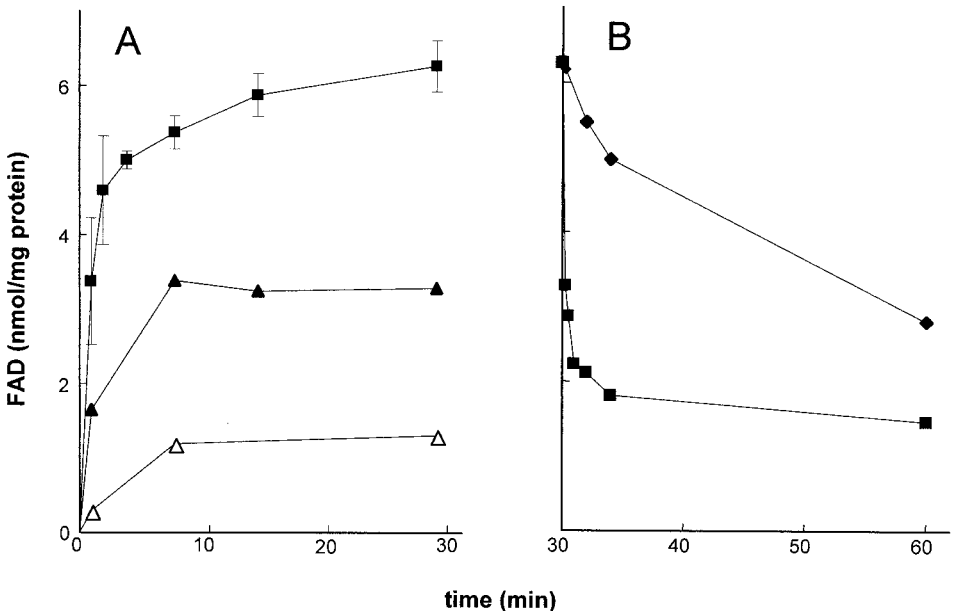

FIG. 1. **FAD uptake and efflux in rat liver microsomal vesicles.** *A*, time course of FAD uptake. Microsomes (1 mg of protein/ml) were incubated in buffer A in the presence of 1 mM FAD at room temperature. At the indicated time intervals, 0.1-ml samples were taken, filtered, and washed with 2 ml of buffer A containing 1 mM DIDS. Vesicles retained by the filters were solubilized with 2% Triton X-100, and FAD was measured fluorimetrically (■). Parallel samples were incubated in the presence of the pore-forming compound alamethicin (0.05 mg/mg of protein; Δ) or were washed with a DIDS-free buffer (▲). As shown in *B*, for the measurement of FAD efflux, microsomes (10 mg of protein/ml) were incubated in buffer A in the presence of 1 mM FAD for 30 min at room temperature. Then the solution was diluted 10-fold by the addition of FAD-free buffer A containing (♦) or not containing (■) DIDS. Rapid filtration measurements were done as before. Data are means of triplicate measurements from two independent experiments or means ± S.D., $n = 6$.

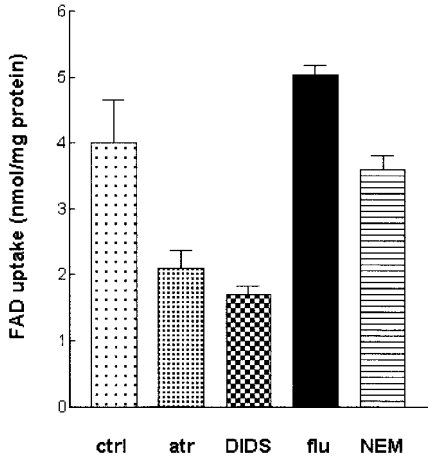

FIG. 2. **Effect of putative inhibitors on FAD uptake in rat liver microsomes.** Microsomes (1 mg of protein/ml) were incubated in buffer A in the presence of the indicated compounds for 5 min at room temperature. After the addition of 1 mM FAD, the vesicles were incubated for a further 5 min, and then FAD uptake was detected by the rapid filtration method as described in the legend for Fig. 1. 1 mM DIDS was used. Data are means ± S.D., $n = 4–6$. The abbreviations are: *ctrl*, control; *atr*, atractyloside (0.25 mM); *flu*, flufenamic acid (10 mM); *NEM*, *N*-ethylmaleimide (10 mM).

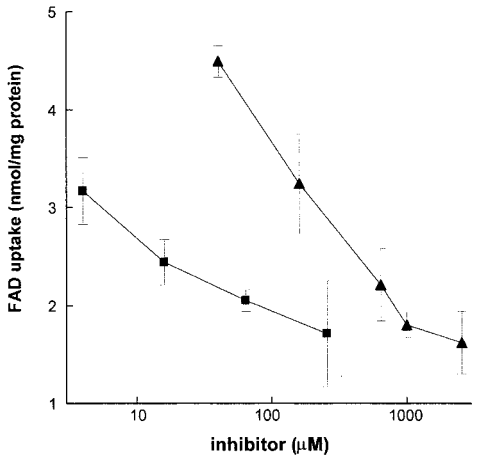

FIG. 3. **Concentration-dependent inhibition of FAD uptake by atractyloside and DIDS.** Microsomes (1 mg of protein/ml) were preincubated in the presence of the indicated concentrations of atractyloside (■) and DIDS (▲) for 5 min at room temperature. After the addition of 1 mM FAD, the vesicles were incubated for a further 5 min, and then FAD uptake was detected by the rapid filtration method as described in the legend for Fig. 1. Data are means ± S.D., $n = 6$.

flavoprotein Ero1p. The latter protein, in addition to having a tightly bound FAD moiety, can bind free FAD; this binding is essential for its function. FAD is synthesized in the cytosol but can readily enter the ER lumen and promote Ero1p-catalyzed oxidation (2, 18).

Since mammalian cells are known to possess Ero1p analogues (19–22), it can be proposed that FAD transport across the endoplasmic reticulum membrane has a crucial role in the oxidative protein folding of these cells. In accordance with this proposal, we have found that FAD can easily reach the intraluminal space of rat liver microsomal vesicles (Table I). As demonstrated in rapid filtration experiments, the transport of FAD is bidirectional (Fig. 1). The calculated intravesicular FAD concentration surpasses the extravesicular concentration in the steady-state phase of the uptake (Fig. 1). The phenomenon can be caused by an intravesicular metabolism, which is further supported by high pressure liquid chromatography analysis of the samples.[2] The transport could be inhibited in a concentration-dependent manner by the anion transport blocker DIDS and by atractyloside (Figs. 1–3), which also inhibits mitochondrial FAD transport (29). Taken together, our observations support the existence of a protein-mediated FAD

[2] A. Szarka, unpublished observation.

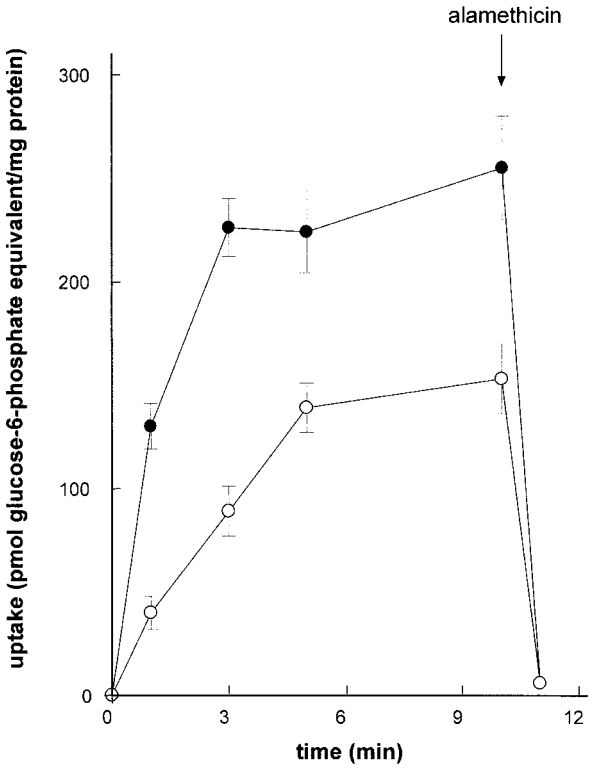

FIG. 4. **Effect of FAD on intravesicular glucose-6-phosphate oxidation in rat liver microsomes.** Glucose-6-phosphate oxidation was estimated on the basis of the intraluminal accumulation of radioactivity upon [^{14}C]glucose-6-phosphate addition. Microsomes (2 mg of protein/ml) were preincubated in the presence (●) or in the absence (○) of 1 mM FAD for 5 min at room temperature. Intravesicular accumulation of radioactivity upon [^{14}C]glucose-6-phosphate (10 μM) addition was measured by rapid filtration. At 10 min, the pore-forming compound alamethicin (0.1 mg/ml; *arrow*) was added to the incubates. Data are means ± S.D., $n = 4–6$.

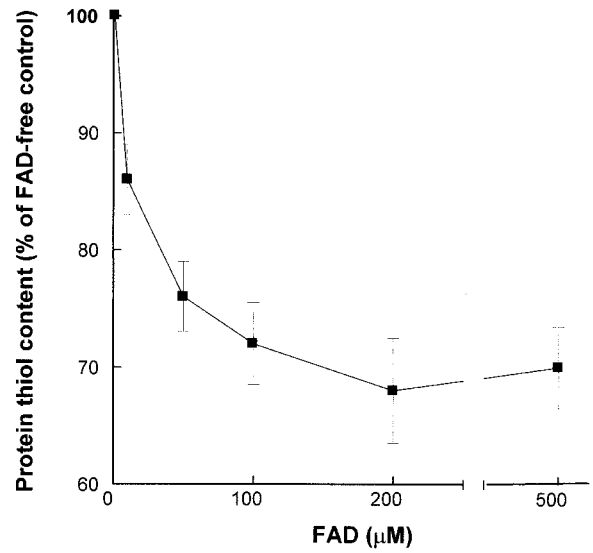

FIG. 5. **Effect of FAD on protein thiol oxidation in rat liver microsomes.** Microsomes (2.5 mg of protein/ml) were incubated in the presence of various concentrations of FAD for 20 min at 37 °C. Protein thiol contents were measured at the end of the incubations. Data are expressed as % of control, means ± S.D., $n = 5$.

transport in the hepatic endoplasmic reticulum.

The presence of FAD in the microsomal lumen has also been shown indirectly. FAD added to the microsomal vesicles promoted the intraluminal oxidation of glucose-6-phosphate (Fig. 4) and protein thiols (Fig. 5). A direct link between FAD transport and FAD-dependent protein thiol oxidation was also demonstrated; the inhibition of the uptake by atractyloside prevented the oxidation of protein thiols (Table II). Similarly, our observations reporting a larger intravesicular FAD space than that of water suggest the utilization of FAD in the ER lumen, beyond a simple turnover of the compound by repeated uptake and efflux steps.

TABLE II
Effect of the inhibition of FAD transport on protein thiol oxidation in rat liver microsomes

Microsomes (3 mg of protein/ml) were incubated for 30 minutes at 37 °C. Protein thiol contents were measured at the end of the incubations. Data are means ± S.D., $n = 6$.

Treatment	Microsomal protein thiol content
	nmol/mg of protein
None	239.8 ± 12.0
FAD (50 μM)	211.0 ± 22.5** [a]
Atractyloside (0.5 mM)	238.0 ± 27.0
FAD (50 μM) + atractyloside (0.5 mM)	230.6 ± 9.9* [b]

[a] **, $p < 0.001$ for FAD-treated versus control.
[b] *, $p < 0.05$ for FAD + atractyloside-treated versus FAD-treated vesicles.

FAD transport through the endomembranes is a poorly characterized process; neither of the responsible transporters is known at the molecular level. FAD transport has only been detected in the inner mitochondral membrane so far (29). That mitochondrial transporter is probably an antiporter, which can be inhibited by atractyloside at low micromolar concentration (29). The features of FAD transport reported here (inhibition by DIDS, relative insensitivity toward atractyloside, slight inhibition by *N*-ethylmaleimide) are similar to that of the ATP/ADP antiporter of the endoplasmic reticulum (33, 34). The possibility is raised that FAD transport is a function of ATP/ADP antiporters in both the mitochondria and the endoplasmic reticulum.

In conclusion, the present findings show that cytosolic free FAD may promote disulfide bond formation in the lumen of the endoplasmic reticulum of mammalian cells. FAD transport through the endoplasmic reticulum membrane can be an important factor to link disulfide formation to the nutritional, metabolic, and energetic status of the cell.

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